CHM 331 ADVANCED ANALYTICAL CHEMISTRY 1



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CHM 331 Advanced Analytical Chemistry 1

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Glossary

Detailed Licensing

Licensing

*A detailed breakdown of this resource's licensing can be found in **Back Matter/Detailed Licensing**.*

CHAPTER OVERVIEW

1: Introduction to Analytical Chemistry

Chemistry is the study of matter, including its composition, its structure, its physical properties, and its reactivity. Although there are many ways to study chemistry, traditionally we divide it into five areas: organic chemistry, inorganic chemistry, biochemistry, physical chemistry, and analytical chemistry. This division is historical and, perhaps, arbitrary, as suggested by current interest in interdisciplinary areas, such as bioanalytical chemistry and organometallic chemistry. Nevertheless, these five areas remain the simplest division that spans the discipline of chemistry.

Each of these traditional areas of chemistry brings a unique perspective to how a chemist makes sense of the diverse array of elements, ions, and molecules (both small and large) that make up our physical environment. An undergraduate chemistry course, therefore, is much more than a collection of facts; it is, instead, the means by which we learn to see the chemical world from a different perspective. In keeping with this spirit, this chapter introduces you to the field of analytical chemistry and highlights the unique perspectives that analytical chemists bring to the study of chemistry.

1.1: What is Analytical Chemistry
1.2: The Analytical Perspective
1.3: Common Analytical Problems
1.4: Problems
1.5: Additional Resources
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Thumbnail: Several graduated cylinders of various thickness and heights with white side markings in front of a large beaker. They are all filled about halfway with red or blue chemical compounds. The blue ink is showing signs of Brownian motion when dissolving into water. Image used with permission (CC BY-SA 3.0; Horia Varlan from Bucharest, Romania).

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1.1: What is Analytical Chemistry

"Analytical chemistry is what analytical chemists do."

This quote is attributed to C. N. Reilly (1925-1981) on receipt of the 1965 Fisher Award in Analytical Chemistry. Reilly, who was a professor of chemistry at the University of North Carolina at Chapel Hill, was one of the most influential analytical chemists of the last half of the twentieth century.

For another view of what constitutes analytical chemistry, see the article "Quo Vadis, Analytical Chemistry?", the full reference for which is Valcárcel, M. *Anal. Bioanal. Chem.* **2016**, *408*, 13-21.

Let's begin with a deceptively simple question: What is analytical chemistry? Like all areas of chemistry, analytical chemistry is so broad in scope and so much in flux that it is difficult to find a simple definition more revealing than that quoted above. In this chapter we will try to expand upon this simple definition by saying a little about what analytical chemistry is, as well as a little about what analytical chemistry is not.

Analytical chemistry often is described as the area of chemistry responsible for characterizing the composition of matter, both qualitatively (Is there lead in this paint chip?) and quantitatively (How much lead is in this paint chip?). As we shall see, this description is misleading.

Most chemists routinely make qualitative and quantitative measurements. For this reason, some scientists suggest that analytical chemistry is not a separate branch of chemistry, but simply the application of chemical knowledge [Ravey, M. *Spectroscopy*, **1990**, *5(7)*, 11]. In fact, you probably have performed many such quantitative and qualitative analyses in other chemistry courses.

> You might, for example, have determined the concentration of acetic acid in vinegar using an acid–base titration, or used a qualitative analysis scheme to identify which of several metal ions are in an aqueous sample.

Defining analytical chemistry as the application of chemical knowledge ignores the unique perspective that an analytical chemist bring to the study of chemistry. The craft of analytical chemistry is found not in performing a routine analysis on a routine sample—a task we appropriately call chemical analysis—but in improving established analytical methods, in extending these analytical methods to new types of samples, and in developing new analytical methods to measure chemical phenomena [de Haseth, J. *Spectroscopy*, **1990**, *5(7)*, 11].

Here is one example of the distinction between analytical chemistry and chemical analysis. A mining engineer evaluates an ore by comparing the cost of removing the ore from the earth with the value of its contents, which they estimate by analyzing a sample of the ore. The challenge of developing and validating a quantitative analytical method is the analytical chemist's responsibility; the routine, daily application of the analytical method is the job of the chemical analyst.

The Seven Stages of an Analytical Method

1. Conception of analytical method (birth).
2. Successful demonstration that the analytical method works.
3. Establishment of the analytical method's capabilities.
4. Widespread acceptance of the analytical method.
5. Continued development of the analytical method leads to significant improvements.
6. New cycle through steps 3–5.
7. Analytical method can no longer compete with newer analytical methods (death).

Steps 1–3 and 5 are the province of analytical chemistry; step 4 is the realm of chemical analysis.

The seven stages of an analytical method listed here are modified from Fassel, V. A. *Fresenius' Z. Anal. Chem.* **1986**, *324*, 511–518 and Hieftje, G. M. *J. Chem. Educ.* **2000**, *77*, 577–583.

Another difference between analytical chemistry and chemical analysis is that an analytical chemist works to improve and to extend established analytical methods. For example, several factors complicate the quantitative analysis of nickel in ores, including nickel's unequal distribution within the ore, the ore's complex matrix of silicates and oxides, and the presence of other metals that may interfere with the analysis. Figure 1.1.1 outlines one standard analytical method in use during the late nineteenth century [Fresenius. C. R. *A System of Instruction in Quantitative Chemical Analysis*; John Wiley and Sons: New York, 1881]. The need for many reactions, digestions, and filtrations makes this analytical method both time-consuming and difficult to perform accurately.

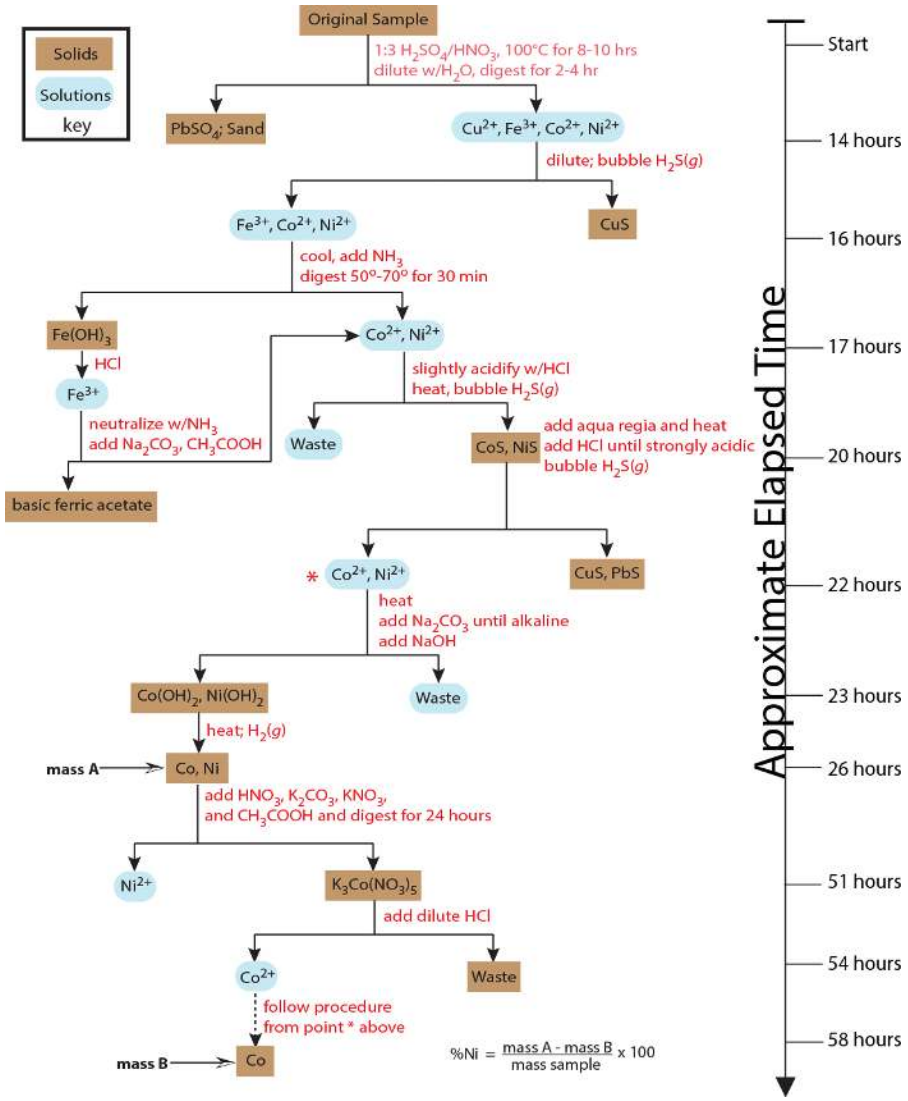


Figure 1.1.1 : Fresenius' analytical scheme for the gravimetric analysis of Ni in ores. After each step, the solid and the solution are separated by gravity filtration. Note that the mass of nickel is not determined directly. Instead, Co and Ni first are isolated and weighed together (**mass A**), and then Co is isolated and weighed separately (**mass B**). The timeline shows that it takes approximately 58 hours to analyze one sample. This scheme is an example of a gravimetric analysis, which is explored further in Chapter 8.

The discovery, in 1905, that dimethylglyoxime (dmg) selectively precipitates Ni^{2+} and Pd^{2+} led to an improved analytical method for the quantitative analysis of nickel [Kolthoff, I. M.; Sandell, E. B. *Textbook of Quantitative Inorganic Analysis*, 3rd Ed., The Macmillan Company: New York, 1952]. The resulting analysis, which is outlined in Figure 1.1.2 , requires fewer manipulations and less time. By the 1970s, flame atomic absorption spectrometry replaced gravimetry as the standard method for analyzing nickel in ores, resulting in an even more rapid analysis [Van Loon, J. C. *Analytical Atomic Absorption Spectroscopy*, Academic Press: New York, 1980]. Today, the standard analytical method utilizes an inductively coupled plasma optical emission spectrometer.

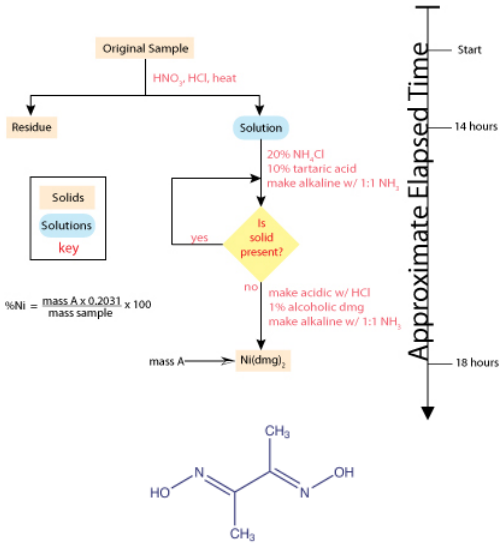


Figure 1.1.2 : Gravimetric analysis for Ni in ores by precipitating $Ni(dmg)_2$. The timeline shows that it takes approximately 18 hours to analyze a single sample, substantially less than 58 hours for the method in Figure 1.1.1 . The factor of 0.2301 in the equation for %Ni accounts for the difference in the formula weights of Ni and $Ni(dmg)_2$; see Chapter 8 for further details. The structure of dmg is shown below the method's flow chart.

Perhaps a more appropriate description of analytical chemistry is “the science of inventing and applying the concepts, principles, and...strategies for measuring the characteristics of chemical systems” [Murray, R. W. *Anal. Chem.* **1991**, *63*, 271A]. Analytical chemists often work at the extreme edges of analysis, extending and improving the ability of all chemists to make meaningful measurements on smaller samples, on more complex samples, on shorter time scales, and on species present at lower concentrations. Throughout its history, analytical chemistry has provided many of the tools and methods necessary for research in other traditional areas of chemistry, as well as fostering multidisciplinary research in, to name a few, medicinal chemistry, clinical chemistry, toxicology, forensic chemistry, materials science, geochemistry, and environmental chemistry.

> To an analytical chemist, the process of making a useful measurement is critical; if the measurement is not of central importance to the work, then it is not analytical chemistry.

You will come across numerous examples of analytical methods in this textbook, most of which are routine examples of chemical analysis. It is important to remember, however, that nonroutine problems prompted analytical chemists to develop these methods.

> An editorial in *Analytical Chemistry* entitled “Some Words about Categories of Manuscripts” highlights nicely what makes a research endeavor relevant to modern analytical chemistry. The full citation is Murray, R. W. *Anal. Chem.* **2008**, *80*, 4775; for a more recent editorial, see “The Scope of *Analytical Chemistry*” by Sweedler, J. V. et. al. *Anal. Chem.* **2015**, *87*, 6425.

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1.2: The Analytical Perspective

Having noted that each area of chemistry brings a unique perspective to the study of chemistry, let's ask a second deceptively simple question: What is the analytical perspective? Many analytical chemists describe this perspective as an analytical approach to solving problems.

> For different viewpoints on the analytical approach see (a) Beilby, A. L. *J. Chem. Educ.* **1970**, *47*, 237-238; (b) Lucchesi, C. A. *Am. Lab.* **1980**, October, 112-119; (c) Atkinson, G. F. *J. Chem. Educ.* **1982**, *59*, 201-202; (d) Pardue, H. L.; Woo, J. *J. Chem. Educ.* **1984**, *61*, 409-412; (e) Guarnieri, M. *J. Chem. Educ.* **1988**, *65*, 201-203, (f) Strobel, H. A. *Am. Lab.* **1990**, October, 17-24.

Although there likely are as many descriptions of the analytical approach as there are analytical chemists, it is convenient to define it as the five-step process shown in Figure 1.2.1 .

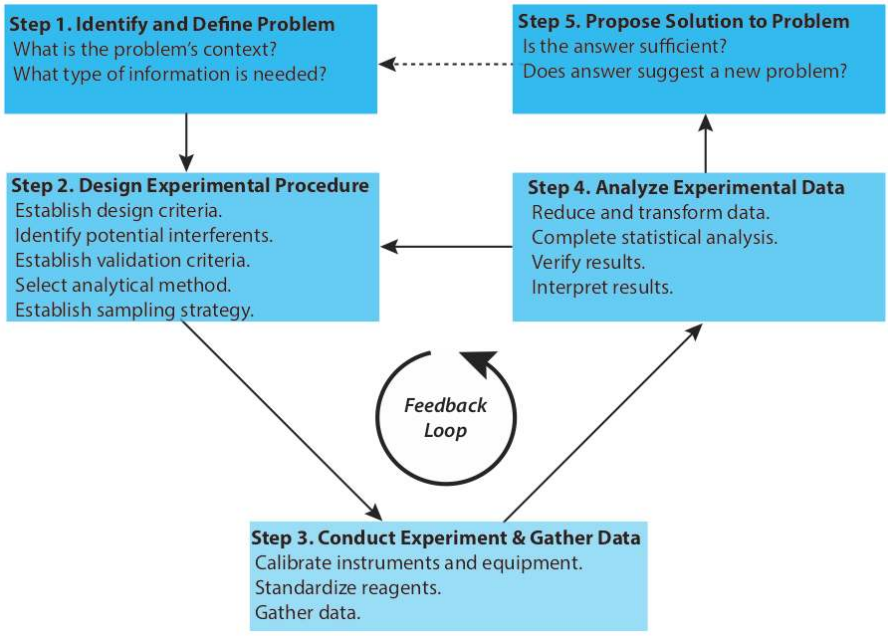


Figure 1.2.1 : Flow diagram showing one view of the analytical approach to solving problems (modified after Atkinson, G. F. *J. Chem. Educ.* **1982**, *59*, 201-202).

Three general features of this approach deserve our attention. First, in steps 1 and 5 analytical chemists have the opportunity to collaborate with individuals outside the realm of analytical chemistry. In fact, many problems on which analytical chemists work originate in other fields. Second, the heart of the analytical approach is a feedback loop (steps 2, 3, and 4) in which the result of one step requires that we reevaluate the other steps. Finally, the solution to one problem often suggests a new problem.

Analytical chemistry begins with a problem, examples of which include evaluating the amount of dust and soil ingested by children as an indicator of environmental exposure to particulate based pollutants, resolving contradictory evidence regarding the toxicity of perfluoro polymers during combustion, and developing rapid and sensitive detectors for chemical and biological weapons. At this point the analytical approach involves a collaboration between the analytical chemist and the individual or agency working on the problem. Together they determine what information is needed and clarify how the problem relates to broader research goals or policy issues, both essential to the design of an appropriate experimental procedure.

> These examples are taken from a series of articles, entitled the "Analytical Approach," which for many years was a regular feature of the journal *Analytical Chemistry*.

To design the experimental procedure the analytical chemist considers criteria, such as the required accuracy, precision, sensitivity, and detection limit, the urgency with which results are needed, the cost of a single analysis, the number of samples to analyze, and the amount of sample available for analysis. Finding an appropriate balance between these criteria frequently is complicated by their interdependence. For example, improving precision may require a larger amount of sample than is available. Consideration also is given to how to collect, store, and prepare samples, and to whether chemical or physical interferences will affect the analysis. Finally a good experimental procedure may yield useless information if there is no method for validating the results.

The most visible part of the analytical approach occurs in the laboratory. As part of the validation process, appropriate chemical and physical standards are used to calibrate equipment and to standardize reagents.

The data collected during the experiment are then analyzed. Frequently the data first is reduced or transformed to a more readily analyzable form and then a statistical treatment of the data is used to evaluate accuracy and precision, and to validate the procedure. Results are compared to the original design criteria and the experimental design is reconsidered, additional trials are run, or a solution to the problem is proposed. When a solution is proposed, the results are subject to an external evaluation that may result in a new problem and the beginning of a new cycle.

Chapter 3 introduces you to the language of analytical chemistry. You will find terms such accuracy, precision, and sensitivity defined there. Chapter 4 introduces the statistical analysis of data. Calibration and standardization methods, including a discussion of linear regression, are covered in Chapter 5. See Chapter 7 for a discussion of how to collect, store, and prepare samples for analysis. See Chapter 14 for a discussion about how to validate an analytical method.

As noted earlier some scientists question whether the analytical approach is unique to analytical chemistry. Here, again, it helps to distinguish between a chemical analysis and analytical chemistry. For an analytically-oriented scientist, such as a physical organic chemist or a public health officer, the primary emphasis is how the analysis supports larger research goals that involve fundamental studies of chemical or physical processes, or that improve access to medical care. The essence of analytical chemistry, however, is in developing new tools for solving problems, and in defining the type and quality of information available to other scientists.

? Exercise 1.2.1

As an exercise, let's adapt our model of the analytical approach to the development of a simple, inexpensive, portable device for completing bioassays in the field. Before continuing, locate and read the article

"Simple Telemedicine for Developing Regions: Camera Phones and Paper-Based Microfluidic Devices for Real-Time, Off-Site Diagnosis"

by Andres W. Martinez, Scott T. Phillips, Emanuel Carriho, Samuel W. Thomas III, Hayat Sindi, and George M. Whitesides. You will find it on pages 3699-3707 in Volume 80 of the journal *Analytical Chemistry*, which was published in 2008. As you read the article, pay particular attention to how it emulates the analytical approach and consider the following questions:

1. What is the analytical problem and why is it important?
2. What criteria did the authors consider in designing their experiments? What is the basic experimental procedure?
3. What interferences were considered and how did they overcome them? How did the authors calibrate the assay?
4. How did the authors validate their experimental method?
5. Is there evidence that steps 2, 3, and 4 in Figure 1.2.1 are repeated?
6. Was there a successful conclusion to the analytical problem?

Don't let the technical details in the paper overwhelm you; if you skim over these you will find the paper both well-written and accessible.

Answer

What is the analytical problem and why is it important?

A medical diagnoses often relies on the results of a clinical analysis. When you visit a doctor, they may draw a sample of your blood and send it to the lab for analysis. In some cases the result of the analysis is available in 10-15 minutes. What is

possible in a developed country, such as the United States, may not be feasible in a country with less access to expensive lab equipment and with fewer trained personnel available to run the tests and to interpret the results. The problem addressed in this paper, therefore, is the development of a reliable device for rapidly performing a clinical assay under less than ideal circumstances.

What criteria did the authors consider in designing their experiments?

In considering a solution to this problem, the authors identify seven important criteria for the analytical method: (1) it must be inexpensive; (2) it must operate without the need for much electricity, so that it can be used in remote locations; (3) it must be adaptable to many types of assays; (4) its must not require a highly skilled technician; (5) it must be quantitative; (6) it must be accurate; and (7) it must produce results rapidly.

What is the basic experimental procedure?

The authors describe how they developed a paper-based microfluidic device that allows anyone to run an analysis simply by dipping the device into a sample (synthetic urine, in this case). The sample moves by capillary action into test zones containing reagents that react with specific species (glucose and protein, for this prototype device). The reagents react to produce a color whose intensity is proportional to the species' concentration. A digital photograph of the microfluidic device is taken using a cell phone camera and sent to an off-site physician who uses image editing software to analyze the photograph and to interpret the assay's result.

What interferences were considered and how did they overcome them?

In developing this analytical method the authors considered several chemical or physical interferences. One concern was the possibility of non-specific interactions between the paper and the glucose or protein, which might lead to non-uniform image in the test zones. A careful analysis of the distribution of glucose and protein in the test zones showed that this was not a problem. A second concern was the possibility that particulate materials in the sample might interfere with the analyses. Paper is a natural filter for particulate materials and the authors found that samples containing dust, sawdust, and pollen do not interfere with the analysis for glucose. Pollen, however, is an interferent for the protein analysis, presumably because it, too, contains protein.

How did the author's calibrate the assay?

To calibrate the device the authors analyzed a series of standard solutions that contained known concentrations of glucose and protein. Because an image's intensity depends upon the available light, a standard sample is run with the test samples, which allows a single calibration curve to be used for samples collected under different lighting conditions.

How did the author's validate their experimental method?

The test device contains two test zones for each analyte, which allows for duplicate analyses and provides one level of experimental validation. To further validate the device, the authors completed 12 analyses at each of three known concentrations of glucose and protein, obtaining acceptable accuracy and precision in all cases.

Is there any evidence of repeating steps 2, 3, and 4 in Figure 1.2.1?

Developing this analytical method required several cycles through steps 2, 3, and 4 of the analytical approach. Examples of this feedback loop include optimizing the shape of the test zones and evaluating the importance of sample size.

Was there a successful conclusion to the analytical problem?

Yes. The authors were successful in meeting their goals by developing and testing an inexpensive, portable, and easy-to-use device for running clinical samples in developing countries.

This exercise provides you with an opportunity to think about the analytical approach in the context of a real analytical problem. Practice exercises such as this provide you with a variety of challenges ranging from simple review problems to more open-ended exercises. You will find answers to practice exercises at the end of each chapter.

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1.3: Common Analytical Problems

Many problems in analytical chemistry begin with the need to identify what is present in a sample. This is the scope of a ***qualitative analysis***, examples of which include identifying the products of a chemical reaction, screening an athlete's urine for a performance-enhancing drug, or determining the spatial distribution of Pb on the surface of an airborne particulate. An early challenge for analytical chemists was developing simple chemical tests to identify inorganic ions and organic functional groups. The classical laboratory courses in inorganic and organic qualitative analysis, still taught at some schools, are based on this work.

> See, for example, the following laboratory textbooks: (a) Sorum, C. H.; Lagowski, J. J. *Introduction to Semimicro Qualitative Analysis*, 5th Ed.; Prentice-Hall: Englewood, NJ, 1977; (b) Shriner, R. L.; Fuson, R. C.; Curtin, D. Y. *The Systematic Identification of Organic Compounds*, 5th Ed.; John Wiley and Sons: New York, 1964.

Modern methods for qualitative analysis rely on instrumental techniques, such as infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS). Because these qualitative applications are covered adequately elsewhere in the undergraduate curriculum, typically in organic chemistry, they receive no further consideration in this text.

Perhaps the most common analytical problem is a ***quantitative analysis***, examples of which include the elemental analysis of a newly synthesized compound, measuring the concentration of glucose in blood, or determining the difference between the bulk and the surface concentrations of Cr in steel. Much of the analytical work in clinical, pharmaceutical, environmental, and industrial labs involves developing new quantitative methods to detect trace amounts of chemical species in complex samples. Most of the examples in this text are of quantitative analyses.

Another important area of analytical chemistry, which receives some attention in this text, are methods for characterizing physical and chemical properties. The determination of chemical structure, of equilibrium constants, of particle size, and of surface structure are examples of a ***characterization analysis***.

The purpose of a qualitative, a quantitative, or a characterization analysis is to solve a problem associated with a particular sample. The purpose of a ***fundamental analysis***, on the other hand, is to improve our understanding of the theory that supports an analytical method and to understand better an analytical method's limitations.

> A good resource for current examples of qualitative, quantitative, characterization, and fundamental analyses is *Analytical Chemistry's* annual review issue that highlights fundamental and applied research in analytical chemistry. Examples of review articles in the 2015 issue include "Analytical Chemistry in Archaeological Research," "Recent Developments in Paper-Based Microfluidic Devices," and "Vibrational Spectroscopy: Recent Developments to Revolutionize Forensic Science."

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1.4: Problems

1. For each of the following problems indicate whether its solution requires a qualitative analysis, a quantitative analysis, a characterization analysis, and/or a fundamental analysis. More than one type of analysis may be appropriate for some problems.
   a. The residents in a neighborhood near a hazardous-waste disposal site are concerned that it is leaking contaminants into their groundwater.
   b. An art museum is concerned that a recently acquired oil painting is a forgery.
   c. Airport security needs a more reliable method for detecting the presence of explosive materials in luggage.
   d. The structure of a newly discovered virus needs to be determined.
   e. A new visual indicator is needed for an acid–base titration.
   f. A new law requires a method for evaluating whether automobiles are emitting too much carbon monoxide.
2. Read the article "When Machine Tastes Coffee: Instrumental Approach to Predict the Sensory Profile of Espresso Coffee," which discusses work completed at the Nestlé Research Center in Lausanne, Switzerland. You will find the article on pages 1574-1581 in Volume 80 of *Analytical Chemistry*, published in 2008. Prepare an essay that summarizes the nature of the problem and how it was solved. Do not worry about the nitty-gritty details of the mathematical model developed by the authors, which relies on a combination of an analysis of variance (ANOVA), a topic we will consider in Chapter 14, and a principle component regression (PCR), at topic that we will not consider in this text. Instead, focus on the results of the model by examining the visualizations in Figure 3 and Figure 4 of the paper. As a guide, refer to Figure 1.2.1 in this chapter for a model of the analytical approach to solving problems. Use this link to access the article's abstract from the journal's web site. If your institution has an on-line subscription you also will be able to download a PDF version of the article.

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1.5: Additional Resources

The role of analytical chemistry within the broader discipline of chemistry has been discussed by many prominent analytical chemists; several notable examples are listed here.

- Baiulescu, G. E.; Patroescu, C; Chalmers, R. A. *Education and Teaching in Analytical Chemistry*, Ellis Horwood: Chichester, 1982.
- de Haseth, J. "What is Analytical Chemistry?," *Spectroscopy* **1990**, *5*, 19–21.
- Heiftje, G. M. "The Two Sides of Analytical Chemistry," *Anal. Chem.* **1985**, *57*, 256A–267A.
- Heiftje, G. M. "But is it analytical chemistry?," *Am. Lab.* **1993**, October, 53–61.
- Kissinger, P. T. "Analytical Chemistry—What is It? Why Teach It?," *Trends Anal. Chem.* **1992**, *11*, 57–57.
- Laitinen, H. A.; Ewing, G. (eds.) *A History of Analytical Chemistry*, The Division of Analytical Chemistry of the American Chemical Society: Washington, D. C., 1972.
- Laitinen, H. A. "Analytical Chemistry in a Changing World," *Anal. Chem.* **1980**, *52*, 605A–609A.
- Laitinen, H. A. "History of Analytical Chemistry in the U. S. A.," *Talanta*, **1989**, *36*, 1–9.
- McLafferty, F. W. "Analytical Chemistry: Historic and Modern," *Acc. Chem. Res.* **1990**, *23*, 63–64.
- Mottola, H. A. "The Interdisciplinary and Multidisciplinary Nature of Contemporary Analytical Chemistry and its Core Components," *Anal. Chim. Acta* **1991**, *242*, 1–3.
- Noble, D. "From Wet Chemistry to Instrumental Analysis: A Perspective on Analytical Sciences," *Anal. Chem.* **1994**, *66*, 251A–263A.
- Tyson, J. *Analysis: What Analytical Chemists Do*, Royal Society of Chemistry: Cambridge, England 1988.

For additional discussion of clinical assays based on paper-based microfluidic devices, see the following papers.

- Ellerbee, A. K.; Phillips, S. T.; Siegel, A. C.; Mirica, K. A.; Martinez, A. W.; Striehl, P.; Jain, N.; Prentiss, M.; Whitesides, G. M. "Quantifying Colorimetric Assays in Paper-Based Microfluidic Devices by Measuring the Transmission of Light Through Paper," *Anal. Chem.* **2009**, *81*, 8447–8452.
- Martinez, A. W.; Phillips, S. T.; Whitesides, G. M. "Diagnostics for the Developing World: Microfluidic Paper-Based Analytical Devices," *Anal. Chem.* **2010**, *82*, 3–10.

This textbook provides one introduction to the discipline of analytical chemistry. There are other textbooks for introductory courses in analytical chemistry and you may find it useful to consult them when you encounter a difficult concept; often a fresh perspective will help crystallize your understanding. The textbooks listed here are excellent resources.

- Enke, C. *The Art and Science of Chemical Analysis*, Wiley: New York.
- Christian, G. D.; Dasgupta, P, K.; Schug; K. A. *Analytical Chemistry*, Wiley: New York.
- Harris, D. *Quantitative Chemical Analysis*, W. H. Freeman and Company: New York.
- Kellner, R.; Mermet, J.-M.; Otto, M.; Valcárcel, M.; Widmer, H. M. *Analytical Chemistry*, Wiley- VCH: Weinheim, Germany.
- Rubinson, J. F.; Rubinson, K. A. *Contemporary Chemical Analysis*, Prentice Hall: Upper Saddle River, NJ.
- Skoog, D. A.; West, D. M.; Holler, F. J. *Fundamentals of Analytical Chemistry*, Saunders: Philadelphia.

To explore the practice of modern analytical chemistry there is no better resource than the primary literature. The following journals publish broadly in the area of analytical chemistry.

- Analytical and Bioanalytical Chemistry
- Analytical Chemistry
- Analytical Chimica Acta
- Analyst
- Talanta

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1.6: Chapter Summary and Key Terms

Chapter Summary

Analytical chemists work to improve the ability of chemists and other scientists to make meaningful measurements. The need to work with smaller samples, with more complex materials, with processes occurring on shorter time scales, and with species present at lower concentrations challenges analytical chemists to improve existing analytical methods and to develop new ones.

Typical problems on which analytical chemists work include qualitative analyses (What is present?), quantitative analyses (How much is present?), characterization analyses (What are the sample's chemical and physical properties?), and fundamental analyses (How does this method work and how can it be improved?).

Key Terms

- characterization analysis
- fundamental analysis
- qualitative analysis
- quantitative analysis

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CHAPTER OVERVIEW

2: Basic Tools of Analytical Chemistry

In the chapters that follow we will explore many aspects of analytical chemistry. In the process we will consider important questions, such as “How do we extract useful results from experimental data?”, “How do we ensure our results are accurate?”, “How do we obtain a representative sample?”, and “How do we select an appropriate analytical technique?” Before we consider these and other questions, we first must review some basic tools of importance to analytical chemists.

2.1: Measurements in Analytical Chemistry
2.2: Concentration
2.3: Stoichiometric Calculations
2.4: Basic Equipment
2.5: Preparing Solutions
2.6: Spreadsheets and Computational Software
2.7: The Laboratory Notebook
2.8: Problems
2.9: Additional Resources
2.10: Chapter Summary and Key Terms

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2.1: Measurements in Analytical Chemistry

Analytical chemistry is a quantitative science. Whether determining the concentration of a species, evaluating an equilibrium constant, measuring a reaction rate, or drawing a correlation between a compound's structure and its reactivity, analytical chemists engage in "measuring important chemical things" [Murray, R. W. *Anal. Chem.* **2007**, *79*, 1765]. In this section we review briefly the basic units of measurement and the proper use of significant figures.

Units of Measurement

A measurement usually consists of a unit and a number that expresses the quantity of that unit. We can express the same physical measurement with different units, which creates confusion if we are not careful to specify the unit. For example, the mass of a sample that weighs 1.5 g is equivalent to 0.0033 lb or to 0.053 oz. To ensure consistency, and to avoid problems, scientists use the common set of fundamental base units listed in Table 2.1.1 . These units are called SI units after the Système International d'Unités.

> It is important for scientists to agree upon a common set of units. In 1999, for example, NASA lost a Mars Orbiter spacecraft because one engineering team used English units in their calculations and another engineering team used metric units. As a result, the spacecraft came too close to the planet's surface, causing its propulsion system to overheat and fail.
>
> Some measurements, such as absorbance, do not have units. Because the meaning of a unitless number often is unclear, some authors include an artificial unit. It is not unusual to see the abbreviation AU—short for absorbance unit—following an absorbance value, which helps clarify that the measurement is an absorbance value.

Table 2.1.1 : Fundamental Base SI Units

Measurement	Unit	Symbol	Definition (1 unit is...)
mass	kilogram	kg	...the mass of the international prototype, a Pt-Ir object housed at the Bureau International de Poids and Measures at Sèvres, France. (Note: The mass of the international prototype changes at a rate of approximately 1 μg per year due to reversible surface contamination. The reference mass, therefore, is determined immediately after its cleaning using a specified procedure. Current plans call for retiring the international prototype and defining the kilogram in terms of Planck's constant; see this link for more details.)
distance	meter	m	...the distance light travels in $(299\ 792\ 458)^{-1}$ seconds.
temperature	kelvin	K	...equal to $(273.16)^{-1}$, where 273.16 K is the triple point of water (where its solid, liquid, and gaseous forms are in equilibrium. and where 0 is the lowest possible temperature).
time	second	s	...the time it takes for 9 192 631 770 periods of radiation corresponding to a specific transition of the ^{133}Cs atom.
current	ampere	A	...the current producing a force of 2×10^{-7} N/m between two straight parallel conductors of infinite length separated by one meter (in a vacuum).
amount of substance	mole	mol	...the amount of a substance containing as many particles as there are atoms in exactly 0.012 kilogram of ^{12}C.
light	candela	cd	...the luminous intensity of a source with a monochromatic frequency of 540×10^{12} hertz and a radiant power of $(683)^{-1}$ watts per steradian.

There is some disagreement on the use of "amount of substance" to describe the measurement for which the mole is the base SI unit; see "What's in a Name? Amount of Substance, Chemical Amount, and Stoichiometric Amount," the full reference for

which is Giunta, C. J. *J. Chem. Educ.* **2016**, *93*, 583–586.

We define other measurements using these fundamental SI units. For example, we measure the quantity of heat produced during a chemical reaction in joules, (J), where 1 J is equivalent to 1 m kg/s . Table 2.1.2 provides a list of some important derived SI units, as well as a few common non-SI units.

Table 2.1.2 : Derived SI Units and Non-SI Units of Importance to Analytical Chemistry

Measurement	Unit	Symbol	Equivalent SI Units
length	angstrom (non-SI)	Å	$1\ Å = 1 \times 10^{-10}$ m
volume	liter (non-SI)	L	$1\ L = 10^{-3}\ m^3$
force	newton (SI)	N	$1\ N = 1\ m{\cdot}kg/s^2$
pressure	pascal (SI) atmosphere (non-SI)	Pa atm	$1\ Pa = 1\ N/m^3 = 1\ kg/(m{\cdot}s^2)$ 1 atm = 101 325 Pa
energy, work, heat	joule (SI) calorie (non-SI) electron volt (non-SI)	J cal eV	$1\ J = 1\ N{\cdot}m = 1\ m^2{\cdot}kg/s^2$ 1 cal = 4.184 J $1\ eV = 1.602\ 177\ 33 \times 10^{-19}$ J
power	watt (SI)	W	$1\ W = 1\ J/s = 1\ m^2{\cdot}kg/s^3$
charge	coulomb (SI)	C	$1\ C = 1\ A{\cdot}s$
potential	volt (SI)	V	$1\ V = 1\ W/A = 1\ m^2{\cdot}kg/(s^3{\cdot}A)$
frequency	hertz (SI)	Hz	$1\ Hz = s^{-1}$
temperature	Celcius (non-SI)	°C	°C = K – 273.15

Chemists frequently work with measurements that are very large or very small. A mole contains 602 213 670 000 000 000 000 000 particles and some analytical techniques can detect as little as 0.000 000 000 000 001 g of a compound. For simplicity, we express these measurements using scientific notation; thus, a mole contains $6.022\ 136\ 7 \times 10^{23}$ particles, and the detected mass is 1×10^{-15} g. Sometimes we wish to express a measurement without the exponential term, replacing it with a prefix (Table 2.1.3). A mass of $\mathbf{1 \times 10^{-15}}$ g, for example, is the same as 1 fg, or femtogram.

Writing a lengthy number with spaces instead of commas may strike you as unusual. For a number with more than four digits on either side of the decimal point, however, the recommendation from the International Union of Pure and Applied Chemistry is to use a thin space instead of a comma.

Table 2.1.3 : Common Prefixes for Exponential Notation

Prefix	Symbol	Factor	Prefix	Symbol	Factor	Prefix	Symbol	Factor
yotta	Y	10^{24}	kilo	k	10^{3}	micro	μ	10^{-6}
zetta	Z	10^{21}	hecto	h	10^{2}	nano	n	10^{-9}
eta	E	10^{18}	deka	da	10^{1}	pico	p	10^{-12}
peta	P	10^{15}	—	—	10^{0}	femto	f	10^{-15}
tera	T	10^{12}	deci	d	10^{-1}	atto	a	10^{-18}
giga	G	10^{9}	centi	c	10^{-2}	zepto	z	10^{-21}
mega	M	10^{6}	milli	m	10^{-3}	yocto	y	10^{-24}

Uncertainty in Measurements

A measurement provides information about both its magnitude and its uncertainty. Consider, for example, the three photos in Figure 2.1.1 , taken at intervals of approximately 1 sec after placing a sample on the balance. Assuming the balance is properly calibrated, we are certain that the sample's mass is more than 0.5729 g and less than 0.5731 g. We are uncertain, however, about the sample's mass in the last decimal place since the final two decimal places fluctuate between 29, 30, and 31. The best we can do is to report the sample's mass as 0.5730 g ± 0.0001 g, indicating both its magnitude and its absolute uncertainty.



Figure 2.1.1 : When weighing a sample on a balance, the measurement fluctuates in the final decimal place. We record this sample's mass as 0.5730 g ± 0.0001 g.

Significant Figures

A measurement's significant figures convey information about a measurement's magnitude and uncertainty. The number of significant figures in a measurement is the number of digits known exactly plus one digit whose value is uncertain. The mass shown in Figure 2.1.1 , for example, has four significant figures, three which we know exactly and one, the last, which is uncertain.

Suppose we weigh a second sample, using the same balance, and obtain a mass of 0.0990 g. Does this measurement have 3, 4, or 5 significant figures? The zero in the last decimal place is the one uncertain digit and is significant. The other two zeros, however, simply indicates the decimal point's location. Writing the measurement in scientific notation, $\mathbf{9.90 \times 10^{-2}}$, clarifies that there are three significant figures in 0.0990.

> In the measurement **0.0**99**0** g, the zero in **green** is a significant digit and the zeros in **red** are not significant digits.

Example 2.1.1

How many significant figures are in each of the following measurements? Convert each measurement to its equivalent scientific notation or decimal form.

a. 0.0120 mol HCl
b. 605.3 mg $CaCO_3$
c. $\mathbf{1.043 \times 10^{-4}}$ mol Ag^+
d. $\mathbf{9.3 \times 10^{4}}$ mg NaOH

Solution

(a) Three significant figures; $\mathbf{1.20 \times 10^{-2}}$ mol HCl.

(b) Four significant figures; $\mathbf{6.053 \times 10^{2}}$ mg $CaCO_3$.

(c) Four significant figures; 0.000 104 3 mol Ag^+.

(d) Two significant figures; 93 000 mg NaOH.

There are two special cases when determining the number of significant figures in a measurement. For a measurement given as a logarithm, such as pH, the number of significant figures is equal to the number of digits to the right of the decimal point. Digits to

the left of the decimal point are not significant figures since they indicate only the power of 10. A pH of 2.45, therefore, contains two significant figures.

> The log of 2.8×10^2 is 2.45. The log of 2.8 is 0.45 and the log of 10^2 is 2. The 2 in 2.45, therefore, only indicates the power of 10 and is not a significant digit.

An exact number, such as a stoichiometric coefficient, has an infinite number of significant figures. A mole of $CaCl_2$, for example, contains exactly two moles of chloride ions and one mole of calcium ions. Another example of an exact number is the relationship between some units. There are, for example, exactly 1000 mL in 1 L. Both the 1 and the 1000 have an infinite number of significant figures.

Using the correct number of significant figures is important because it tells other scientists about the uncertainty of your measurements. Suppose you weigh a sample on a balance that measures mass to the nearest ±0.1 mg. Reporting the sample's mass as 1.762 g instead of 1.7623 g is incorrect because it does not convey properly the measurement's uncertainty. Reporting the sample's mass as 1.76231 g also is incorrect because it falsely suggests an uncertainty of ±0.01 mg.

Significant Figures in Calculations

Significant figures are also important because they guide us when reporting the result of an analysis. When we calculate a result, the answer cannot be more certain than the least certain measurement in the analysis. Rounding an answer to the correct number of significant figures is important.

For addition and subtraction, we round the answer to the last decimal place in common for each measurement in the calculation. The exact sum of 135.621, 97.33, and 21.2163 is 254.1673. Since the last decimal place common to all three numbers is the hundredth's place

$$\begin{array}{r} 135.621 \\ 97.33 \\ 21.2163 \\ \hline 254.1673 \end{array}$$

we round the result to 254.17.

> The last common decimal place shared by 135.621, 97.33, and 21.2163 is shown in **red**.

When working with scientific notation, first convert each measurement to a common exponent before determining the number of significant figures. For example, the sum of 6.17×10^7, 4.3×10^5, and 3.23×10^4 is 6.22×10^7.

$$\begin{array}{rl} 6.17 & \times 10^7 \\ 0.043 & \times 10^7 \\ \underline{0.00323} & \underline{\times 10^7} \\ 6.21623 & \times 10^7 \end{array}$$

> The last common decimal place shared by 6.17×10^7, 4.3×10^5 and 3.23×10^4 is shown in **red**.

For multiplication and division, we round the answer to the same number of significant figures as the measurement with the fewest number of significant figures. For example, when we divide the product of 22.91 and 0.152 by 16.302, we report the answer as 0.214 (three significant figures) because 0.152 has the fewest number of significant figures.

$$\frac{22.91 \times 0.152}{16.302} = 0.2136 = 0.214$$

There is no need to convert measurements in scientific notation to a common exponent when multiplying or dividing.

It is important to recognize that the rules presented here for working with significant figures are generalizations. What actually is conserved is uncertainty, not the number of significant figures. For example, the following calculation

$$101/99 = 1.02$$

is correct even though it violates the general rules outlined earlier. Since the relative uncertainty in each measurement is approximately 1% (101 ± 1 and 99 ± 1), the relative uncertainty in the final answer also is approximately 1%. Reporting the answer as 1.0 (two significant figures), as required by the general rules, implies a relative uncertainty of 10%, which is too large. The correct answer, with three significant figures, yields the expected relative uncertainty. Chapter 4 presents a more thorough treatment of uncertainty and its importance in reporting the result of an analysis.

Finally, to avoid "round-off" errors, it is a good idea to retain at least one extra significant figure throughout any calculation. Better yet, invest in a good scientific calculator that allows you to perform lengthy calculations without the need to record intermediate values. When your calculation is complete, round the answer to the correct number of significant figures using the following simple rules.

1. Retain the least significant figure if it and the digits that follow are less than halfway to the next higher digit. For example, rounding 12.442 to the nearest tenth gives 12.4 since 0.442 is less than half way between 0.400 and 0.500.
2. Increase the least significant figure by 1 if it and the digits that follow are more than halfway to the next higher digit. For example, rounding 12.476 to the nearest tenth gives 12.5 since 0.476 is more than halfway between 0.400 and 0.500.
3. If the least significant figure and the digits that follow are exactly halfway to the next higher digit, then round the least significant figure to the nearest even number. For example, rounding 12.450 to the nearest tenth gives 12.4, while rounding 12.550 to the nearest tenth gives 12.6. Rounding in this manner ensures that we round up as often as we round down.

? Exercise 2.1.1

For a problem that involves both addition and/or subtraction, and multiplication and/or division, be sure to account for significant figures at each step of the calculation. With this in mind, report the result of this calculation to the correct number of significant figures.

$$\frac{0.250 \times (9.93 \times 10^{-3}) - 0.100 \times (1.927 \times 10^{-2})}{9.93 \times 10^{-3} + 1.927 \times 10^{-2}} =$$

Answer

The correct answer to this exercise is 1.9×10^{-2}. To see why this is correct, let's work through the problem in a series of steps. Here is the original problem

$$\frac{0.250 \times (9.93 \times 10^{-3}) - 0.100 \times (1.927 \times 10^{-2})}{9.93 \times 10^{-3} + 1.927 \times 10^{-2}} =$$

Following the correct order of operations we first complete the two multiplications in the numerator. In each case the answer has three significant figures, although we retain an extra digit, highlight in **red**, to avoid round-off errors.

$$\frac{2.482 \times 10^{-3} - 1.927 \times 10^{-3}}{9.93 \times 10^{-3} + 1.927 \times 10^{-2}} =$$

Completing the subtraction in the numerator leaves us with two significant figures since the last significant digit for each value is in the hundredths place.

$$\frac{0.555 \times 10^{-3}}{9.93 \times 10^{-3} + 1.927 \times 10^{-2}} =$$

The two values in the denominator have different exponents. Because we are adding together these values, we first rewrite them using a common exponent.

$$\frac{0.555 \times 10^{-3}}{0.993 \times 10^{-2} + 1.927 \times 10^{-2}} =$$

The sum in the denominator has four significant figures since each of the addends has three decimal places.

$$\frac{0.555 \times 10^{-3}}{2.920 \times 10^{-2}} =$$

Finally, we complete the division, which leaves us with a result having two significant figures.

$$\frac{0.555 \times 10^{-3}}{2.920 \times 10^{-2}} = 1.9 \times 10^{-2}$$

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2.2: Concentration

Concentration is a general measurement unit that reports the amount of solute present in a known amount of solution or solvent

$$\text{concentration} = \frac{\text{amount of solute}}{\text{amount of solution}} \tag{2.2.1}$$

Although we associate the terms "solute" and "solution" with liquid samples, we can extend their use to gas-phase and solid-phase samples as well. Table 2.2.1 lists the most common units of concentration.

Table 2.2.1 : Common Units for Reporting Concentration

Name	Units	Symbol
molarity	$\frac{\text{moles solute}}{\text{liters solution}}$	M
formality	$\frac{\text{moles solute}}{\text{liters solution}}$	F
normality	$\frac{\text{equivalents solute}}{\text{liters solution}}$	N
molality	$\frac{\text{moles solute}}{\text{kilograms solvent}}$	m
weight percent	$\frac{\text{grams solute}}{\text{100 grams solution}}$	% w/w
volume percent	$\frac{\text{mL solute}}{\text{100 mL solution}}$	% v/v
weight-to-volume percent	$\frac{\text{grams solute}}{\text{100 mL solution}}$	% w/v
parts per million	$\frac{\text{grams solute}}{10^6 \text{ grams solution}}$	ppm
parts per billion	$\frac{\text{grams solute}}{10^9 \text{ grams solution}}$	ppb

An alternative expression for weight percent is

$$\frac{\text{grams solute}}{\text{grams solution}} \times 100$$

You can use similar alternative expressions for volume percent and for weight-to-volume percent.

Molarity and Formality

Both molarity and formality express concentration as moles of solute per liter of solution; however, there is a subtle difference between them. ***Molarity*** is the concentration of a particular chemical species. ***Formality***, on the other hand, is a substance's total concentration without regard to its specific chemical form. There is no difference between a compound's molarity and formality if it dissolves without dissociating into ions. The formal concentration of a solution of glucose, for example, is the same as its molarity.

For a compound that ionizes in solution, such as $CaCl_2$, molarity and formality are different. When we dissolve 0.1 moles of $CaCl_2$ in 1 L of water, the solution contains 0.1 moles of Ca^{2+} and 0.2 moles of Cl^-. The molarity of $CaCl_2$, therefore, is zero since there is no undissociated $CaCl_2$ in solution; instead, the solution is 0.1 M in Ca^{2+} and 0.2 M in Cl^-. The formality of $CaCl_2$, however, is 0.1 F since it represents the total amount of $CaCl_2$ in solution. This more rigorous definition of molarity, for better or worse, largely is ignored in the current literature, as it is in this textbook. When we state that a solution is 0.1 M $CaCl_2$ we understand it to consist of Ca^{2+} and Cl^- ions. We will reserve the unit of formality to situations where it provides a clearer description of solution chemistry.

Molarity is used so frequently that we use a symbolic notation to simplify its expression in equations and in writing. Square brackets around a species indicate that we are referring to that species' molarity. Thus, $[Ca^{2+}]$ is read as "the molarity of calcium

ions."

> For a solute that dissolves without undergoing ionization, molarity and formality have the same value. A solution that is 0.0259 M in glucose, for example, is 0.0259 F in glucose as well.

Normality

Normality is a concentration unit that no longer is in common use; however, because you may encounter normality in older handbooks of analytical methods, it is helpful to understand its meaning. ***Normality*** defines concentration in terms of an equivalent, which is the amount of one chemical species that reacts stoichiometrically with another chemical species. Note that this definition makes an equivalent, and thus normality, a function of the chemical reaction in which the species participates. Although a solution of H_2SO_4 has a fixed molarity, its normality depends on how it reacts. You will find a more detailed treatment of normality in Appendix 1.

> One handbook that still uses normality is *Standard Methods for the Examination of Water and Wastewater*, a joint publication of the American Public Health Association, the American Water Works Association, and the Water Environment Federation. This handbook is one of the primary resources for the environmental analysis of water and wastewater.

Molality

Molality is used in thermodynamic calculations where a temperature independent unit of concentration is needed. Molarity is based on the volume of solution that contains the solute. Since density is a temperature dependent property, a solution's volume, and thus its molar concentration, changes with temperature. By using the solvent's mass in place of the solution's volume, the resulting concentration becomes independent of temperature.

Weight, Volume, and Weight-to-Volume Percent

Weight percent (% w/w), ***volume percent*** (% v/v) and ***weight-to-volume percent*** (% w/v) express concentration as the units of solute present in 100 units of solution. A solution that is 1.5% w/v NH_4NO_3, for example, contains 1.5 gram of NH_4NO_3 in 100 mL of solution.

Parts Per Million and Parts Per Billion

Parts per million (ppm) and ***parts per billion*** (ppb) are ratios that give the grams of solute in, respectively, one million or one billion grams of sample. For example, a sample of steel that is 450 ppm in Mn contains 450 μg of Mn for every gram of steel. If we approximate the density of an aqueous solution as 1.00 g/mL, then we can express solution concentrations in ppm or ppb using the following relationships.

$$\text{ppm} = \frac{\mu\text{g}}{\text{g}} = \frac{\text{mg}}{\text{L}} = \frac{\mu\text{g}}{\text{mL}} \quad \text{ppb} = \frac{\text{ng}}{\text{g}} = \frac{\mu\text{g}}{\text{L}} = \frac{\text{ng}}{\text{mL}}$$

For gases a part per million usually is expressed as a volume ratio; for example, a helium concentration of 6.3 ppm means that one liter of air contains 6.3 μL of He.

> You should be careful when using parts per million and parts per billion to express the concentration of an aqueous solute. The difference between a solute's concentration in mg/L and ng/g, for example, is significant if the solution's density is not 1.00 g/mL. For this reason many organizations advise against using the abbreviation ppm and ppb (see section 7.10.3 at www.nist.gov). If in doubt, include the exact units, such as 0.53 μg Pb^{2+}/L for the concentration of lead in a sample of seawater.

Converting Between Concentration Units

The most common ways to express concentration in analytical chemistry are molarity, weight percent, volume percent, weight-to-volume percent, parts per million and parts per billion. The general definition of concentration in Equation 2.2.1 makes it is easy to convert between concentration units.

Example 2.2.1

A concentrated solution of ammonia is 28.0% w/w NH_3 and has a density of 0.899 g/mL. What is the molar concentration of NH_3 in this solution?

Solution

$$\frac{28.0\text{ g NH}_3}{100\text{ g soln}} \times \frac{0.899\text{ g soln}}{\text{ml soln}} \times \frac{1\text{ mol NH}_3}{17.03\text{ g NH}_3} \times \frac{1000\text{mL}}{\text{L}} = 14.8\text{ M}$$

Example 2.2.2

The maximum permissible concentration of chloride ion in a municipal drinking water supply is 2.50×10^2 ppm Cl^-. When the supply of water exceeds this limit it often has a distinctive salty taste. What is the equivalent molar concentration of Cl^-?

Solution

$$\frac{2.50 \times 10^2\text{ mg Cl}^-}{\text{L}} \times \frac{1\text{ g}}{1000\text{ mg}} \times \frac{1\text{ mol Cl}^-}{35.453\text{ gCl}^-} = 7.05 \times 10^{-3}\text{ M}$$

Exercise 2.2.1

Which solution—0.50 M NaCl or 0.25 M $SrCl_2$—has the larger concentration when expressed in mg/mL?

Answer

The concentrations of the two solutions are

$$\frac{0.50\text{ mol NaCl}}{\text{L}} \times \frac{58.44\text{ g NaCl}}{\text{mol NaCl}} \times \frac{10^6\,\mu\text{g}}{\text{g}} \times \frac{1\text{L}}{1000\text{ mL}} = 2.9 \times 10^4\,\mu\text{g/mL NaCl}$$

$$\frac{0.25\text{ mol SrCl}_2}{\text{L}} \times \frac{158.5\text{ g SrCl}_2}{\text{mol SrCl}_2} \times \frac{10^6\,\mu\text{g}}{\text{g}} \times \frac{1\text{L}}{1000\text{ mL}} = 4.0 \times 10^4\,\mu\text{g/ml SrCl}_2$$

The solution of $SrCl_2$ has the larger concentration when it is expressed in µg/mL instead of in mol/L.

p-Functions

Sometimes it is inconvenient to use the concentration units in Table 2.2.1 . For example, during a chemical reaction a species' concentration may change by many orders of magnitude. If we want to display the reaction's progress graphically we might wish to plot the reactant's concentration as a function of the volume of a reagent added to the reaction. Such is the case in Figure 2.2.1 for the titration of HCl with NaOH. The *y*-axis on the left-side of the figure displays the $[H^+]$ as a function of the volume of NaOH. The initial $[H^+]$ is 0.10 M and its concentration after adding 80 mL of NaOH is 4.3×10^{-13} M. We easily can follow the change in $[H^+]$ for the addition of the first 50 mL of NaOH; however, for the remaining volumes of NaOH the change in $[H^+]$ is too small to see.

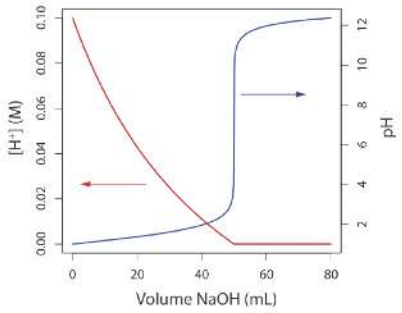


Figure 2.2.1 : Two curves showing the progress of a titration of 50.0 mL of 0.10 M HCl with 0.10 M NaOH. The $[H^+]$ is shown on the left *y*-axis and the pH on the right *y*-axis.

When working with concentrations that span many orders of magnitude, it often is more convenient to express concentration using a p-function. The p-function of *X* is written as p*X* and is defined as

$$\mathrm{p}X = -\log(X)$$

The pH of a solution that is 0.10 M H^+ for example, is

$$\mathrm{pH} = -\log[\mathrm{H^+}] = -\log(0.10) = 1.00$$

and the pH of 4.3×10^{-13} M H^+ is

$$\mathrm{pH} = -\log[\mathrm{H^+}] = -\log(4.3 \times 10^{-13}) = 12.37$$

Figure 2.2.1 shows that plotting pH as a function of the volume of NaOH provides more useful information about how the concentration of H^+ changes during the titration.

A more appropriate equation for pH is $\mathrm{pH} = -\log(a_{\mathrm{H^+}})$ where $a_{\mathrm{H^+}}$ is the activity of the hydrogen ion. See Chapter 6.9 for more details. For now the approximate equation $\mathrm{pH} = -\log[\mathrm{H^+}]$ is sufficient.

Example 2.2.3

What is pNa for a solution of 1.76×10^{-3} M Na_3PO_4?

Solution

Since each mole of Na_3PO_4 contains three moles of Na^+, the concentration of Na^+ is

$$[\mathrm{Na^+}] = (1.76 \times 10^{-3}\ \mathrm{M}) \times \frac{3\ \mathrm{mol\ Na^+}}{\mathrm{mol\ Na_3PO_4}} = 5.28 \times 10^{-3}\ \mathrm{M}$$

and pNa is

$$\mathrm{pNa} = -\log[\mathrm{Na^+}] = -\log(5.28 \times 10^{-3}) = 2.277$$

Remember that a pNa of 2.777 has three, not four, significant figures; the 2 that appears in the one's place indicates the power of 10 when we write $[Na^+]$ as 0.528×10^{-2} M.

Example 2.2.4

What is the $[H^+]$ in a solution that has a pH of 5.16?

Solution

The concentration of H^+ is

$$\mathrm{pH} = -\log[\mathrm{H^+}] = 5.16$$

$$\log[H^+] = -5.16$$

$$[H^+] = 10^{-5.16} = 6.9 \times 10^{-6}\ M$$

Recall that if $\log(X) = a$, then $X = 10^a$.

Exercise 2.2.2

What are the values for pNa and pSO_4 if we dissolve 1.5 g Na_2SO_4 in a total solution volume of 500.0 mL?

Answer

The concentrations of Na^+ and SO_4^{2-} are

$$\frac{1.5\ g\ Na_2SO_4}{0.500 L} \times \frac{1\ mol\ Na_2SO_4}{142.0\ g\ Na_2SO_4} \times \frac{2\ mol\ Na^+}{mol\ mol Na_2SO_4} = 4.23 \times 10^{-2}\ M\ Na^+$$

$$\frac{1.5\ g\ Na_2SO_4}{0.500 L} \times \frac{1\ mol\ Na_2SO_4}{142.0\ g\ Na_2SO_4} \times \frac{1\ mol\ SO_4^{2-}}{mol\ mol Na_2SO_4} = 2.11 \times 10^{-2}\ M\ SO_4^{2-}$$

The pNa and pSO_4 values are

$$pNa = -\log(4.23 \times 10^{-2}\ M\ Na^+) = 1.37$$

$$pSO_4 = -\log(2.11 \times 10^{-2}\ M\ SO_4^{2-}) = 1.68$$

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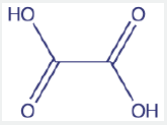
2.3: Stoichiometric Calculations

A balanced reaction, which defines the stoichiometric relationship between the moles of reactants and the moles of products, provides the basis for many analytical calculations. Consider, for example, an analysis for oxalic acid, $H_2C_2O_4$, in which Fe^{3+} oxidizes oxalic acid to CO_2

$$2Fe^{3+}(aq) + H_2C_2O_4(aq) + 2H_2O(l) \longrightarrow 2Fe^{2+}(aq) + 2CO_2(g) + 2H_3O^+(aq)$$

The balanced reaction shows us that one mole of oxalic acid reacts with two moles of Fe^{3+}. As shown in the following example, we can use this balanced reaction to determine the amount of $H_2C_2O_4$ in a sample of rhubarb if we know the moles of Fe^{3+} needed to react completely with oxalic acid.

In sufficient amounts, oxalic acid, the structure for which is shown to the right, is toxic. At lower physiological concentrations it leads to the formation of kidney stones. The leaves of the rhubarb plant contain relatively high concentrations of oxalic acid. The stalk, which many individuals enjoy eating, contains much smaller concentrations of oxalic acid.



In the examples that follow, note that we retain an extra significant figure throughout the calculation, rounding to the correct number of significant figures at the end. We will follow this convention in any calculation that involves more than one step. If we forget that we are retaining an extra significant figure, we might report the final answer with one too many significant figures. Here we mark the extra digit in **red** for emphasis. Be sure you pick a system for keeping track of significant figures.

Example 2.3.1

The amount of oxalic acid in a sample of rhubarb was determined by reacting with Fe^{3+}. After extracting a 10.62 g of rhubarb with a solvent, oxidation of the oxalic acid required 36.44 mL of 0.0130 M Fe^{3+}. What is the weight percent of oxalic acid in the sample of rhubarb?

Solution

We begin by calculating the moles of Fe^{3+} used in the reaction

$$\frac{0.0130 \text{ mol } Fe^{3+}}{\text{L}} \times 0.03644 \text{ M} = 4.737 \times 10^{-4} \text{ mol } Fe^{3+}$$

The moles of oxalic acid reacting with the Fe^{3+}, therefore, is

$$4.737 \times 10^{-4} \text{ mol } Fe^{3+} \times \frac{1 \text{ mol } H_2C_2O_4}{2 \text{ mol } Fe^{3+}} = 2.368 \times 10^{-4} \text{ mol } H_2C_2O_4$$

Converting the moles of oxalic acid to grams of oxalic acid

$$2.368 \times 10^{-4} \text{ mol } H_2C_2O_4 \times \frac{90.03 \text{ g } H_2C_2O_4}{\text{mol } H_2C_2O_4} = 2.132 \times 10^{-2} \text{ g } H_2C_2O_4$$

and calculating the weight percent gives the concentration of oxalic acid in the sample of rhubarb as

$$\frac{2.132 \times 10^{-2} \text{ g } H_2C_2O_4}{10.62 \text{ g rhubarb}} \times 100 = 0.201 H_2C_2O_4$$

Exercise 2.3.1

You can dissolve a precipitate of AgBr by reacting it with $Na_2S_2O_3$, as shown here.

$$AgBr(s) + 2Na_2S_2O_3(aq) \longrightarrow Ag(S_2O_3)_2^{3-}(aq) + Br^-(aq) + 4Na^+(aq)$$

How many mL of 0.0138 M $Na_2S_2O_3$ do you need to dissolve 0.250 g of AgBr?

Answer

First, we find the moles of AgBr

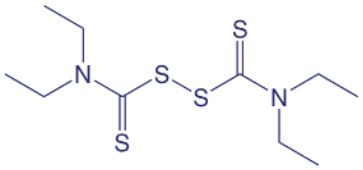
$$0.250 \text{ g AgBr} \times \frac{1 \text{ mol AgBr}}{187.8 \text{ g AgBr}} = 1.331 \times 10^{-3} \text{ mol AgBr}$$

and then the moles and volume of $Na_2S_2O_3$

$$1.331 \times 10^{-3} \text{ mol AgBr} \times \frac{2 \text{ mol } Na_2S_2O_3}{\text{mol AgBr}} = 2.662 \times 10^{-3} \text{ mol } Na_2S_2O_3$$

$$2.662 \times 10^{-3} \text{ mol } Na_2S_2O_3 \times \frac{1 \text{ L}}{0.0138 \text{ mol } Na_2S_2O_3} \times \frac{1000 \text{ mL}}{\text{L}} = 193 \text{ mL}$$

The analyte in Example 2.3.1 , oxalic acid, is in a chemically useful form because there is a reagent, Fe^{3+}, that reacts with it quantitatively. In many analytical methods, we first must convert the analyte into a more accessible form before we can complete the analysis. For example, one method for the quantitative analysis of disulfiram, $C_{10}H_{20}N_2S_4$—the active ingredient in the drug Antabuse, and whose structure is shown to the right —requires that we first convert the sulfur to SO_2 by combustion, and then oxidize the SO_2 to H_2SO_4 by bubbling it through a solution of H_2O_2. When the conversion is complete, the amount of H_2SO_4 is determined by titrating with NaOH.



To convert the moles of NaOH used in the titration to the moles of disulfiram in the sample, we need to know the stoichiometry of each reaction. Writing a balanced reaction for H_2SO_4 and NaOH is straightforward

$$H_2SO_4(aq) + 2NaOH(aq) \longrightarrow 2H_2O(l) + Na_2SO_4(aq)$$

but the balanced reactions for the oxidations of $C_{10}H_{20}N_2S_4$ to SO_2, and of SO_2 to H_2SO_4 are not as immediately obvious. Although we can balance these redox reactions, it is often easier to deduce the overall stoichiometry by using a little chemical logic.

Example 2.3.2

An analysis for disulfiram, $C_{10}H_{20}N_2S_4$, in Antabuse is carried out by oxidizing the sulfur to H_2SO_4 and titrating the H_2SO_4 with NaOH. If a 0.4613-g sample of Antabuse requires 34.85 mL of 0.02500 M NaOH to titrate the H_2SO_4, what is the %w/w disulfiram in the sample?

Solution

Calculating the moles of H_2SO_4 is easy—first, we calculate the moles of NaOH used in the titration

$$(0.02500 \text{ M}) \times (0.03485 \text{ L}) = 8.7125 \times 10^{-4} \text{ mol NaOH}$$

and then we use the titration reaction's stoichiometry to calculate the corresponding moles of H_2SO_4.

$$8.7125 \times 10^{-4} \text{ mol NaOH} \times \frac{1 \text{ mol } H_2SO_4}{2 \text{ mol NaOH}} = 4.3562 \times 10^{-4} \text{ mol } H_2SO_4$$

Here is where we use a little chemical logic. Instead of balancing the reactions for the combustion of $C_{10}H_{20}N_2S_4$ to SO_2 and for the subsequent oxidation of SO_2 to H_2SO_4, we recognize that a conservation of mass requires that all the sulfur in $C_{10}H_{20}N_2S_4$ ends up in the H_2SO_4; thus

$$4.3562 \times 10^{-4} \text{ mol } H_2SO_4 \times \frac{1 \text{ mol S}}{\text{mol } H_2SO_4} \times \frac{1 \text{ mol } C_{10}H_{20}N_2S_4}{4 \text{ mol S}} = 1.0890 \times 10^{-4} \text{ mol } C_{10}H_{20}N_2S_4$$

$$1.0890 \times 10^{-4} \text{ mol } C_{10}H_{20}N_2S_4 \times \frac{296.54 \text{ g } C_{10}H_{20}N_2S_4}{\text{mol } C_{10}H_{20}N_2S_4} = 0.032293 \text{ g } C_{10}H_{20}N_2S_4$$

$$\frac{0.032293 \text{ g } C_{10}H_{20}N_2S_4}{0.4613 \text{ g sample}} \times 100 = 7.000 C_{10}H_{20}N_2S_4$$

A conservation of mass is the essence of stoichiometry!

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2.4: Basic Equipment

The array of equipment available for making analytical measurements and working with analytical samples is impressive, ranging from the simple and inexpensive, to the complex and expensive. With three exceptions—the measurement of mass, and the measurement of volume —we will postpone the discussion of equipment to later chapters where its application to specific analytical methods is relevant.

Equipment for Measuring Mass

An object's mass is measured using a digital electronic analytical balance (Figure **2.4.1**). An electromagnet levitates the sample pan above a permanent cylindrical magnet. When we place an object on the sample pan, it displaces the sample pan downward by a force equal to the product of the sample's mass and its acceleration due to gravity. The balance detects this downward movement and generates a counterbalancing force by increasing the current to the electromagnet. The current needed to return the balance to its original position is proportional to the object's mass. A typical electronic balance has a capacity of 100–200 g, and can measure mass to the nearest ±0.01 mg to ±1 mg.



Figure **2.4.1**: The photo shows a typical digital electronic balance capable of determining mass to the nearest ±0.1 mg. The sticker inside the balance's wind shield is its annual calibration certification. For a review of other types of electronic balances, see Schoonover, R. M. *Anal. Chem.* **1982**, *54*, 973A-980A.

Although we tend to use interchangeably, the terms "weight" and "mass," there is an important distinction between them. Mass is the absolute amount of matter in an object, measured in grams. Weight, *W*, is a measure of the gravitational force, *g*, acting on that mass, *m*:

$$W = m \times g$$

An object has a fixed mass but its weight depends upon the acceleration due to gravity, which varies subtly from location-to-location.

A balance measures an object's weight, not its mass. Because weight and mass are proportional to each other, we can calibrate a balance using a standard weight whose mass is traceable to the standard prototype for the kilogram. A properly calibrated balance gives an accurate value for an object's mass; see Appendix 9 for more details on calibrating a balance.

If the sample is not moisture sensitive, a clean and dry container is placed on the balance. The container's mass is called the ***tare*** and most balances allow you to set the container's tare to a mass of zero. The sample is transferred to the container, the new mass is measured and the sample's mass determined by subtracting the tare. A sample that absorbs moisture from the air is treated differently. The sample is placed in a covered weighing bottle and their combined mass is determined. A portion of the sample is removed and the weighing bottle and the remaining sample are reweighed. The difference between the two masses gives the sample's mass.

Several important precautions help to minimize errors when we determine an object's mass. To minimize the effect of vibrations, the balance is placed on a stable surface and in a level position. Because the sensitivity of an analytical balance is sufficient to measure the mass of a fingerprint, materials often are handled using tongs or laboratory tissues. Volatile liquid samples must be weighed in a covered container to avoid the loss of sample by evaporation. To minimize fluctuations in mass due to air currents, the balance pan often is housed within a wind shield, as seen in Figure **2.4.1**. A sample that is cooler or warmer than the surrounding air will create a convective air currents that affects the measurement of its mass. For this reason, bring your samples to room

temperature before determining their mass. Finally, samples dried in an oven are stored in a desiccator to prevent them from reabsorbing moisture from the atmosphere.

Equipment for Measuring Volume

Analytical chemists use a variety of glassware to measure volume, including graduated cylinders, volumetric pipets, and volumetric flasks. The choice of what type of glassware to use depends on how accurately and how precisely we need to know the sample's volume and whether we are interested in containing or delivering the sample.

A ***graduated cylinder*** is the simplest device for delivering a known volume of a liquid reagent (Figure **2.4.2**). The graduated scale allows you to deliver any volume up to the cylinder's maximum. Typical accuracy is ±1% of the maximum volume. A 100-mL graduated cylinder, for example, is accurate to ±1 mL.



Figure 2.4.2: An example of a 250-mL graduated cylinder.

A ***volumetric pipet*** provides a more accurate method for delivering a known volume of solution. Several different styles of pipets are available, two of which are shown in Figure **2.4.3**. Transfer pipets provide the most accurate means for delivering a known volume of solution. A transfer pipet delivering less than 100 mL generally is accurate to the hundredth of a mL. Larger transfer pipets are accurate to a tenth of a mL. For example, the 10-mL transfer pipet in Figure **2.4.3** will deliver 10.00 mL with an accuracy of ±0.02 mL.

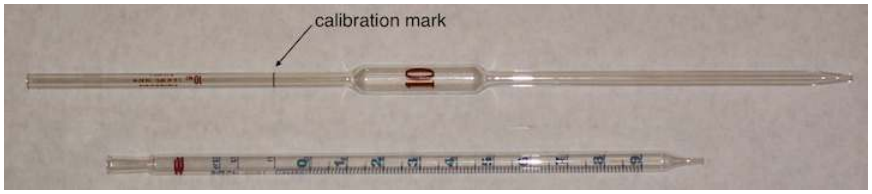


Figure 2.4.3: Two examples of 10-mL volumetric pipets. The pipet on the top is a transfer pipet and the pipet on the bottom is a Mohr measuring pipet. The transfer pipet delivers a single volume of 10.00 mL when filled to its calibration mark. The Mohr pipet has a mark every 0.1 mL, allowing for the delivery of variable volumes. It also has additional graduations at 11 mL, 12 mL, and 12.5 mL.

Scientists at the Brookhaven National Laboratory used a germanium nanowire to make a pipet that delivers a 35 zeptoliter (10^{-21} L) drop of a liquid gold-germanium alloy. You can read about this work in the April 21, 2007 issue of *Science News*.

To fill a transfer pipet, use a rubber suction bulb to pull the solution up past the calibration mark (*Never use your mouth to suck a solution into a pipet!*). After replacing the bulb with your finger, adjust the solution's level to the calibration mark and dry the outside of the pipet with a laboratory tissue. Allow the pipet's contents to drain into the receiving container with the pipet's tip touching the inner wall of the container. A small portion of the liquid remains in the pipet's tip and is not be blown out. With some measuring pipets any solution remaining in the tip must be blown out.

Delivering microliter volumes of liquids is not possible using transfer or measuring pipets. Digital micropipets (Figure **2.4.4**), which come in a variety of volume ranges, provide for the routine measurement of microliter volumes.



Figure 2.4.4: A set of two digital micropipets. The pipet on the top delivers volumes between 0.5 mL and 10 µL and the pipet on the bottom delivers volumes between 10 mL and 100 µL.

Graduated cylinders and pipets deliver a known volume of solution. A ***volumetric flask***, on the other hand, contains a specific volume of solution (Figure **2.4.5**). When filled to its calibration mark, a volumetric flask that contains less than 100 mL generally is accurate to the hundredth of a mL, whereas larger volumetric flasks are accurate to the tenth of a mL. For example, a 10-mL volumetric flask contains 10.00 mL ± 0.02 mL and a 250-mL volumetric flask contains 250.0 mL ± 0.12 mL.



Figure 2.4.5: A collection of volumetric flasks with volumes of 10 mL, 50 mL, 100, mL, 250 mL, and 500 mL.

Because a volumetric flask contains a solution, it is used to prepare a solution with an accurately known concentration. Transfer the reagent to the volumetric flask and add enough solvent to bring the reagent into solution. Continuing adding solvent in several portions, mixing thoroughly after each addition, and then adjust the volume to the flask's calibration mark using a dropper. Finally, complete the mixing process by inverting and shaking the flask at least 10 times.

If you look closely at a volumetric pipet or a volumetric flask you will see markings similar to those shown in Figure **2.4.6**. The text of the markings, which reads

$$10 \text{ mL T. D. at } 20\ ^{o}\text{C} \pm 0.02 \text{ mL}$$

indicates that the pipet is calibrated to deliver (T. D.) 10 mL of solution with an uncertainty of ±0.02 mL at a temperature of 20 ^{o}C. The temperature is important because glass expands and contracts with changes in temperatures; thus, the pipet's accuracy is less than ±0.02 mL at a higher or a lower temperature. For a more accurate result, you can calibrate your volumetric glassware at the temperature you are working by weighing the amount of water contained or delivered and calculating the volume using its temperature dependent density.



Figure 2.4.6: Close-up of the 10-mL transfer pipet from Figure 2.4.3.

A volumetric flask has similar markings, but uses the abbreviation T. C. for “to contain” in place of T. D.

You should take three additional precautions when you work with pipets and volumetric flasks. First, the volume delivered by a pipet or contained by a volumetric flask assumes that the glassware is clean. Dirt and grease on the inner surface prevent liquids from draining evenly, leaving droplets of liquid on the container’s walls. For a pipet this means the delivered volume is less than the calibrated volume, while drops of liquid above the calibration mark mean that a volumetric flask contains more than its calibrated volume. Commercially available cleaning solutions are available for cleaning pipets and volumetric flasks.

Second, when filling a pipet or volumetric flask the liquid’s level must be set exactly at the calibration mark. The liquid’s top surface is curved into a meniscus, the bottom of which should align with the glassware’s calibration mark (Figure **2.4.7**). When adjusting the meniscus, keep your eye in line with the calibration mark to avoid parallax errors. If your eye level is above the calibration mark you will overfill the pipet or the volumetric flask and you will underfill them if your eye level is below the calibration mark.



Figure 2.4.7: Proper position of the solution’s meniscus relative to the volumetric flask’s calibration mark.

Finally, before using a pipet or volumetric flask rinse it with several small portions of the solution whose volume you are measuring. This ensures the removal of any residual liquid remaining in the pipet or volumetric flask.

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2.5: Preparing Solutions

Preparing a solution of known concentration is perhaps the most common activity in any analytical lab. The method for measuring out the solute and the solvent depend on the desired concentration and how exact the solution's concentration needs to be known. Pipets and volumetric flasks are used when we need to know a solution's exact concentration; graduated cylinders, beakers, and/or reagent bottles suffice when a concentrations need only be approximate. Two methods for preparing solutions are described in this section.

Preparing Stock Solutions

A ***stock solution*** is prepared by weighing out an appropriate portion of a pure solid or by measuring out an appropriate volume of a pure liquid, placing it in a suitable flask, and diluting to a known volume. Exactly how one measure's the reagent depends on the desired concentration unit. For example, to prepare a solution with a known molarity you weigh out an appropriate mass of the reagent, dissolve it in a portion of solvent, and bring it to the desired volume. To prepare a solution where the solute's concentration is a volume percent, you measure out an appropriate volume of solute and add sufficient solvent to obtain the desired total volume.

Example 2.5.1

Describe how to prepare the following three solutions: (a) 500 mL of approximately 0.20 M NaOH using solid NaOH; (b) 1 L of 150.0 ppm Cu^{2+} using Cu metal; and (c) 2 L of 4% v/v acetic acid using concentrated glacial acetic acid (99.8% w/w acetic acid).

Solution

(a) Because the desired concentration is known to two significant figures, we do not need to measure precisely the mass of NaOH or the volume of solution. The desired mass of NaOH is

$$\frac{0.20 \text{ mol NaOH}}{\text{L}} \times \frac{40.0 \text{ g NaOH}}{\text{mol NaOH}} \times 0.50 \text{ L} = 4.0 \text{ g NaOH}$$

To prepare the solution, place 4.0 grams of NaOH, weighed to the nearest tenth of a gram, in a bottle or beaker and add approximately 500 mL of water.

(b) Since the desired concentration of Cu^{2+} is given to four significant figures, we must measure precisely the mass of Cu metal and the final solution volume. The desired mass of Cu metal is

$$\frac{150.0 \text{ mg Cu}}{\text{L}} \times 1.000 \text{ M} \times \frac{1 \text{ g}}{1000 \text{ mg}} = 0.1500 \text{ g Cu}$$

To prepare the solution, measure out exactly 0.1500 g of Cu into a small beaker and dissolve it using a small portion of concentrated HNO_3. To ensure a complete transfer of Cu^{2+} from the beaker to the volumetric flask—what we call a ***quantitative transfer***—rinse the beaker several times with small portions of water, adding each rinse to the volumetric flask. Finally, add additional water to the volumetric flask's calibration mark.

(c) The concentration of this solution is only approximate so it is not necessary to measure exactly the volumes, nor is it necessary to account for the fact that glacial acetic acid is slightly less than 100% w/w acetic acid (it is approximately 99.8% w/w). The necessary volume of glacial acetic acid is

$$\frac{4 \text{ mL } CH_3COOH}{100 \text{ mL}} \times 2000 \text{ mL} = 80 \text{ mL } CH_3COOH$$

To prepare the solution, use a graduated cylinder to transfer 80 mL of glacial acetic acid to a container that holds approximately 2 L and add sufficient water to bring the solution to the desired volume.

Exercise 2.5.1

Provide instructions for preparing 500 mL of 0.1250 M $KBrO_3$.

Answer

Preparing 500 mL of 0.1250 M $KBrO_3$ requires

$$0.5000\ \text{L} \times \frac{0.1250\ \text{mol KBrO}_3}{\text{L}} \times \frac{167.00\ \text{g KBrO}_3}{\text{mol KBrO}_3} = 10.44\ \text{g KBrO}_3$$

Because the concentration has four significant figures, we must prepare the solution using volumetric glassware. Place a 10.44 g sample of $KBrO_3$ in a 500-mL volumetric flask and fill part way with water. Swirl to dissolve the $KBrO_3$ and then dilute with water to the flask's calibration mark.

Preparing Solutions by Dilution

Solutions are often prepared by diluting a more concentrated stock solution. A known volume of the stock solution is transferred to a new container and brought to a new volume. Since the total amount of solute is the same before and after dilution, we know that

$$C_o \times V_o = C_d \times V_d \tag{2.5.1}$$

where C_o is the stock solution's concentration, V_o is the volume of stock solution being diluted, C_d is the dilute solution's concentration, and V_d is the volume of the dilute solution. Again, the type of glassware used to measure V_o and V_d depends on how precisely we need to know the solution's concentration.

Note that Equation 2.5.1 applies only to those concentration units that are expressed in terms of the solution's volume, including molarity, formality, normality, volume percent, and weight-to-volume percent. It also applies to weight percent, parts per million, and parts per billion if the solution's density is 1.00 g/mL. We cannot use Equation 2.5.1 if we express concentration in terms of molality as this is based on the mass of solvent, not the volume of solution. See Rodríquez-López, M.; Carrasquillo, A. *J. Chem. Educ.* **2005**, *82*, 1327-1328 for further discussion.

Example 2.5.2

A laboratory procedure calls for 250 mL of an approximately 0.10 M solution of NH_3. Describe how you would prepare this solution using a stock solution of concentrated NH_3 (14.8 M).

Solution

Substituting known values into Equation 2.5.1

$$14.8\ \text{M} \times V_o = 0.10\ \text{M} \times 250\ \text{mL}$$

and solving for V_o gives 1.7 mL. Since we are making a solution that is approximately 0.10 M NH_3, we can use a graduated cylinder to measure the 1.7 mL of concentrated NH_3, transfer the NH_3 to a beaker, and add sufficient water to give a total volume of approximately 250 mL.

Although usually we express molarity as mol/L, we can express the volumes in mL if we do so both for both V_o and V_d.

Exercise 2.5.2

To prepare a standard solution of Zn^{2+} you dissolve a 1.004 g sample of Zn wire in a minimal amount of HCl and dilute to volume in a 500-mL volumetric flask. If you dilute 2.000 mL of this stock solution to 250.0 mL, what is the concentration of Zn^{2+}, in μg/mL, in your standard solution?

Answer

The first solution is a stock solution, which we then dilute to prepare the standard solution. The concentration of Zn^{2+} in the stock solution is

$$\frac{1.004\ \text{g Zn}^{2+}}{500.0\ \text{mL}} \times \frac{10^6\ \mu\text{g}}{\text{g}} = 2008\ \mu\text{g Zn}^{2+}/\text{mL}$$

To find the concentration of the standard solution we use Equation 2.5.1

$$\frac{2008\ \mu g\ Zn^{2+}}{mL} \times 2.000\ mL = C_d \times 250.0\ mL$$

where C_d is the standard solution's concentration. Solving gives a concentration of 16.06 μg Zn^{2+}/mL.

As shown in the following example, we can use Equation 2.5.1 to calculate a solution's original concentration using its known concentration after dilution.

Example 2.5.3

A sample of an ore was analyzed for Cu^{2+} as follows. A 1.25 gram sample of the ore was dissolved in acid and diluted to volume in a 250-mL volumetric flask. A 20 mL portion of the resulting solution was transferred by pipet to a 50-mL volumetric flask and diluted to volume. An analysis of this solution gives the concentration of Cu^{2+} as 4.62 μg/mL. What is the weight percent of Cu in the original ore?

Solution

Substituting known values (with significant figures appropriate for pipets and volumetric flasks) into Equation 2.5.1

$$(C_{Cu})_o \times 20.00\ mL = 4.62\ \mu g/mL\ Cu^{2+} \times 50.00\ mL$$

and solving for $(C_{Cu})_o$ gives the original concentration as 11.55 μg/mL Cu^{2+}. To calculate the grams of Cu^{2+} we multiply this concentration by the total volume

$$\frac{11.55 \mu g\ Cu^{2+}}{mL} \times 250.0\ mL \times \frac{1\ g}{10^6\ \mu g} = 2.888 \times 10^{-3}\ g\ Cu^{2+}$$

The weight percent Cu is

$$\frac{2.888 \times 10^{-3}\ g\ Cu^{2+}}{1.25\ g\ sample} \times 100 = 0.231 Cu^{2+}$$

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2.6: Spreadsheets and Computational Software

Analytical chemistry is a quantitative discipline. Whether you are completing a statistical analysis, trying to optimize experimental conditions, or exploring how a change in pH affects a compound's solubility, the ability to work with complex mathematical equations is essential. Spreadsheets, such as Microsoft Excel are an important tool for analyzing your data and for preparing graphs of your results. Scattered throughout this textbook you will find instructions for using spreadsheets.

> If you do not have access to Microsoft Excel or another commercial spreadsheet package, you might considering using Calc, a freely available open-source spreadsheet that is part of the OpenOffice.org software package at www.openoffice.org, or Google Sheets.

Although spreadsheets are useful, they are not always well suited for working with scientific data. If you plan to pursue a career in chemistry, you may wish to familiarize yourself with a more sophisticated computational software package, such as the freely available open-source program that goes by the name R, or commercial programs such as Mathematica or Matlab. You will find instructions for using R scattered throughout this textbook.

> You can download the current version of R from www.r-project.org. Click on the link for Download: CRAN and find a local mirror site. Click on the link for the mirror site and then use the link for Linux, MacOS X, or Windows under the heading "Download and Install R."

Despite the power of spreadsheets and computational programs, *don't forget that the most important software is behind your eyes and between your ears*. The ability to think intuitively about chemistry is a critically important skill. In many cases you will find that it is possible to determine if an analytical method is feasible or to approximate the optimum conditions for an analytical method without resorting to complex calculations. Why spend time developing a complex spreadsheet or writing software code when a "back-of-the-envelope" estimate will do the trick? Once you know the general solution to your problem, you can use a spreadsheet or a computational program to work out the specifics. Throughout this textbook we will introduce tools to help develop your ability to think intuitively.

> For an interesting take on the importance of intuitive thinking, see *Are You Smart Enough to Work at Google?* by William Poundstone (Little, Brown and Company, New York, 2012).

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2.7: The Laboratory Notebook

Finally, we can not end a chapter on the basic tools of analytical chemistry without mentioning the laboratory notebook. A laboratory notebook is your most important tool when working in the lab. If kept properly, you should be able to look back at your laboratory notebook several years from now and reconstruct the experiments on which you worked.

Your instructor will provide you with detailed instructions on how he or she wants you to maintain your notebook. Of course, you should expect to bring your notebook to the lab. Everything you do, measure, or observe while working in the lab should be recorded in your notebook as it takes place. Preparing data tables to organize your data will help ensure that you record the data you need, and that you can find the data when it is time to calculate and analyze your results. Writing a narrative to accompany your data will help you remember what you did, why you did it, and why you thought it was significant. Reserve space for your calculations, for analyzing your data, and for interpreting your results. Take your notebook with you when you do research in the library.

Maintaining a laboratory notebook may seem like a great deal of effort, but if you do it well you will have a permanent record of your work. Scientists working in academic, industrial and governmental research labs rely on their notebooks to provide a written record of their work. Questions about research carried out at some time in the past can be answered by finding the appropriate pages in the laboratory notebook. A laboratory notebook is also a legal document that helps establish patent rights and proof of discovery.

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2.8: Problems

1. Indicate how many significant figures are in each of the following numbers.
   a. 903 b. 0.903 c. 1.0903
   d. 0.0903 e. 0.09030 f. 9.03×10^2
2. Round each of the following to three significant figures.
   a. 0.89377 b. 0.89328 c. 0.89350
   d. 0.8997 e. 0.08907
3. Round each to the stated number of significant figures.
   a. the atomic weight of carbon to 4 significant figures b. the atomic weight of oxygen to 3 significant figures
   c. Avogadro's number to 4 significant figures d. Faraday's constant to 3 significant figures
4. Report results for the following calculations to the correct number of significant figures.
   a. 4.591 + 0.2309 + 67.1 = b. 313 – 273.15 = c. 712 × 8.6 =
   d. 1.43/0.026 = e. (8.314 × 298)/96 485 = f. $\log(6.53\times10^{-5})$ =
   g. $10^{-7.14}$ = h. $(6.51 \times 10^{-5}) \times (8.14 \times 10^{-9})$
5. A 12.1374 g sample of an ore containing Ni and Co is carried through Fresenius' analytical scheme, as shown in Figure 1.1.1. At point A the combined mass of Ni and Co is 0.2306 g, while at point B the mass of Co is 0.0813 g. Report the weight percent Ni in the ore to the correct number of significant figures.
6. Figure 1.1.2 shows an analytical method for the analysis of Ni in ores based on the precipitation of Ni^{2+} using dimethylglyoxime. The formula for the precipitate is $Ni(C_4H_7N_2O_2)_2$. Calculate the precipitate's formula weight to the correct number of significant figures.
7. An analyst wishes to add 256 mg of Cl^- to a reaction mixture. How many mL of 0.217 M $BaCl_2$ is this?
8. The concentration of lead in an industrial waste stream is 0.28 ppm. What is its molar concentration?
9. Commercially available concentrated hydrochloric acid is 37.0% w/w HCl. Its density is 1.18 g/mL. Using this information calculate (a) the molarity of concentrated HCl, and (b) the mass and volume, in mL, of a solution that contains 0.315 moles of HCl.
10. The density of concentrated ammonia, which is 28.0% w/w NH_3, is 0.899 g/mL. What volume of this reagent should you dilute to 1.0×10^3 mL to make a solution that is 0.036 M in NH_3?
11. A 250.0 mL aqueous solution contains 45.1 μg of a pesticide. Express the pesticide's concentration in weight-to-volume percent, in parts per million, and in parts per billion.
12. A city's water supply is fluoridated by adding NaF. The desired concentration of F^- is 1.6 ppm. How many mg of NaF should you add per gallon of treated water if the water supply already is 0.2 ppm in F^-?
13. What is the pH of a solution for which the concentration of H^+ is 6.92×10^{-6} M? What is the $[H^+]$ in a solution whose pH is 8.923?
14. When using a graduated cylinder, the absolute accuracy with which you can deliver a given volume is ±1% of the cylinder's maximum volume. What are the absolute and the relative uncertainties if you deliver 15 mL of a reagent using a 25 mL graduated cylinder? Repeat for a 50 mL graduated cylinder.
15. Calculate the molarity of a potassium dichromate solution prepared by placing 9.67 grams of $K_2Cr_2O_7$ in a 100-mL volumetric flask, dissolving, and diluting to the calibration mark.
16. For each of the following explain how you would prepare 1.0 L of a solution that is 0.10 M in K^+. Repeat for concentrations of 1.0×10^2 ppm K^+ and 1.0% w/v K^+.
    a. KCl b. K_2SO_4 c. $K_3Fe(CN)_6$
17. A series of dilute NaCl solutions are prepared starting with an initial stock solution of 0.100 M NaCl. Solution A is prepared by pipeting 10 mL of the stock solution into a 250-mL volumetric flask and diluting to volume. Solution B is prepared by pipeting 25 mL of solution A into a 100-mL volumetric flask and diluting to volume. Solution C is prepared by pipeting 20 mL of solution B into a 500-mL volumetric flask and diluting to volume. What is the molar concentration of NaCl in solutions A, B and C?
18. Calculate the molar concentration of NaCl, to the correct number of significant figures, if 1.917 g of NaCl is placed in a beaker and dissolved in 50 mL of water measured with a graduated cylinder. If this solution is quantitatively transferred to a 250-mL volumetric flask and diluted to volume, what is its concentration to the correct number of significant figures?

19. What is the molar concentration of NO_3^- in a solution prepared by mixing 50.0 mL of 0.050 M KNO_3 with 40.0 mL of 0.075 M $NaNO_3$? What is pNO_3 for the mixture?
20. What is the molar concentration of Cl^- in a solution prepared by mixing 25.0 mL of 0.025 M NaCl with 35.0 mL of 0.050 M $BaCl_2$? What is pCl for the mixture?
21. To determine the concentration of ethanol in cognac a 5.00 mL sample of the cognac is diluted to 0.500 L. Analysis of the diluted cognac gives an ethanol concentration of 0.0844 M. What is the molar concentration of ethanol in the undiluted cognac?

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2.9: Additional Resources

The following two web sites contain useful information about the SI system of units.

- http://www.bipm.org/en/home/ – The home page for the Bureau International des Poids and Measures.
- http://physics.nist.gov/cuu/Units/index.html – The National Institute of Standards and Technology's introduction to SI units.

For a chemist's perspective on the SI units for mass and amount, consult the following papers.

- Davis, R. S. "What is a Kilogram in the Revised International System of Units (SI)?", *J. Chem. Educ.* **2015**, *92*, 1604–1609.
- Freeman, R. D. "SI for Chemists: Persistent Problems, Solid Solutions," *J. Chem. Educ.* **2003**, *80*, 16–20.
- Gorin, G. "Mole, Mole per Liter, and Molar: A Primer on SI and Related Units for Chemistry Students," *J. Chem. Educ.* **2003**, *80*, 103–104.

Discussions regarding possible changes in the SI base units are reviewed in these articles.

- Chao, L. S.; Schlamminger, S.; Newell, D. B.; Pratt, J. R.; Seifert, F.; Zhang, X.; Sineriz, M. L.; Haddad, D. "A LEGO Watt Balance: An Apparatus to Determine a Mass Based on the New SI," arXiv:1412.1699 [physics.ins-det].
- Fraundorf, P. "A Multiple of 12 for Avogadro," arXiv:1201.5537 [physics.gen-ph].
- Kemsley, J. "Rethinking the Mole and Kilogram," *C&E News*, August 25, 2014, p. 25.

The following are useful resources for maintaining a laboratory notebook and for preparing laboratory reports.

- Coghill, A. M.; Garson, L. M. (eds) *The ACS Style Guide: Effective Communication of Scientific Information*, 3rd Edition, American Chemical Society: Washington, D. C.; 2006.
- Kanare, H. M. *Writing the Laboratory Notebook*, American Chemical Society: Washington, D. C.; 1985.

The following texts provide instructions for using spreadsheets in analytical chemistry.

- de Levie, R. *How to Use Excel® in Analytical Chemistry and in General Scientific Data Analysis*, Cambridge University Press: Cambridge, UK, 2001.
- Diamond, D.; Hanratty, V. C. A., *Spreadsheet Applications in Chemistry*, Wiley-Interscience: New York, 1997.
- Feiser, H. *Concepts and Calculations in Analytical Chemistry: A Spreadsheet Approach*, CRC Press: Boca Raton, FL, 1992.

The following classic textbook emphasizes the application of intuitive thinking to the solving of problems.

- Harte, J. *Consider a Spherical Cow: A Course in Environmental Problem Solving*, University Science Books: Sausalito, CA, 1988.

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2.10: Chapter Summary and Key Terms

Chapter Summary

There are a few basic numerical and experimental tools with which you must be familiar. Fundamental measurements in analytical chemistry, such as mass, use base SI units, such as the kilogram. Other units, such as energy, are defined in terms of these base units. When reporting a measurement, we must be careful to include only those digits that are significant, and to maintain the uncertainty implied by these significant figures when trans- forming measurements into results.

The relative amount of a constituent in a sample is expressed as a concentration. There are many ways to express concentration, the most common of which are molarity, weight percent, volume percent, weight-to-volume percent, parts per million and parts per billion. Concentrations also can be expressed using p-functions.

Stoichiometric relationships and calculations are important in many quantitative analyses. The stoichiometry between the reactants and the products of a chemical reaction are given by the coefficients of a balanced chemical reaction.

Balances, volumetric flasks, pipets, and ovens are standard pieces of equipment that you will use routinely in the analytical lab. You should be familiar with the proper way to use this equipment. You also should be familiar with how to prepare a stock solution of known concentration, and how to prepare a dilute solution from a stock solution.

Key Terms

analytical balance	concentration	desiccant
desiccator	dilution	formality
graduated cylinder	meniscus	molality
molarity	normality	parts per million
parts per billion	p-function	quantitative transfer
scientific notation	significant figures	SI units
stock solution	tare	volume percent
volumetric flask	volumetric pipet	weight percent
weight-to-volume percent		

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CHAPTER OVERVIEW

3: Evaluating Analytical Data

When we use an analytical method we make three separate evaluations of experimental error. First, before we begin the analysis we evaluate potential sources of errors to ensure they will not adversely effect our results. Second, during the analysis we monitor our measurements to ensure that errors remain acceptable. Finally, at the end of the analysis we evaluate the quality of the measurements and results, and compare them to our original design criteria. This chapter provides an introduction to sources of error, to evaluating errors in analytical measurements, and to the statistical analysis of data.

3.1: Characterizing Measurements and Results
3.2: Characterizing Experimental Errors
3.3: Propagation of Uncertainty
3.4: The Distribution of Measurements and Results
3.5: Statistical Analysis of Data
3.6: Statistical Methods for Normal Distributions
3.7: Detection Limits
3.8: Using Excel and R to Analyze Data
3.9: Problems
3.10: Additional Resources
3.11: Chapter Summary and Key Terms

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3.1: Characterizing Measurements and Results

Let's begin by choosing a simple quantitative problem that requires a single measurement: What is the mass of a penny? You probably recognize that our statement of the problem is too broad. For example, are we interested in the mass of a United States penny or of a Canadian penny, or is the difference relevant? Because a penny's composition and size may differ from country to country, let's narrow our problem to pennies from the United States.

There are other concerns we might consider. For example, the United States Mint produces pennies at two locations (Figure 4.1.1). Because it seems unlikely that a penny's mass depends on where it is minted, we will ignore this concern. Another concern is whether the mass of a newly minted penny is different from the mass of a circulating penny. Because the answer this time is not obvious, let's further narrow our question and ask "What is the mass of a circulating United States Penny?"



Figure 4.1.1 : An uncirculated 2005 Lincoln head penny. The "D" below the date indicates that this penny was produced at the United States Mint at Denver, Colorado. Pennies produced at the Philadelphia Mint do not have a letter below the date. Source: United States Mint image.

A good way to begin our analysis is to gather some preliminary data. Table 4.1.1 shows masses for seven pennies collected from my change jar. In examining this data we see that our question does not have a simple answer. That is, we can not use the mass of a single penny to draw a specific conclusion about the mass of any other penny (although we might reasonably conclude that all pennies weigh at least 3 g). We can, however, characterize this data by reporting the spread of the individual measurements around a central value.

Table 4.1.1 : Masses of Seven Circulating U. S. Pennies

Penny	Mass (g)
1	3.080
2	3.094
3	3.107
4	3.056
5	3.112
6	3.174
7	3.198

Measures of Central Tendency

One way to characterize the data in Table 4.1.1 is to assume that the masses of individual pennies are scattered randomly around a central value that is the best estimate of a penny's expected, or "true" mass. There are two common ways to estimate central tendency: the mean and the median.

Mean

The ***mean***, $\overline{X}$, is the numerical average for a data set. We calculate the mean by dividing the sum of the individual values by the size of the data set

$$\overline{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

where X_i is the i^{th} measurement, and n is the size of the data set.

Example 4.1.1

What is the mean for the data in Table 4.1.1 ?

Solution

To calculate the mean we add together the results for all measurements

$$3.080 + 3.094 + 3.107 + 3.056 + 3.112 + 3.174 + 3.198 = 21.821 \text{ g}$$

and divide by the number of measurements

$$\overline{X} = \frac{21.821 \text{ g}}{7} = 3.117 \text{ g}$$

The mean is the most common estimate of central tendency. It is not a robust estimate, however, because a single extreme value—one much larger or much smaller than the remainder of the data—influences strongly the mean's value [Rousseeuw, P. J. *J. Chemom.* **1991**, *5*, 1–20]. For example, if we accidently record the third penny's mass as 31.07 g instead of 3.107 g, the mean changes from 3.117 g to 7.112 g!

> An estimate for a statistical parameter is robust if its value is not affected too much by an unusually large or an unusually small measurement.

Median

The ***median***, $\widetilde{X}$, is the middle value when we order our data from the smallest to the largest value. When the data has an odd number of values, the median is the middle value. For an even number of values, the median is the average of the $n/2$ and the $(n/2) + 1$ values, where n is the size of the data set.

> When $n = 5$, the median is the third value in the ordered data set; for $n = 6$, the median is the average of the third and fourth members of the ordered data set.

Example 4.1.2

What is the median for the data in Table 4.1.1 ?

Solution

To determine the median we order the measurements from the smallest to the largest value

$$3.056 \quad 3.080 \quad 3.094 \quad 3.107 \quad 3.112 \quad 3.174 \quad 3.198$$

Because there are seven measurements, the median is the fourth value in the ordered data; thus, the median is 3.107 g.

As shown by Example 4.1.1 and Example 4.1.2 , the mean and the median provide similar estimates of central tendency when all measurements are comparable in magnitude. The median, however, is a more robust estimate of central tendency because it is less sensitive to measurements with extreme values. For example, if we accidently record the third penny's mass as 31.07 g instead of 3.107 g, the median's value changes from 3.107 g to 3.112 g.

Measures of Spread

If the mean or the median provides an estimate of a penny's expected mass, then the spread of individual measurements about the mean or median provides an estimate of the difference in mass among pennies or of the uncertainty in measuring mass with a balance. Although we often define the spread relative to a specific measure of central tendency, its magnitude is independent of the central value. Although shifting all measurements in the same direction by adding or subtracting a constant value changes the mean or median, it does not change the spread. There are three common measures of spread: the range, the standard deviation, and the variance.

Problem 13 at the end of the chapter asks you to show that this is true.

Range

The ***range***, *w*, is the difference between a data set's largest and smallest values.

$$w = X_{\text{largest}} - X_{\text{smallest}}$$

The range provides information about the total variability in the data set, but does not provide information about the distribution of individual values. The range for the data in Table 4.1.1 is

$$w = 3.198\ \text{g} - 3.056\ \text{g} = 0.142\ \text{g}$$

Standard Deviation

The ***standard deviation***, *s*, describes the spread of individual values about their mean, and is given as

$$s = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \overline{X})^2}{n-1}} \tag{3.1.1}$$

where X_i is one of the *n* individual values in the data set, and $\overline{X}$ is the data set's mean value. Frequently, we report the relative standard deviation, s_r, instead of the absolute standard deviation.

$$s_r = \frac{s}{\overline{X}}$$

The percent relative standard deviation, $\%s_r$, is $s_r \times 100$.

The relative standard deviation is important because it allows for a more meaningful comparison between data sets when the individual measurements differ significantly in magnitude. Consider again the data in Table 4.1.1 . If we multiply each value by 10, the absolute standard deviation will increase by 10 as well; the relative standard deviation, however, is the same.

Example 4.1.3

Report the standard deviation, the relative standard deviation, and the percent relative standard deviation for the data in Table 4.1.1 ?

Solution

To calculate the standard deviation we first calculate the difference between each measurement and the data set's mean value (3.117), square the resulting differences, and add them together to find the numerator of Equation 3.1.1

$$\begin{aligned}
(3.080 - 3.117)^2 &= (-0.037)^2 = 0.001369 \\
(3.094 - 3.117)^2 &= (-0.023)^2 = 0.000529 \\
(3.107 - 3.117)^2 &= (-0.010)^2 = 0.000100 \\
(3.056 - 3.117)^2 &= (-0.061)^2 = 0.003721 \\
(3.112 - 3.117)^2 &= (-0.005)^2 = 0.000025 \\
(3.174 - 3.117)^2 &= (+0.057)^2 = 0.003249 \\
(3.198 - 3.117)^2 &= (+0.081)^2 = \underline{0.006561} \\
&\qquad\qquad\qquad\quad 0.015554
\end{aligned}$$

For obvious reasons, the numerator of Equation 3.1.1 is called a sum of squares. Next, we divide this sum of squares by *n* – 1, where *n* is the number of measurements, and take the square root.

$$s = \sqrt{\frac{0.015554}{7-1}} = 0.051\ \text{g}$$

Finally, the relative standard deviation and percent relative standard deviation are

$$s_r = \frac{0.051\ \text{g}}{3.117\ \text{g}} = 0.016$$

$$\%s_r = (0.016) \times 100 = 1.6\%$$

It is much easier to determine the standard deviation using a scientific calculator with built in statistical functions.

Many scientific calculators include two keys for calculating the standard deviation. One key calculates the standard deviation for a data set of *n* samples drawn from a larger collection of possible samples, which corresponds to Equation 3.1.1. The other key calculates the standard deviation for all possible samples. The latter is known as the population's standard deviation, which we will cover later in this chapter. Your calculator's manual will help you determine the appropriate key for each.

Variance

Another common measure of spread is the ***variance***, which is the square of the standard deviation. We usually report a data set's standard deviation, rather than its variance, because the mean value and the standard deviation share the same unit. As we will see shortly, the variance is a useful measure of spread because its values are additive.

Example 4.1.4

What is the variance for the data in Table 4.1.1 ?

Solution

The variance is the square of the absolute standard deviation. Using the standard deviation from Example 4.1.3 gives the variance as

$$s^2 = (0.051)^2 = 0.0026$$

Exercise 4.1.1

The following data were collected as part of a quality control study for the analysis of sodium in serum; results are concentrations of Na^+ in mmol/L.

140 143 141 137 132 157 143 149 118 145

Report the mean, the median, the range, the standard deviation, and the variance for this data. This data is a portion of a larger data set from Andrew, D. F.; Herzberg, A. M. *Data: A Collection of Problems for the Student and Research Worker*, Springer-Verlag:New York, 1985, pp. 151–155.

Answer

Mean: To find the mean we add together the individual measurements and divide by the number of measurements. The sum of the 10 concentrations is 1405. Dividing the sum by 10, gives the mean as 140.5, or 1.40×10^2 mmol/L.

Median: To find the median we arrange the 10 measurements from the smallest concentration to the largest concentration; thus

118 132 137 140 141 143 143 145 149 157

The median for a data set with 10 members is the average of the fifth and sixth values; thus, the median is (141 + 143)/2, or 142 mmol/L.

Range: The range is the difference between the largest value and the smallest value; thus, the range is 157 – 118 = 39 mmol/L.

Standard Deviation: To calculate the standard deviation we first calculate the absolute difference between each measurement and the mean value (140.5), square the resulting differences, and add them together. The differences are

–0.5 2.5 0.5 –3.5 –8.5 16.5 2.5 8.5 –22.5 4.5

and the squared differences are

$$0.25 \quad 6.25 \quad 0.25 \quad 12.25 \quad 72.25 \quad 272.25 \quad 6.25 \quad 72.25 \quad 506.25 \quad 20.25$$

The total sum of squares, which is the numerator of Equation 3.1.1, is 968.50. The standard deviation is

$$s = \sqrt{\frac{968.50}{10-1}} = 10.37 \approx 10.4$$

Variance: The variance is the square of the standard deviation, or 108.

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3.2: Characterizing Experimental Errors

Characterizing a penny's mass using the data in Table 4.1.1 suggests two questions. First, does our measure of central tendency agree with the penny's expected mass? Second, why is there so much variability in the individual results? The first of these questions addresses the accuracy of our measurements and the second addresses the precision of our measurements. In this section we consider the types of experimental errors that affect accuracy and precision.

Errors That Affect Accuracy

Accuracy is how close a measure of central tendency is to its expected value, μ. We express accuracy either as an absolute error, e

$$e = \overline{X} - \mu \tag{3.2.1}$$

or as a percent relative error, $\%e$

$$\%e = \frac{\overline{X} - \mu}{\mu} \times 100 \tag{3.2.2}$$

Although Equation 3.2.1 and Equation 3.2.2 use the mean as the measure of central tendency, we also can use the median.

> The convention for representing a statistical parameter is to use a Roman letter for a value calculated from experimental data, and a Greek letter for its corresponding expected value. For example, the experimentally determined mean is $\overline{X}$ and its underlying expected value is μ. Likewise, the experimental standard deviation is s and the underlying expected value is σ.

We identify as determinate an error that affects the accuracy of an analysis. Each source of a ***determinate error*** has a specific magnitude and sign. Some sources of determinate error are positive and others are negative, and some are larger in magnitude and others are smaller in magnitude. The cumulative effect of these determinate errors is a net positive or negative error in accuracy.

> It is possible, although unlikely, that the positive and negative determinate errors will offset each other, producing a result with no net error in accuracy.

We assign determinate errors into four categories—sampling errors, method errors, measurement errors, and personal errors—each of which we consider in this section.

Sampling Errors

A determinate sampling error occurs when our sampling strategy does not provide a us with a representative sample. For example, if we monitor the environmental quality of a lake by sampling from a single site near a point source of pollution, such as an outlet for industrial effluent, then our results will be misleading. To determine the mass of a U. S. penny, our strategy for selecting pennies must ensure that we do not include pennies from other countries.

> An awareness of potential sampling errors especially is important when we work with heterogeneous materials. Strategies for obtaining representative samples are covered in Chapter 5.

Method Errors

In any analysis the relationship between the signal, S_{total}, and the absolute amount of analyte, n_A, or the analyte's concentration, C_A, is

$$S_{total} = k_A n_A + S_{mb} \tag{3.2.3}$$

$$S_{total} = k_A C_A + S_{mb} \tag{3.2.4}$$

where k_A is the method's sensitivity for the analyte and S_{mb} is the signal from the method blank. A ***method error*** exists when our value for k_A or for S_{mb} is in error. For example, a method in which S_{total} is the mass of a precipitate assumes that k is defined by a

pure precipitate of known stoichiometry. If this assumption is not true, then the resulting determination of n_A or C_A is inaccurate. We can minimize a determinate error in k_A by calibrating the method. A method error due to an interferent in the reagents is minimized by using a proper method blank.

Measurement Errors

The manufacturers of analytical instruments and equipment, such as glassware and balances, usually provide a statement of the item's maximum ***measurement error***, or ***tolerance***. For example, a 10-mL volumetric pipet (Figure 4.2.1) has a tolerance of ±0.02 mL, which means the pipet delivers an actual volume within the range 9.98–10.02 mL at a temperature of 20 °C. Although we express this tolerance as a range, the error is determinate; that is, the pipet's expected volume, μ, is a fixed value within this stated range.



Figure 4.2.1 : Close-up of a 10-mL volumetric pipet showing that it has a tolerance of ±0.02 mL at 20 °C.

Volumetric glassware is categorized into classes based on its relative accuracy. Class A glassware is manufactured to comply with tolerances specified by an agency, such as the National Institute of Standards and Technology or the American Society for Testing and Materials. The tolerance level for Class A glassware is small enough that normally we can use it without calibration. The tolerance levels for Class B glassware usually are twice that for Class A glassware. Other types of volumetric glassware, such as beakers and graduated cylinders, are not used to measure volume accurately. Table 4.2.1 provides a summary of typical measurement errors for Class A volumetric glassware. Tolerances for digital pipets and for balances are provided in Table 4.2.2 and Table 4.2.3 .

Table 4.2.1 : Measurement Errors for Type A Volumetric Glassware

Transfer Pipets		Volumetric Flasks		Burets	
Capacity (mL)	Tolerance (mL)	Capacity (mL)	Tolerance (mL)	Capacity (mL)	Tolerance (mL)
1	±0.006	5	±0.02	10	±0.02
2	±0.006	10	±0.02	25	±0.03
5	±0.01	25	±0.03	50	±0.05
10	±0.02	50	±0.05		
20	±0.03	100	±0.08		
25	±0.03	250	±0.12		
50	±0.05	500	±0.20		
100	±0.08	1000	±0.30		
		2000			

Table 4.2.2 : Measurement Errors for Digital Pipets

Pipet Range	Volume (mL or μL)	Percent Measurement Error
10–100 μL	10	±3.0%
	50	±1.0%
	100	±0.8%
100–1000 μL	100	±3.0%
	500	±1.0%
	1000	±0.6%
1–10 mL	1	±3.0%
	5	±0.8%
	10	±0.6%

The tolerance values for the volumetric glassware in Table 4.2.1 are from the ASTM E288, E542, and E694 standards. The measurement errors for the digital pipets in Table 4.2.2 are from www.eppendorf.com.

Table 4.2.3 : Measurement Errors for Selected Balances

Balance	Capacity (g)	Measurement Error
Precisa 160M	160	±1 mg
A & D ER 120M	120	±0.1 mg
Metler H54	160	±0.01 mg

We can minimize a determinate measurement error by calibrating our equipment. Balances are calibrated using a reference weight whose mass we can trace back to the SI standard kilogram. Volumetric glassware and digital pipets are calibrated by determining the mass of water delivered or contained and using the density of water to calculate the actual volume. It is never safe to assume that a calibration does not change during an analysis or over time. One study, for example, found that repeatedly exposing volumetric glassware to higher temperatures during machine washing and oven drying, led to small, but significant changes in the glassware's calibration [Castanheira, I.; Batista, E.; Valente, A.; Dias, G.; Mora, M.; Pinto, L.; Costa, H. S. *Food Control* **2006**, *17*, 719–726]. Many instruments drift out of calibration over time and may require frequent recalibration during an analysis.

Personal Errors

Finally, analytical work is always subject to ***personal error***, examples of which include the ability to see a change in the color of an indicator that signals the endpoint of a titration, biases, such as consistently overestimating or underestimating the value on an instrument's readout scale, failing to calibrate instrumentation, and misinterpreting procedural directions. You can minimize personal errors by taking proper care.

Identifying Determinate Errors

Determinate errors often are difficult to detect. Without knowing the expected value for an analysis, the usual situation in any analysis that matters, we often have nothing to which we can compare our experimental result. Nevertheless, there are strategies we can use to detect determinate errors.

The magnitude of a constant ***determinate error*** is the same for all samples and is more significant when we analyze smaller samples. Analyzing samples of different sizes, therefore, allows us to detect a constant determinate error. For example, consider a quantitative analysis in which we separate the analyte from its matrix and determine its mass. Let's assume the sample is 50.0% w/w analyte. As we see in Table 4.2.4 , the expected amount of analyte in a 0.100 g sample is 0.050 g. If the analysis has a positive

constant determinate error of 0.010 g, then analyzing the sample gives 0.060 g of analyte, or an apparent concentration of 60.0% w/w. As we increase the size of the sample the experimental results become closer to the expected result. An upward or downward trend in a graph of the analyte's experimental concentration versus the sample's mass (Figure 4.2.2) is evidence of a constant determinate error.

Table 4.2.4 : Effect of a Constant Determinate Error on the Analysis of a Sample That is 50.0% w/w Analyte

Mass of Sample (g)	Expected Mass of Analyte (g)	Constant Error (g)	Experimental Mass of Analyte (g)	Experimental Concentration of Analyte (% w/w)
0.100	0.050	0.010	0.060	60.0
0.200	0.100	0.010	0.110	55.0
0.400	0.200	0.010	0.210	52.5
0.800	0.400	0.010	0.410	51.2
1.600	0.800	0.010	0.810	50.6

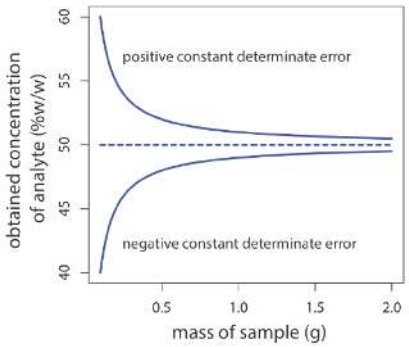


Figure 4.2.2 : Effect of a constant positive determinate error of +0.01 g and a constant negative determinate error of –0.01 g on the determination of an analyte in samples of varying size. The analyte's expected concentration of 50% w/w is shown by the dashed line.

A ***proportional determinate error***, in which the error's magnitude depends on the amount of sample, is more difficult to detect because the result of the analysis is independent of the amount of sample. Table 4.2.5 outlines an example that shows the effect of a positive proportional error of 1.0% on the analysis of a sample that is 50.0% w/w in analyte. Regardless of the sample's size, each analysis gives the same result of 50.5% w/w analyte.

Table 4.2.5 : Effect of a Proportional Determinate Error on the Analysis of a Sample That is 50.0% w/w Analyte

Mass of Sample (g)	Expected Mass of Analyte (g)	Proportional Error (%)	Experimental Mass of Analyte (g)	Experimental Concentration of Analyte (% w/w)
0.100	0.050	1.00	0.0505	50.5
0.200	0.100	1.00	0.101	50.5
0.400	0.200	1.00	0.202	50.5
0.800	0.400	1.00	0.404	50.5
1.600	0.800	1.00	0.808	50.5

One approach for detecting a proportional determinate error is to analyze a standard that contains a known amount of analyte in a matrix similar to our samples. Standards are available from a variety of sources, such as the National Institute of Standards and

Technology (where they are called Standard Reference Materials) or the American Society for Testing and Materials. Table 4.2.6 , for example, lists certified values for several analytes in a standard sample of *Gingko biloba* leaves. Another approach is to compare our analysis to an analysis carried out using an independent analytical method that is known to give accurate results. If the two methods give significantly different results, then a determinate error is the likely cause.

Table 4.2.6 : Certified Concentrations for SRM 3246: *Gingko bilbo* (Leaves)

Class of Analyte	Analyte	Mass Fraction (mg/g or ng/g)
Flavonoids/Ginkgolide B (mass fraction in mg/g)	Qurecetin	2.69 ± 0.31
	Kaempferol	3.02 ± 0.41
	Isorhamnetin	$0.517 \pm 0.0.99$
	Total Aglycones	6.22 ± 0.77
Selected Terpenes (mass fraction in mg/g)	Ginkgolide A	0.57 ± 0.28
	Ginkgolide B	0.470 ± 0.090
	Ginkgolide C	0.59 ± 0.22
	Ginkgolide J	0.18 ± 0.10
	Bilobalide	1.52 ± 0.40
	Total Terpene Lactones	3.3 ± 1.1
Selected Toxic Elements (mass fraction in ng/g)	Cadmium	20.8 ± 1.0
	Lead	995 ± 30
	Mercury	23.08 ± 0.17

> The primary purpose of this Standard Reference Material is to validate analytical methods for determining flavonoids, terpene lactones, and toxic elements in *Ginkgo biloba* or other materials with a similar matrix. Values are from the official Certificate of Analysis available at www.nist.gov.

Constant and proportional determinate errors have distinctly different sources, which we can define in terms of the relationship between the signal and the moles or concentration of analyte (Equation 3.2.3 and Equation 3.2.4). An invalid method blank, S_{mb}, is a constant determinate error as it adds or subtracts the same value to the signal. A poorly calibrated method, which yields an invalid sensitivity for the analyte, k_A, results in a proportional determinate error.

Errors that Affect Precision

As we saw in Section 4.1, precision is a measure of the spread of individual measurements or results about a central value, which we express as a range, a standard deviation, or a variance. Here we draw a distinction between two types of precision: repeatability and reproducibility. ***Repeatability*** is the precision when a single analyst completes an analysis in a single session using the same solutions, equipment, and instrumentation. ***Reproducibility***, on the other hand, is the precision under any other set of conditions, including between analysts or between laboratory sessions for a single analyst. Since reproducibility includes additional sources of variability, the reproducibility of an analysis cannot be better than its repeatability.

> The ratio of the standard deviation associated with reproducibility to the standard deviation associated with repeatability is called the Horowitz ratio. For a wide variety of analytes in foods, for example, the median Horowtiz ratio is 2.0 with larger

values for fatty acids and for trace elements; see Thompson, M.; Wood, R. “The ‘Horowitz Ratio’–A Study of the Ratio Between Reproducibility and Repeatability in the Analysis of Foodstuffs,” *Anal. Methods*, **2015**, *7*, 375–379.

Errors that affect precision are indeterminate and are characterized by random variations in their magnitude and their direction. Because they are random, positive and negative ***indeterminate errors*** tend to cancel, provided that we make a sufficient number of measurements. In such situations the mean and the median largely are unaffected by the precision of the analysis.

Sources of Indeterminate Error

We can assign indeterminate errors to several sources, including collecting samples, manipulating samples during the analysis, and making measurements. When we collect a sample, for instance, only a small portion of the available material is taken, which increases the chance that small-scale inhomogeneities in the sample will affect repeatability. Individual pennies, for example, may show variations in mass from several sources, including the manufacturing process and the loss of small amounts of metal or the addition of dirt during circulation. These variations are sources of indeterminate sampling errors.

During an analysis there are many opportunities to introduce indeterminate method errors. If our method for determining the mass of a penny includes directions for cleaning them of dirt, then we must be careful to treat each penny in the same way. Cleaning some pennies more vigorously than others might introduce an indeterminate method error.

Finally, all measuring devices are subject to indeterminate measurement errors due to limitations in our ability to read its scale. For example, a buret with scale divisions every 0.1 mL has an inherent indeterminate error of ±0.01–0.03 mL when we estimate the volume to the hundredth of a milliliter (Figure 4.2.3).

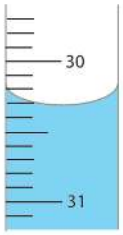


Figure 4.2.3 : Close-up of a buret showing the difficulty in estimating volume. With scale divisions every 0.1 mL it is difficult to read the actual volume to better than ±0.01–0.03 mL.

Evaluating Indeterminate Error

Indeterminate errors associated with our analytical equipment or instrumentation generally are easy to estimate if we measure the standard deviation for several replicate measurements, or if we monitor the signal’s fluctuations over time in the absence of analyte (Figure 4.2.4) and calculate the standard deviation. Other sources of indeterminate error, such as treating samples inconsistently, are more difficult to estimate.

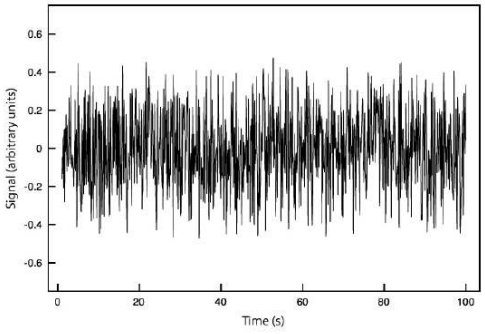


Figure 4.2.4 : Background noise in an instrument showing the random fluctuations in the signal.

To evaluate the effect of an indeterminate measurement error on our analysis of the mass of a circulating United States penny, we might make several determinations of the mass for a single penny (Table 4.2.7). The standard deviation for our original experiment (see Table 4.1.1) is 0.051 g, and it is 0.0024 g for the data in Table 4.2.7 . The significantly better precision when we determine the

mass of a single penny suggests that the precision of our analysis is not limited by the balance. A more likely source of indeterminate error is a variability in the masses of individual pennies.

Table 4.2.7 : Replicate Determinations of the Mass of a Single Circulating U. S. Penny

Replicate	Mass (g)	Replicate	Mass (g)
1	3.025	6	3.023
2	3.024	7	3.022
3	3.028	8	3.021
4	3.027	9	3.026
5	3.028	10	3.024

In Section 4.5 we will discuss a statistical method—the *F*-test—that you can use to show that this difference is significant.

Error and Uncertainty

Analytical chemists make a distinction between error and uncertainty [Ellison, S.; Wegscheider, W.; Williams, A. *Anal. Chem.* **1997**, *69*, 607A–613A]. ***Error*** is the difference between a single measurement or result and its expected value. In other words, error is a measure of ***bias***. As discussed earlier, we divide errors into determinate and indeterminate sources. Although we can find and correct a source of determinate error, the indeterminate portion of the error remains.

Uncertainty expresses the range of possible values for a measurement or result. Note that this definition of uncertainty is not the same as our definition of precision. We calculate precision from our experimental data and use it to estimate the magnitude of indeterminate errors. Uncertainty accounts for all errors—both determinate and indeterminate—that reasonably might affect a measurement or a result. Although we always try to correct determinate errors before we begin an analysis, the correction itself is subject to uncertainty.

Here is an example to help illustrate the difference between precision and uncertainty. Suppose you purchase a 10-mL Class A pipet from a laboratory supply company and use it without any additional calibration. The pipet's tolerance of ±0.02 mL is its uncertainty because your best estimate of its expected volume is 10.00 mL ± 0.02 mL. This uncertainty primarily is determinate. If you use the pipet to dispense several replicate samples of a solution and determine the volume of each sample, the resulting standard deviation is the pipet's precision. Table 4.2.8 shows results for ten such trials, with a mean of 9.992 mL and a standard deviation of ±0.006 mL. This standard deviation is the precision with which we expect to deliver a solution using a Class A 10-mL pipet. In this case the pipet's published uncertainty of ±0.02 mL is worse than its experimentally determined precision of ±0.006 ml. Interestingly, the data in Table 4.2.8 allows us to calibrate this specific pipet's delivery volume as 9.992 mL. If we use this volume as a better estimate of the pipet's expected volume, then its uncertainty is ±0.006 mL. As expected, calibrating the pipet allows us to decrease its uncertainty [Kadis, R. *Talanta* **2004**, *64*, 167–173].

Table 4.2.8 : Experimental Results for Volume Dispensed by a 10–mL Class A Transfer Pipet

Replicate	Volume (ml)	Replicate	Volume (mL)
1	10.002	6	9.983
2	9.993	7	9.991
3	9.984	8	9.990
4	9.996	9	9.988
5	9.989	10	9.999

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3.3: Propagation of Uncertainty

Suppose we dispense 20 mL of a reagent using the Class A 10-mL pipet whose calibration information is given in Table 4.2.8. If the volume and uncertainty for one use of the pipet is 9.992 ± 0.006 mL, what is the volume and uncertainty if we use the pipet twice?

As a first guess, we might simply add together the volume and the maximum uncertainty for each delivery; thus

$$(9.992 \text{ mL} + 9.992 \text{ mL}) \pm (0.006 \text{ mL} + 0.006 \text{ mL}) = 19.984 \pm 0.012 \text{ mL}$$

It is easy to appreciate that combining uncertainties in this way overestimates the total uncertainty. Adding the uncertainty for the first delivery to that of the second delivery assumes that with each use the indeterminate error is in the same direction and is as large as possible. At the other extreme, we might assume that the uncertainty for one delivery is positive and the other is negative. If we subtract the maximum uncertainties for each delivery,

$$(9.992 \text{ mL} + 9.992 \text{ mL}) \pm (0.006 \text{ mL} - 0.006 \text{ mL}) = 19.984 \pm 0.000 \text{ mL}$$

we clearly underestimate the total uncertainty.

So what is the total uncertainty? From the discussion above, we reasonably expect that the total uncertainty is greater than ±0.000 mL and that it is less than ±0.012 mL. To estimate the uncertainty we use a mathematical technique known as the propagation of uncertainty. Our treatment of the propagation of uncertainty is based on a few simple rules.

A Few Symbols

A ***propagation of uncertainty*** allows us to estimate the uncertainty in a result from the uncertainties in the measurements used to calculate that result. For the equations in this section we represent the result with the symbol R, and we represent the measurements with the symbols A, B, and C. The corresponding uncertainties are u_R, u_A, u_B, and u_C. We can define the uncertainties for A, B, and C using standard deviations, ranges, or tolerances (or any other measure of uncertainty), as long as we use the same form for all measurements.

> The requirement that we express each uncertainty in the same way is a critically important point. Suppose you have a range for one measurement, such as a pipet's tolerance, and standard deviations for the other measurements. All is not lost. There are ways to convert a range to an estimate of the standard deviation. See Appendix 2 for more details.

Uncertainty When Adding or Subtracting

When we add or subtract measurements we propagate their absolute uncertainties. For example, if the result is given by the equation

$$R = A + B - C$$

the the absolute uncertainty in R is

$$u_R = \sqrt{u_A^2 + u_B^2 + u_C^2} \tag{3.3.1}$$

Example 3.3.1

If we dispense 20 mL using a 10-mL Class A pipet, what is the total volume dispensed and what is the uncertainty in this volume? First, complete the calculation using the manufacturer's tolerance of 10.00 mL±0.02 mL, and then using the calibration data from Table 4.2.8.

Solution

To calculate the total volume we add the volumes for each use of the pipet. When using the manufacturer's values, the total volume is

$$V = 10.00 \text{ mL} + 10.00 \text{ mL} = 20.00 \text{ mL}$$

and when using the calibration data, the total volume is

$$V = 9.992 \text{ mL} + 9.992 \text{ mL} = 19.984 \text{ mL}$$

Using the pipet's tolerance as an estimate of its uncertainty gives the uncertainty in the total volume as

$$u_R = (0.02)^2 + (0.02)^2 = 0.028 \text{ mL} = 0.028 \text{ mL}$$

and using the standard deviation for the data in Table 4.2.8 gives an uncertainty of

$$u_R = (0.006)^2 + (0.006)^2 = 0.0085 \text{ mL}$$

Rounding the volumes to four significant figures gives 20.00 mL ± 0.03 mL when we use the tolerance values, and 19.98 ± 0.01 mL when we use the calibration data.

Uncertainty When Multiplying or Dividing

When we multiple or divide measurements we propagate their relative uncertainties. For example, if the result is given by the equation

$$R = \frac{A \times B}{C}$$

then the relative uncertainty in R is

$$\frac{u_R}{R} \sqrt{\left(\frac{u_A}{A}\right)^2 + \left(\frac{u_B}{B}\right)^2 + \left(\frac{u_C}{C}\right)^2} \tag{3.3.2}$$

Example 3.3.2

The quantity of charge, Q, in coulombs that passes through an electrical circuit is

$$Q = i \times t$$

where i is the current in amperes and t is the time in seconds. When a current of 0.15 A ± 0.01 A passes through the circuit for 120 s ± 1 s, what is the total charge and its uncertainty?

Solution

The total charge is

$$Q = (0.15 \text{ A}) \times (120 \text{ s}) = 18 \text{ C}$$

Since charge is the product of current and time, the relative uncertainty in the charge is

$$u_R = \sqrt{\left(\frac{0.01}{0.15}\right)^2 + \left(\frac{1}{120}\right)^2} = 0.0672$$

and the charge's absolute uncertainty is

$$u_R = R \times 0.0672 = (18 \text{ C}) \times (0.0672) = 1.2 \text{ C}$$

Thus, we report the total charge as 18 C ± 1 C.

Uncertainty for Mixed Operations

Many chemical calculations involve a combination of adding and subtracting, and of multiply and dividing. As shown in the following example, we can calculate the uncertainty by separately treating each operation using Equation 3.3.1 and Equation 3.3.2 as needed.

Example 3.3.3

For a concentration technique, the relationship between the signal and the an analyte's concentration is

$$S_{total} = k_A C_A + S_{mb}$$

What is the analyte's concentration, C_A, and its uncertainty if S_{total} is 24.37 ± 0.02, S_{mb} is 0.96 ± 0.02, and k_A is $0.186 \pm 0.003 \text{ ppm}^{-1}$?

Solution

Rearranging the equation and solving for C_A

$$C_A = \frac{S_{total} - S_{mb}}{k_A} = \frac{24.37 - 0.96}{0.186 \text{ ppm}^{-1}} = \frac{23.41}{0.186 \text{ ppm}^{-1}} = 125.9 \text{ ppm}$$

gives the analyte's concentration as 126 ppm. To estimate the uncertainty in C_A, we first use Equation 3.3.1 to determine the uncertainty for the numerator.

$$u_R = \sqrt{(0.02)^2 + (0.02)^2} = 0.028$$

The numerator, therefore, is 23.41 ± 0.028. To complete the calculation we use Equation 3.3.2 to estimate the relative uncertainty in C_A.

$$\frac{u_R}{R} = \sqrt{\left(\frac{0.028}{23.41}\right)^2 + \left(\frac{0.003}{0.186}\right)^2} = 0.0162$$

The absolute uncertainty in the analyte's concentration is

$$u_R = (125.9 \text{ ppm}) \times (0.0162) = 2.0 \text{ ppm}$$

Thus, we report the analyte's concentration as 126 ppm ± 2 ppm.

Exercise 3.3.1

To prepare a standard solution of Cu^{2+} you obtain a piece of copper from a spool of wire. The spool's initial weight is 74.2991 g and its final weight is 73.3216 g. You place the sample of wire in a 500-mL volumetric flask, dissolve it in 10 mL of HNO_3, and dilute to volume. Next, you pipet a 1 mL portion to a 250-mL volumetric flask and dilute to volume. What is the final concentration of Cu^{2+} in mg/L, and its uncertainty? Assume that the uncertainty in the balance is ±0.1 mg and that you are using Class A glassware.

Answer

The first step is to determine the concentration of Cu^{2+} in the final solution. The mass of copper is

$$74.2991 \text{ g} - 73.3216 \text{ g} = 0.9775 \text{ g Cu}$$

The 10 mL of HNO_3 used to dissolve the copper does not factor into our calculation. The concentration of Cu^{2+} is

$$\frac{0.9775 \text{ g Cu}}{0.5000 \text{ L}} \times \frac{1.000 \text{ mL}}{250.0 \text{ mL}} \times \frac{1000 \text{ mg}}{\text{g}} = 7.820 \text{ mg } Cu^{2+}/\text{L}$$

Having found the concentration of Cu^{2+}, we continue with the propagation of uncertainty. The absolute uncertainty in the mass of Cu wire is

$$u_{\text{g Cu}} = \sqrt{(0.0001)^2 + (0.0001)^2} = 0.00014 \text{ g}$$

The relative uncertainty in the concentration of Cu^{2+} is

$$\frac{u_{\text{mg/L}}}{7.820 \text{ mg/L}} = \sqrt{\left(\frac{0.00014}{0.9775}\right)^2 + \left(\frac{0.20}{500.0}\right)^2 + \left(\frac{0.006}{1.000}\right)^2 + \left(\frac{0.12}{250.0}\right)^2} = 0.00603$$

Solving for $u_{\text{mg/L}}$ gives the uncertainty as 0.0472. The concentration and uncertainty for Cu^{2+} is 7.820 mg/L ± 0.047 mg/L.

Uncertainty for Other Mathematical Functions

Many other mathematical operations are common in analytical chemistry, including the use of powers, roots, and logarithms. Table **3.3.1** provides equations for propagating uncertainty for some of these function where A and B are independent measurements and where k is a constant whose value has no uncertainty.

Table 3.3.1: Propagation of Uncertainty for Selected Mathematical Functions

Function	u_R	Function	u_R
$R = kA$	$u_R = ku_A$	$R = \ln(A)$	$u_R = \frac{u_A}{A}$
$R = A + B$	$u_R = \sqrt{u_A^2 + u_B^2}$	$R = \log(A)$	$u_R = 0.4343 \times \frac{u_A}{A}$
$R = A - B$	$u_R = \sqrt{u_A^2 + u_B^2}$	$R = e^A$	$\frac{u_R}{R} = u_A$
$R = A \times B$	$u_R = \sqrt{\left(\frac{u_A}{A}\right)^2 + \left(\frac{u_B}{B}\right)^2}$	$R = 10^A$	$\frac{u_R}{R} = 2.303 \times u_A$
$R = \frac{A}{B}$	$u_R = \sqrt{\left(\frac{u_A}{A}\right)^2 + \left(\frac{u_B}{B}\right)^2}$	$R = A^k$	$\frac{u_R}{R} = k \times \frac{u_A}{A}$

Example 3.3.4

If the pH of a solution is 3.72 with an absolute uncertainty of ±0.03, what is the [H^+] and its uncertainty?

Solution

The concentration of H^+ is

$$[H^+] = 10^{-pH} = 10^{-3.72} = 1.91 \times 10^{-4}\ M$$

or 1.9×10^{-4} M to two significant figures. From Table 3.3.1 the relative uncertainty in [H^+] is

$$\frac{u_R}{R} = 2.303 \times u_A = 2.303 \times 0.03 = 0.069$$

The uncertainty in the concentration, therefore, is

$$(1.91 \times 10^{-4}\ M) \times (0.069) = 1.3 \times 10^{-5}\ M$$

We report the [H^+] as $1.9(\pm 0.1) \times 10^{-4}$ M, which is equivalent to $1.9 \times 10^{-4}\ M\ \pm 0.1 \times 10^{-4}\ M$.

Exercise 3.3.2

A solution of copper ions is blue because it absorbs yellow and orange light. Absorbance, *A*, is defined as

$$A = -\log T = -\log\left(\frac{P}{P_o}\right)$$

where, *T* is the transmittance, P_o is the power of radiation as emitted from the light source and *P* is its power after it passes through the solution. What is the absorbance if P_o is 3.80×10^2 and *P* is 1.50×10^2? If the uncertainty in measuring P_o and *P* is 15, what is the uncertainty in the absorbance?

Answer

The first step is to calculate the absorbance, which is

$$A = -\log T = -\log\frac{P}{P_o} = -\log\frac{1.50 \times 10^2}{3.80 \times 10^2} = 0.4037 \approx 0.404$$

Having found the absorbance, we continue with the propagation of uncertainty. First, we find the uncertainty for the ratio P/P_o, which is the transmittance, T.

$$\frac{u_T}{T} = \sqrt{\left(\frac{15}{3.80 \times 10^2}\right)^2 + \left(\frac{15}{1.50 \times 10^2}\right)^2} = 0.1075$$

Finally, from Table 3.3.1 the uncertainty in the absorbance is

$$u_A = 0.4343 \times \frac{u_T}{T} = (0.4343) \times (0.1075) = 4.669 \times 10^{-2}$$

The absorbance and uncertainty is 0.40 ± 0.05 absorbance units.

The Basis Behind the Equations for the Propagation of Error and Extension to other Calculated Results

Now let's look at a general case of x = fn(p,q,r,…) and we assume p, q, r,… can fluctuate randomly and be treated as independent variables.

Then for any individual measurement of x, say x_i then

$$dx_i = fn\,(\partial p_i, \partial q_i, \partial r_i, ...)$$

Now from calculus we know that the total variation in x as a function of the variations in p,q,r,… comes from the partial derivative of x with respect to each variable, p,q,r,… so

$$dx = \left(\frac{\partial x}{\partial p}\right)_v dp + \left(\frac{\partial x}{\partial q}\right)_v dq + \left(\frac{\partial x}{\partial r}\right)_v dr + ...$$

To develop a relationship between the standard deviation of x; (s_x) and the standard deviations of p,q,r,…; (s_p,s_q,s_r,…) we square the above expression and sum over i = 1 to n values

$$\sum_{i=1}^{n} (dx_i)^2 = \sum_{i=1}^{n} \left(\left(\frac{\partial x}{\partial p}\right)_v dp_i + \left(\frac{\partial x}{\partial q}\right)_v dq_i + \left(\frac{\partial x}{\partial r}\right)_v dr_i + ...\right)^2$$

Expanding the right hand side of this last equation we will get

square terms are always positive and always sum to a positive value, such as

$$\left(\frac{\partial x}{\partial p}\right)^2 (dp)^2; \left(\frac{\partial x}{\partial q}\right)^2 (dq)^2; \text{ etc}$$

and **cross terms,** such as the one shown below, can be positive or negative and that will sum to 0 as n get large and as long as the variables are independent

$$\left(\frac{\partial x}{\partial p}\right)\left(\frac{\partial x}{\partial q}\right) dpdq$$

Focusing only on the square terms and the sum of those terms for i = 1 to n

$$\sum_{i=1}^{n} (dx_i)^2 = \left(\frac{\partial x}{\partial p}\right)^2 \sum_{i=1}^{n} (dp_i)^2 + \left(\frac{\partial x}{\partial q}\right)^2 \sum_{i=1}^{n} (dq_i)^2 + \left(\frac{\partial x}{\partial r}\right)^2 \sum_{i=1}^{n} (dr_i)^2 + ...$$

If we now divide each term by n-1 then we have

$$\frac{\sum_{i=1}^{n}(dx_i)^2}{n-1} = \frac{\left(\frac{\partial x}{\partial p}\right)^2 \sum_{i=1}^{n}(dp_i)^2}{n-1} + \frac{\left(\frac{\partial x}{\partial q}\right)^2 \sum_{i=1}^{n}(dq_i)^2}{n-1} + \frac{\left(\frac{\partial x}{\partial r}\right)^2 \sum_{i=1}^{n}(dr_i)^2}{n-1} + ...$$

And if we note that each term $(dx_i)^2 = (x_i - \bar{x})^2$ and likewise $(dq_i)^2 = (q_i - \bar{q})^2$

then we get the important and generally applicable result in terms of the variances (u^2)

$$u_x^2 = \left(\frac{\partial x}{\partial p}\right)^2 u_p^2 + \left(\frac{\partial x}{\partial q}\right)^2 u_q^2 + \left(\frac{\partial x}{\partial r}\right)^2 u_r^2 + ...$$

Example 3.3.5:

Question: What is the uncertainty in the volume of a rectangular solid that has a base = 2.00 +/- 0.05 cm on a side and a height = 5.50 +/- 0.10 cm?

Answer: In this case $V = b^2h$ and $V = (2.00)^2 \times 5.50 = 22.0\ cm^3$

To get the uncertainty we get the partial derivatives of V with respect ot b and h

$\left(\frac{\partial V}{\partial b}\right)_h = 2bh$ and $\left(\frac{\partial V}{\partial h}\right)_b = b^2$

so following our general result

$$u_V^2 = \left(\frac{\partial V}{\partial b}\right)^2 u_b^2 + \left(\frac{\partial V}{\partial h}\right)^2 u_b^2$$

and after substitution

$u_V^2 = [(2 \times 2.00 \times 5.50)^2 \times (0.05)^2] + [(2.00)^2 \times (0.10)^2] = 1.21 + 0.04 = 1.25$ so $u_V = (1.25)^{0.5} = 1.12\ cm^3$

and the volume of the solid to be 22.0 +/- 1.12 cm^3 that would be reported as 22 +/- 1 cm^3 .

Is Calculating Uncertainty Actually Useful?

Given the effort it takes to calculate uncertainty, it is worth asking whether such calculations are useful. The short answer is, yes. Let's consider three examples of how we can use a propagation of uncertainty to help guide the development of an analytical method.

One reason to complete a propagation of uncertainty is that we can compare our estimate of the uncertainty to that obtained experimentally. For example, to determine the mass of a penny we measure its mass twice—once to tare the balance at 0.000 g and once to measure the penny's mass. If the uncertainty in each measurement of mass is ±0.001 g, then we estimate the total uncertainty in the penny's mass as

$$u_R = \sqrt{(0.001)^2 + (0.001)^2} = 0.0014 \text{ g}$$

If we measure a single penny's mass several times and obtain a standard deviation of ±0.050 g, then we have evidence that the measurement process is out of control. Knowing this, we can identify and correct the problem.

We also can use a propagation of uncertainty to help us decide how to improve an analytical method's uncertainty. In Example 3.3.3, for instance, we calculated an analyte's concentration as 126 ppm ± 2 ppm, which is a percent uncertainty of 1.6%. Suppose we want to decrease the percent uncertainty to no more than 0.8%. How might we accomplish this? Looking back at the calculation, we see that the concentration's relative uncertainty is determined by the relative uncertainty in the measured signal (corrected for the reagent blank)

$$\frac{0.028}{23.41} = 0.0012 \text{ or } 0.12\%$$

and the relative uncertainty in the method's sensitivity, k_A,

$$\frac{0.003 \text{ ppm}^{-1}}{0.186 \text{ ppm}^{-1}} = 0.016 \text{ or } 1.6\%$$

Of these two terms, the uncertainty in the method's sensitivity dominates the overall uncertainty. Improving the signal's uncertainty will not improve the overall uncertainty of the analysis. To achieve an overall uncertainty of 0.8% we must improve the uncertainty in k_A to ±0.0015 ppm^{-1}.

Exercise 3.3.3

Verify that an uncertainty of ±0.0015 ppm^{-1} for k_A is the correct result.

Answer

An uncertainty of 0.8% is a relative uncertainty in the concentration of 0.008; thus, letting u be the uncertainty in k_A

$$0.008 = \sqrt{\left(\frac{0.028}{23.41}\right)^2 + \left(\frac{u}{0.186}\right)^2}$$

Squaring both sides of the equation gives

$$6.4 \times 10^{-5} = \left(\frac{0.028}{23.41}\right)^2 + \left(\frac{u}{0.186}\right)^2$$

Solving for the uncertainty in k_A gives its value as 1.47×10^{-3} or ±0.0015 ppm^{-1}.

Finally, we can use a propagation of uncertainty to determine which of several procedures provides the smallest uncertainty. When we dilute a stock solution usually there are several combinations of volumetric glassware that will give the same final concentration. For instance, we can dilute a stock solution by a factor of 10 using a 10-mL pipet and a 100-mL volumetric flask, or using a 25-mL pipet and a 250-mL volumetric flask. We also can accomplish the same dilution in two steps using a 50-mL pipet and 100-mL volumetric flask for the first dilution, and a 10-mL pipet and a 50-mL volumetric flask for the second dilution. The

overall uncertainty in the final concentration—and, therefore, the best option for the dilution—depends on the uncertainty of the volumetric pipets and volumetric flasks. As shown in the following example, we can use the tolerance values for volumetric glassware to determine the optimum dilution strategy [Lam, R. B.; Isenhour, T. L. *Anal. Chem.* **1980**, *52*, 1158–1161].

Example 3.3.6:

Which of the following methods for preparing a 0.0010 M solution from a 1.0 M stock solution provides the smallest overall uncertainty?

(a) A one-step dilution that uses a 1-mL pipet and a 1000-mL volumetric flask.

(b) A two-step dilution that uses a 20-mL pipet and a 1000-mL volumetric flask for the first dilution, and a 25-mL pipet and a 500-mL volumetric flask for the second dilution.

Solution

The dilution calculations for case (a) and case (b) are

$$\text{case (a): } 1.0\text{ M} \times \frac{1.000\text{ mL}}{1000.0\text{ mL}} = 0.0010\text{ M}$$

$$\text{case (b): } 1.0\text{ M} \times \frac{20.00\text{ mL}}{1000.0\text{ mL}} \times \frac{25.00\text{ mL}}{500.0\text{mL}} = 0.0010\text{ M}$$

Using tolerance values from Table 4.2.1, the relative uncertainty for case (a) is

$$u_R = \sqrt{\left(\frac{0.006}{1.000}\right)^2 + \left(\frac{0.3}{1000.0}\right)^2} = 0.006$$

and for case (b) the relative uncertainty is

$$u_R = \sqrt{\left(\frac{0.03}{20.00}\right)^2 + \left(\frac{0.3}{1000}\right)^2 + \left(\frac{0.03}{25.00}\right)^2 + \left(\frac{0.2}{500.0}\right)^2} = 0.002$$

Since the relative uncertainty for case (b) is less than that for case (a), the two-step dilution provides the smallest overall uncertainty. Of course we must balance the smaller uncertainty for case (b) against the increased opportunity for introducing a determinate error when making two dilutions instead of just one dilution, as in case (a).

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3.4: The Distribution of Measurements and Results

Earlier we reported results for a determination of the mass of a circulating United States penny, obtaining a mean of 3.117 g and a standard deviation of 0.051 g. Table 4.4.1 shows results for a second, independent determination of a penny's mass, as well as the data from the first experiment. Although the means and standard deviations for the two experiments are similar, they are not identical. The difference between the two experiments raises some interesting questions. Are the results for one experiment better than the results for the other experiment? Do the two experiments provide equivalent estimates for the mean and the standard deviation? What is our best estimate of a penny's expected mass? To answer these questions we need to understand how we might predict the properties of all pennies using the results from an analysis of a small sample of pennies. We begin by making a distinction between populations and samples.

Table 4.4.1 : Results for Two Determinations of the Mass of a Circulating U. S. Penny

First Experiment		Second Experiment	
Penny	Mass (g)	Penny	Mass (g)
1	3.080	1	3.052
2	3.094	2	3.141
3	3.107	3	3.083
4	3.056	4	3.083
5	3.112	5	3.048
6	3.174		
7	3.198		
$\overline{X}$	3.117		3.081
s	0.051		0.037

Populations and Samples

A ***population*** is the set of all objects in the system we are investigating. For the data in Table 4.4.1 , the population is all United States pennies in circulation. This population is so large that we cannot analyze every member of the population. Instead, we select and analyze a limited subset, or ***sample*** of the population. The data in Table 4.4.1 , for example, shows the results for two such samples drawn from the larger population of all circulating United States pennies.

Probability Distributions for Populations

Table 4.4.1 provides the means and the standard deviations for two samples of circulating United States pennies. What do these samples tell us about the population of pennies? What is the largest possible mass for a penny? What is the smallest possible mass? Are all masses equally probable, or are some masses more common?

To answer these questions we need to know how the masses of individual pennies are distributed about the population's average mass. We represent the distribution of a population by plotting the probability or frequency of obtaining a specific result as a function of the possible results. Such plots are called ***probability distributions***.

There are many possible probability distributions; in fact, the probability distribution can take any shape depending on the nature of the population. Fortunately many chemical systems display one of several common probability distributions. Two of these distributions, the binomial distribution and the normal distribution, are discussed in this section.

The Binomial Distribution

The binomial distribution describes a population in which the result is the number of times a particular event occurs during a fixed number of trials. Mathematically, the binomial distribution is defined as

$$P(X,N) = \frac{N!}{X!(N-X)!} \times p^X \times (1-p)^{N-X}$$

where $P(X\ ,\ N)$ is the probability that an event occurs X times during N trials, and p is the event's probability for a single trial. If you flip a coin five times, $P(2,5)$ is the probability the coin will turn up "heads" exactly twice.

> The term $N!$ reads as N-factorial and is the product $N \times (N–1) \times (N–2) \times \cdots \times 1$. For example, 4! is $4 \times 3 \times 2 \times 1 = 24$. Your calculator probably has a key for calculating factorials.

A binomial distribution has well-defined measures of central tendency and spread. The expected mean value is

$$\mu = Np$$

and the expected spread is given by the variance

$$\sigma^2 = Np(1-p)$$

or the standard deviation.

$$\sigma = \sqrt{Np(1-p)}$$

The binomial distribution describes a population whose members have only specific, discrete values. When you roll a die, for example, the possible values are 1, 2, 3, 4, 5, or 6. A roll of 3.45 is not possible. As shown in Worked Example 4.4.1 , one example of a chemical system that obeys the binomial distribution is the probability of finding a particular isotope in a molecule.

Example 4.4.1

Carbon has two stable, non-radioactive isotopes, ^{12}C and ^{13}C, with relative isotopic abundances of, respectively, 98.89% and 1.11%.

(a) What are the mean and the standard deviation for the number of ^{13}C atoms in a molecule of cholesterol ($C_{27}H_{44}O$)?

(b) What is the probability that a molecule of cholesterol has no atoms of ^{13}C?

Solution

The probability of finding an atom of ^{13}C in a molecule of cholesterol follows a binomial distribution, where X is the number of ^{13}C atoms, N is the number of carbon atoms in a molecule of cholesterol, and p is the probability that an atom of carbon in ^{13}C.

For (a), the mean number of ^{13}C atoms in a molecule of cholesterol is

$$\mu = Np = 27 \times 0.0111 = 0.300$$

with a standard deviation of

$$\sigma = \sqrt{Np(1-p)} = \sqrt{27 \times 0.0111 \times (1-0.0111)} = 0.544$$

For (b), the probability of finding a molecule of cholesterol without an atom of ^{13}C is

$$P(0,27) = \frac{27!}{0!\ (27-0)!} \times (0.0111)^0 \times (1-0.0111)^{27-0} = 0.740$$

There is a 74.0% probability that a molecule of cholesterol will not have an atom of ^{13}C, a result consistent with the observation that the mean number of ^{13}C atoms per molecule of cholesterol, 0.300, is less than one.

A portion of the binomial distribution for atoms of ^{13}C in cholesterol is shown in Figure 4.4.1 . Note in particular that there is little probability of finding more than two atoms of ^{13}C in any molecule of cholesterol.

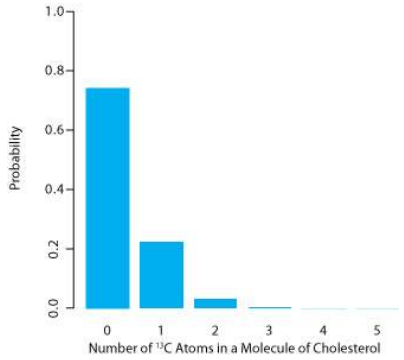


Figure 4.4.1 : Portion of the binomial distribution for the number of naturally occurring ^{13}C atoms in a molecule of cholesterol. Only 3.6% of cholesterol molecules contain more than one atom of ^{13}C, and only 0.33% contain more than two atoms of ^{13}C.

The Normal Distribution

A binomial distribution describes a population whose members have only certain discrete values. This is the case with the number of ^{13}C atoms in cholesterol. A molecule of cholesterol, for example, can have two ^{13}C atoms, but it can not have 2.5 atoms of ^{13}C. A population is continuous if its members may take on any value. The efficiency of extracting cholesterol from a sample, for example, can take on any value between 0% (no cholesterol is extracted) and 100% (all cholesterol is extracted).

The most common continuous distribution is the Gaussian, or ***normal distribution***, the equation for which is

$$f(X) = \frac{1}{\sqrt{2\pi\sigma^2}} e^{-\frac{(X-\mu)^2}{2\sigma^2}}$$

where μ is the expected mean for a population with n members

$$\mu = \frac{\sum_{i=1}^{n} X_i}{n}$$

and σ^2 is the population's variance.

$$\sigma^2 = \frac{\sum_{i=1}^{n} (X_i - \mu)^2}{n} \tag{3.4.1}$$

Examples of three normal distributions, each with an expected mean of 0 and with variances of 25, 100, or 400, respectively, are shown in Figure 4.4.2 . Two features of these normal distribution curves deserve attention. First, note that each normal distribution has a single maximum that corresponds to μ, and that the distribution is symmetrical about this value. Second, increasing the population's variance increases the distribution's spread and decreases its height; the area under the curve, however, is the same for all three distributions.

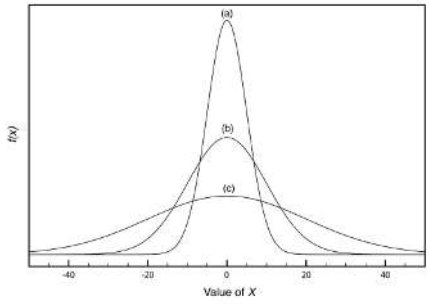


Figure 4.4.2 : Normal distribution curves for: (a) $\mu = 0$; $\sigma^2 = 25$ (b) $\mu = 0$; $\sigma^2 = 100$ (c) $\mu = 0$; $\sigma^2 = 400$.

The area under a normal distribution curve is an important and useful property as it is equal to the probability of finding a member of the population within a particular range of values. In Figure 4.4.2 , for example, 99.99% of the population shown in curve (a) have values of X between –20 and +20. For curve (c), 68.26% of the population's members have values of X between –20 and +20.

Because a normal distribution depends solely on μ and σ^2, the probability of finding a member of the population between any two limits is the same for all normally distributed populations. Figure 4.4.3 , for example, shows that 68.26% of the members of a normal

distribution have a value within the range $\mu \pm 1\sigma$, and that 95.44% of population's members have values within the range $\mu \pm 2\sigma$. Only 0.27% members of a population have values that exceed the expected mean by more than ± 3σ. Additional ranges and probabilities are gathered together in the probability table included in Appendix 3. As shown in Example 4.4.2 , if we know the mean and the standard deviation for a normally distributed population, then we can determine the percentage of the population between any defined limits.

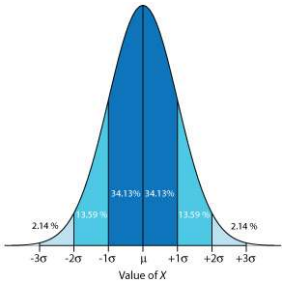


Figure 4.4.3 : Normal distribution curve showing the area under the curve for several different ranges of values of *X*.

Example 4.4.2

The amount of aspirin in the analgesic tablets from a particular manufacturer is known to follow a normal distribution with μ = 250 mg and σ = 5. In a random sample of tablets from the production line, what percentage are expected to contain between 243 and 262 mg of aspirin?

Solution

We do not determine directly the percentage of tablets between 243 mg and 262 mg of aspirin. Instead, we first find the percentage of tablets with less than 243 mg of aspirin and the percentage of tablets having more than 262 mg of aspirin. Subtracting these results from 100%, gives the percentage of tablets that contain between 243 mg and 262 mg of aspirin.

To find the percentage of tablets with less than 243 mg of aspirin or more than 262 mg of aspirin we calculate the deviation, *z*, of each limit from μ in terms of the population's standard deviation, σ

$$z = \frac{X - \mu}{\sigma}$$

where *X* is the limit in question. The deviation for the lower limit is

$$z_{lower} = \frac{243 - 250}{5} = -1.4$$

and the deviation for the upper limit is

$$z_{upper} = \frac{262 - 250}{5} = +2.4$$

Using the table in Appendix 3, we find that the percentage of tablets with less than 243 mg of aspirin is 8.08%, and that the percentage of tablets with more than 262 mg of aspirin is 0.82%. Therefore, the percentage of tablets containing between 243 and 262 mg of aspirin is

$$100.00\% - 8.08\% - 0.82\% = 91.10\%$$

Figure 4.4.4 shows the distribution of aspiring in the tablets, with the area in blue showing the percentage of tablets containing between 243 mg and 262 mg of aspirin.

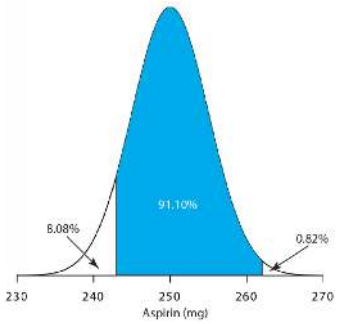


Figure 4.4.4 : Normal distribution for the population of aspirin tablets in Example 4.4.2 . The population's mean and standard deviation are 250 mg and 5 mg, respectively. The shaded area shows the percentage of tablets containing between 243 mg and 262 mg of aspirin.

? Exercise 4.4.1

What percentage of aspirin tablets will contain between 240 mg and 245 mg of aspirin if the population's mean is 250 mg and the population's standard deviation is 5 mg.

Answer

To find the percentage of tablets that contain less than 245 mg of aspirin we first calculate the deviation, z,

$$z = \frac{245 - 250}{5} = -1.00$$

and then look up the corresponding probability in Appendix 3, obtaining a value of 15.87%. To find the percentage of tablets that contain less than 240 mg of aspirin we find that

$$z = \frac{240 - 250}{5} = -2.00$$

which corresponds to 2.28%. The percentage of tablets containing between 240 and 245 mg of aspiring is 15.87% – 2.28% = 13.59%.

Confidence Intervals for Populations

If we select at random a single member from a population, what is its most likely value? This is an important question, and, in one form or another, it is at the heart of any analysis in which we wish to extrapolate from a sample to the sample's parent population. One of the most important features of a population's probability distribution is that it provides a way to answer this question.

Figure 4.4.3 shows that for a normal distribution, 68.26% of the population's members have values within the range $\mu \pm 1\sigma$. Stating this another way, there is a 68.26% probability that the result for a single sample drawn from a normally distributed population is in the interval $\mu \pm 1\sigma$. In general, if we select a single sample we expect its value, X_i is in the range

$$X_i = \mu \pm z\sigma \tag{3.4.2}$$

where the value of z is how confident we are in assigning this range. Values reported in this fashion are called ***confidence intervals***. Equation 3.4.2, for example, is the confidence interval for a single member of a population. Table 4.4.2 gives the confidence intervals for several values of z. For reasons discussed later in the chapter, a 95% confidence level is a common choice in analytical chemistry.

When z = 1, we call this the 68.26% confidence interval.

Table 4.4.2 : Confidence Intervals for a Normal Distribution

z	Confidence Interval
0.50	38.30
1.00	68.26
1.50	86.64
1.96	95.00
2.00	95.44
2.50	98.76
3.00	99.73
3.50	99.95

Example 4.4.3

What is the 95% confidence interval for the amount of aspirin in a single analgesic tablet drawn from a population for which μ is 250 mg and for which σ is 5?

Solution

Using Table 4.4.2 , we find that z is 1.96 for a 95% confidence interval. Substituting this into Equation 3.4.2 gives the confidence interval for a single tablet as

$$X_i = \mu \pm 1.96\sigma = 250 \text{ mg} \pm (1.96 \times 5) = 250 \text{ mg} \pm 10\text{mg}$$

A confidence interval of 250 mg ± 10 mg means that 95% of the tablets in the population contain between 240 and 260 mg of aspirin.

Alternatively, we can rewrite Equation 3.4.2 so that it gives the confidence interval is for μ based on the population's standard deviation and the value of a single member drawn from the population.

$$\mu = X_i \pm z\sigma \tag{3.4.3}$$

Example 4.4.4

The population standard deviation for the amount of aspirin in a batch of analgesic tablets is known to be 7 mg of aspirin. If you randomly select and analyze a single tablet and find that it contains 245 mg of aspirin, what is the 95% confidence interval for the population's mean?

Solution

The 95% confidence interval for the population mean is given as

$$\mu = X_i \pm z\sigma = 245 \text{ mg} \pm (1.96 \times 7) \text{ mg} = 245 \text{ mg} \pm 14 \text{ mg}$$

Therefore, based on this one sample, we estimate that there is 95% probability that the population's mean, μ, lies within the range of 231 mg to 259 mg of aspirin.

> Note the qualification that the prediction for μ is based on one sample; a different sample likely will give a different 95% confidence interval. Our result here, therefore, is an estimate for μ based on this one sample.

It is unusual to predict the population's expected mean from the analysis of a single sample; instead, we collect n samples drawn from a population of known σ, and report the mean, X . The standard deviation of the mean, $\sigma_{\overline{X}}$, which also is known as the ***standard error of the mean***, is

$$\sigma_{\overline{X}} = \frac{\sigma}{\sqrt{n}}$$

The confidence interval for the population's mean, therefore, is

$$\mu = \overline{X} \pm \frac{z\sigma}{\sqrt{n}}$$

Example 4.4.5

What is the 95% confidence interval for the analgesic tablets in Example 4.4.4 , if an analysis of five tablets yields a mean of 245 mg of aspirin?

Solution

In this case the confidence interval is

$$\mu = 245 \text{ mg} \pm \frac{1.96 \times 7}{\sqrt{5}} \text{ mg} = 245 \text{ mg} \pm 6 \text{ mg}$$

We estimate a 95% probability that the population's mean is between 239 mg and 251 mg of aspirin. As expected, the confidence interval when using the mean of five samples is smaller than that for a single sample.

Exercise 4.4.2

An analysis of seven aspirin tablets from a population known to have a standard deviation of 5, gives the following results in mg aspirin per tablet:

$$246 \quad 249 \quad 255 \quad 251 \quad 251 \quad 247 \quad 250$$

What is the 95% confidence interval for the population's expected mean?

Answer

The mean is 249.9 mg aspirin/tablet for this sample of seven tablets. For a 95% confidence interval the value of *z* is 1.96, which makes the confidence interval

$$249.9 \pm \frac{1.96 \times 5}{\sqrt{7}} = 249.9 \pm 3.7 \approx 250 \text{ mg} \pm 4 \text{ mg}$$

Probability Distributions for Samples

In Examples 4.4.2 –4.4.5 we assumed that the amount of aspirin in analgesic tablets is normally distributed. Without analyzing every member of the population, how can we justify this assumption? In a situation where we cannot study the whole population, or when we cannot predict the mathematical form of a population's probability distribution, we must deduce the distribution from a limited sampling of its members.

Sample Distributions and the Central Limit Theorem

Let's return to the problem of determining a penny's mass to explore further the relationship between a population's distribution and the distribution of a sample drawn from that population. The two sets of data in Table 4.4.1 are too small to provide a useful picture of a sample's distribution, so we will use the larger sample of 100 pennies shown in Table 4.4.3 . The mean and the standard deviation for this sample are 3.095 g and 0.0346 g, respectively.

Table 4.4.3 : Masses for a Sample of 100 Circulating U. S. Pennies

Penny	Weight (g)	Penny	Weight (g)	Penny	Weight (g)	Penny	Weight (g)
1	3.126	26	3.073	51	3.101	76	3.086
2	3.140	27	3.084	52	3.049	77	3.123
3	3.092	28	3.148	53	3.082	78	3.115
4	3.095	29	3.047	54	3.142	79	3.055
5	3.080	30	3.121	55	3.082	80	3.057
6	3.065	31	3.116	56	3.066	81	3.097
7	3.117	32	3.005	57	3.128	82	3.066
8	3.034	33	3.115	58	3.112	83	3.113
9	3.126	34	3.103	59	3.085	84	3.102
10	3.057	35	3.086	60	3.086	85	3.033
11	3.053	36	3.103	61	3.084	86	3.112
12	3.099	37	3.049	62	3.104	87	3.103
13	3.065	38	2.998	63	3.107	88	3.198
14	3.059	39	3.063	64	3.093	89	3.103
15	3.068	40	3.055	65	3.126	90	3.126
16	3.060	41	3.181	66	3.138	91	3.111
17	3.078	42	3.108	67	3.131	92	3.126
18	3.125	43	3.114	68	3.120	93	3.052
19	3.090	44	3.121	69	3.100	94	3.113
20	3.100	45	3.105	70	3.099	95	3.085
21	3.055	46	3.078	71	3.097	96	3.117
22	3.105	47	3.147	72	3.091	97	3.142
23	3.063	48	3.104	73	3.077	98	3.031
24	3.083	49	3.146	74	3.178	99	3.083
25	3.065	50	3.095	75	3.054	100	3.104

A ***histogram*** (Figure 4.4.5) is a useful way to examine the data in Table 4.4.3 . To create the histogram, we divide the sample into intervals, by mass, and determine the percentage of pennies within each interval (Table 4.4.4). Note that the sample's mean is the midpoint of the histogram.

Table 4.4.4 : Frequency Distribution for the Data in Table 4.4.3

Mass Interval	Frequency (as % of Sample)	Mass Interval	Frequency (as % of Sample)
2.991 – 3.009	2	3.105 – 3.123	19
3.010 – 3.028	0	3.124 – 3.142	12
3.029 – 3.047	4	3.143 – 3.161	3
3.048 – 3.066	19	3.162 – 3.180	1
3.067 – 3.085	14	3.181 – 3.199	2
3.086 – 3.104	24	3.200 – 3.218	0

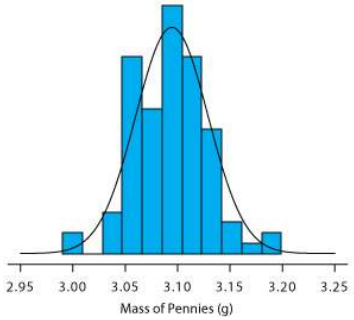


Figure 4.4.5 : The blue bars show a histogram for the data in Table 4.4.3 . The height of each bar corresponds to the percentage of pennies within one of the mass intervals in Table 4.4.4 . Superimposed on the histogram is a normal distribution curve based on the assumption that μ and σ^2 for the population are equivalent to $\overline{X}$ and σ^2 for the sample. The total area of the histogram's bars and the area under the normal distribution curve are equal.

Figure 4.4.5 also includes a normal distribution curve for the population of pennies, based on the assumption that the mean and the variance for the sample are appropriate estimates for the population's mean and variance. Although the histogram is not perfectly symmetric in shape, it provides a good approximation of the normal distribution curve, suggesting that the sample of 100 pennies is normally distributed. It is easy to imagine that the histogram will approximate more closely a normal distribution if we include additional pennies in our sample.

We will not offer a formal proof that the sample of pennies in Table 4.4.3 and the population of all circulating U. S. pennies are normally distributed; however, the evidence in Figure 4.4.5 strongly suggests this is true. Although we cannot claim that the results of all experiments are normally distributed, in most cases our data are normally distributed. According to the ***central limit theorem***, when a measurement is subject to a variety of indeterminate errors, the results for that measurement will approximate a normal distribution [Mark, H.; Workman, J. *Spectroscopy* **1988**, *3*, 44–48]. The central limit theorem holds true even if the individual sources of indeterminate error are not normally distributed. The chief limitation to the central limit theorem is that the sources of indeterminate error must be independent and of similar magnitude so that no one source of error dominates the final distribution.

An additional feature of the central limit theorem is that a distribution of means for samples drawn from a population with any distribution will approximate closely a normal distribution if the size of each sample is sufficiently large. For example, Figure 4.4.6 shows the distribution for two samples of 10 000 drawn from a uniform distribution in which every value between 0 and 1 occurs with an equal frequency. For samples of size n = 1, the resulting distribution closely approximates the population's uniform distribution. The distribution of the means for samples of size n = 10, however, closely approximates a normal distribution.

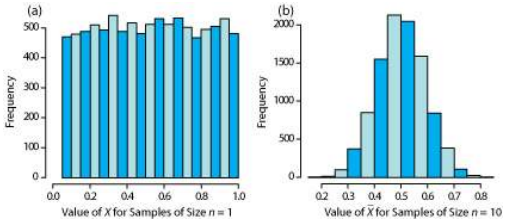


Figure 4.4.6 : Histograms for (a) 10 000 samples of size n = 1 drawn from a uniform distribution with a minimum value of 0 and a maximum value of 1, and (b) the means for 10000 samples of size n = 10 drawn from the same uniform distribution. For (a) the mean of the 10 000 samples is 0.5042, and for (b) the mean of the 10 000 samples is 0.5006. Note that for (a) the distribution closely approximates a uniform distribution in which every possible result is equally likely, and that for (b) the distribution closely approximates a normal distribution.

You might reasonably ask whether this aspect of the central limit theorem is important as it is unlikely that we will complete 10 000 analyses, each of which is the average of 10 individual trials. This is deceiving. When we acquire a sample of soil, for example, it consists of many individual particles each of which is an individual sample of the soil. Our analysis of this sample, therefore, gives the mean for this large number of individual soil particles. Because of this, the central limit theorem is relevant. For a discussion of circumstances where the central limit theorem may not apply, see "Do You Reckon It's Normally Distributed?", the full reference for which is Majewsky, M.; Wagner, M.; Farlin, J. *Sci. Total Environ.* **2016**, *548–549*, 408–409.

Degrees of Freedom

Did you notice the differences between the equation for the variance of a population and the variance of a sample? If not, here are the two equations:

$$\sigma^2 = \frac{\sum_{i=1}^{n} (X_i - \mu)^2}{n}$$

$$s^2 = \frac{\sum_{i=1}^{n} (X_i - \overline{X})^2}{n-1}$$

Both equations measure the variance around the mean, using μ for a population and $\overline{X}$ for a sample. Although the equations use different measures for the mean, the intention is the same for both the sample and the population. A more interesting difference is between the denominators of the two equations. When we calculate the population's variance we divide the numerator by the population's size, n; for the sample's variance, however, we divide by n – 1, where n is the sample's size. Why do we divide by n – 1 when we calculate the sample's variance?

A variance is the average squared deviation of individual results relative to the mean. When we calculate an average we divide the sum by the number of independent measurements, or ***degrees of freedom***, in the calculation. For the population's variance, the degrees of freedom is equal to the population's size, n. When we measure every member of a population we have complete information about the population.

When we calculate the sample's variance, however, we replace μ with $\overline{X}$, which we also calculate using the same data. If there are n members in the sample, we can deduce the value of the n^{th} member from the remaining n – 1 members and the mean. For example, if \(n = 5\) and we know that the first four samples are 1, 2, 3 and 4, and that the mean is 3, then the fifth member of the sample must be

$$X_5 = (\overline{X} \times n) - X_1 - X_2 - X_3 - X_4 = (3 \times 5) - 1 - 2 - 3 - 4 = 5$$

Because we have just four independent measurements, we have lost one degree of freedom. Using n – 1 in place of n when we calculate the sample's variance ensures that s^2 is an unbiased estimator of σ^2.

Here is another way to think about degrees of freedom. We analyze samples to make predictions about the underlying population. When our sample consists of n measurements we cannot make more than n independent predictions about the population. Each time we estimate a parameter, such as the population's mean, we lose a degree of freedom. If there are n

degrees of freedom for calculating the sample's mean, then $n - 1$ degrees of freedom remain when we calculate the sample's variance.

Confidence Intervals for Samples

Earlier we introduced the confidence interval as a way to report the most probable value for a population's mean, μ

$$\mu = \overline{X} \pm \frac{z\sigma}{\sqrt{n}} \tag{3.4.4}$$

where $\overline{X}$ is the mean for a sample of size n, and σ is the population's standard deviation. For most analyses we do not know the population's standard deviation. We can still calculate a confidence interval, however, if we make two modifications to Equation 3.4.4.

The first modification is straightforward—we replace the population's standard deviation, σ, with the sample's standard deviation, s. The second modification is not as obvious. The values of z in Table 4.4.2 are for a normal distribution, which is a function of $sigma^2$, not s^2. Although the sample's variance, s^2, is an unbiased estimate of the population's variance, σ^2, the value of s^2 will only rarely equal σ^2. To account for this uncertainty in estimating σ^2, we replace the variable z in Equation 3.4.4 with the variable t, where t is defined such that $t \geq z$ at all confidence levels.

$$\mu = \overline{X} \pm \frac{ts}{\sqrt{n}} \tag{3.4.5}$$

Values for t at the 95% confidence level are shown in Table 4.4.5 . Note that t becomes smaller as the number of degrees of freedom increases, and that it approaches z as n approaches infinity. The larger the sample, the more closely its confidence interval for a sample (Equation 3.4.5) approaches the confidence interval for the population (Equation 3.4.3). Appendix 4 provides additional values of t for other confidence levels.

Table 4.4.5 : Values of t for a 95% Confidence Interval

Degrees of Freedom	t	Degrees of Freedom	t	Degrees of Freedom	t	Degrees of Freedom	t
1	12.706	6	2.447	12	2.179	30	2.042
2	4.303	7	2.365	14	2.145	40	2.021
3	3.181	8	2.306	16	2.120	60	2.000
4	2.776	9	2.262	18	2.101	100	1.984
5	2.571	10	2.228	20	2.086	\(\infty	1.960

Example 4.4.6

What are the 95% confidence intervals for the two samples of pennies in Table 4.4.1 ?

Solution

The mean and the standard deviation for first experiment are, respectively, 3.117 g and 0.051 g. Because the sample consists of seven measurements, there are six degrees of freedom. The value of t from Table 4.4.5 , is 2.447. Substituting into Equation 3.4.5 gives

$$\mu = 3.117\ \text{g} \pm \frac{2.447 \times 0.051\ \text{g}}{\sqrt{7}} = 3.117\ \text{g} \pm 0.047\ \text{g}$$

For the second experiment the mean and the standard deviation are 3.081 g and 0.073 g, respectively, with four degrees of freedom. The 95% confidence interval is

$$\mu = 3.081\ \text{g} \pm \frac{2.776 \times 0.037\ \text{g}}{\sqrt{5}} = 3.081\ \text{g} \pm 0.046\ \text{g}$$

Based on the first experiment, the 95% confidence interval for the population's mean is 3.070–3.164 g. For the second experiment, the 95% confidence interval is 3.035–3.127 g. Although the two confidence intervals are not identical—remember, each confidence interval provides a different estimate for μ—the mean for each experiment is contained within the other experiment's confidence interval. There also is an appreciable overlap of the two confidence intervals. Both of these observations are consistent with samples drawn from the same population.

Note that our comparison of these two confidence intervals at this point is somewhat vague and unsatisfying. We will return to this point in the next section, when we consider a statistical approach to comparing the results of experiments.

? Exercise 4.4.3

What is the 95% confidence interval for the sample of 100 pennies in Table 4.4.3 ? The mean and the standard deviation for this sample are 3.095 g and 0.0346 g, respectively. Compare your result to the confidence intervals for the samples of pennies in Table 4.4.1 .

Answer

With 100 pennies, we have 99 degrees of freedom for the mean. Although Table 4.4.3 does not include a value for $t(0.05, 99)$, we can approximate its value by using the values for $t(0.05, 60)$ and $t(0.05, 100)$ and by assuming a linear change in its value.

$$t(0.05, 99) = t(0.05, 60) - \frac{39}{40}\{t(0.05, 60) - t(0.05, 100\}$$

$$t(0.05, 99) = 2.000 - \frac{39}{40}\{2.000 - 1.984\} = 1.9844$$

The 95% confidence interval for the pennies is

$$3.095 \pm \frac{1.9844 \times 0.0346}{\sqrt{100}} = 3.095 \text{ g} \pm 0.007 \text{ g}$$

From Example 4.4.6 , the 95% confidence intervals for the two samples in Table 4.4.1 are 3.117 g ± 0.047 g and 3.081 g ± 0.046 g. As expected, the confidence interval for the sample of 100 pennies is much smaller than that for the two smaller samples of pennies. Note, as well, that the confidence interval for the larger sample fits within the confidence intervals for the two smaller samples.

A Cautionary Statement

There is a temptation when we analyze data simply to plug numbers into an equation, carry out the calculation, and report the result. This is never a good idea, and you should develop the habit of reviewing and evaluating your data. For example, if you analyze five samples and report an analyte's mean concentration as 0.67 ppm with a standard deviation of 0.64 ppm, then the 95% confidence interval is

$$\mu = 0.67 \text{ ppm} \pm \frac{2.776 \times 0.64 \text{ ppm}}{\sqrt{5}} = 0.67 \text{ ppm} \pm 0.79 \text{ ppm}$$

This confidence interval estimates that the analyte's true concentration is between –0.12 ppm and 1.46 ppm. Including a negative concentration within the confidence interval should lead you to reevaluate your data or your conclusions. A closer examination of your data may convince you that the standard deviation is larger than expected, making the confidence interval too broad, or you may conclude that the analyte's concentration is too small to report with confidence.

We will return to the topic of detection limits near the end of this chapter.

Here is a second example of why you should closely examine your data: results obtained on samples drawn at random from a normally distributed population must be random. If the results for a sequence of samples show a regular pattern or trend, then the

underlying population either is not normally distributed or there is a time-dependent determinate error. For example, if we randomly select 20 pennies and find that the mass of each penny is greater than that for the preceding penny, then we might suspect that our balance is drifting out of calibration.

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3.5: Statistical Analysis of Data

A confidence interval is a useful way to report the result of an analysis because it sets limits on the expected result. In the absence of determinate error, a confidence interval based on a sample's mean indicates the range of values in which we expect to find the population's mean. When we report a 95% confidence interval for the mass of a penny as 3.117 g ± 0.047 g, for example, we are stating that there is only a 5% probability that the penny's expected mass is less than 3.070 g or more than 3.164 g.

Because a confidence interval is a statement of probability, it allows us to consider comparative questions, such as these: "Are the results for a newly developed method to determine cholesterol in blood significantly different from those obtained using a standard method?" or "Is there a significant variation in the composition of rainwater collected at different sites downwind from a coal-burning utility plant?" In this section we introduce a general approach to the statistical analysis of data. Specific statistical tests are presented in Section 4.6.

> The reliability of significance testing recently has received much attention—see Nuzzo, R. "Scientific Method: Statistical Errors," *Nature*, **2014**, *506*, 150–152 for a general discussion of the issues—so it is appropriate to begin this section by noting the need to ensure that our data and our research question are compatible so that we do not read more into a statistical analysis than our data allows; see Leek, J. T.; Peng, R. D. "What is the Question? *Science*, **2015**, *347*, 1314-1315 for a use-ul discussion of six common research questions.
>
> In the context of analytical chemistry, significance testing often accompanies an exploratory data analysis (Is there a reason to suspect that there is a difference between these two analytical methods when applied to a common sample?) or an inferential data analysis (Is there a reason to suspect that there is a relationship between these two independent measurements?). A statistically significant result for these types of analytical research questions generally leads to the design of additional experiments better suited to making predictions or to explaining an underlying causal relationship. A significance test is the first step toward building a greater understanding of an analytical problem, not the final answer to that problem.

Significance Testing

Let's consider the following problem. To determine if a medication is effective in lowering blood glucose concentrations, we collect two sets of blood samples from a patient. We collect one set of samples immediately before we administer the medication, and collect the second set of samples several hours later. After analyzing the samples, we report their respective means and variances. How do we decide if the medication was successful in lowering the patient's concentration of blood glucose?

One way to answer this question is to construct a normal distribution curve for each sample, and to compare the two curves to each other. Three possible outcomes are shown in Figure 4.5.1 . In Figure 4.5.1 a, there is a complete separation of the two normal distribution curves, which suggests the two samples are significantly different from each other. In Figure 4.5.1 b, the normal distribution curves for the two samples almost completely overlap, which suggests that the difference between the samples is insignificant. Figure 4.5.1 c, however, presents us with a dilemma. Although the means for the two samples seem different, the overlap of their normal distribution curves suggests that a significant number of possible outcomes could belong to either distribution. In this case the best we can do is to make a statement about the probability that the samples are significantly different from each other.

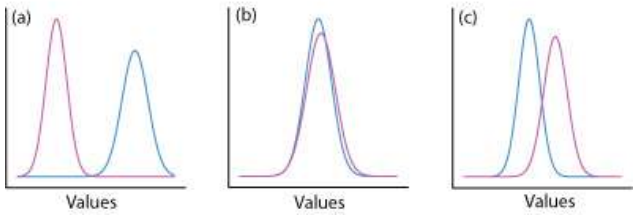


Figure 4.5.1 : Three examples of the possible relationships between the normal distribution curves for two samples. In (a) the curves do not overlap, which suggests that the samples are significantly different from each other. In (b) the two curves are almost identical, suggesting the samples are indistinguishable. The partial overlap of the curves in (c) means that the best we can do is evaluate the probability that there is a difference between the samples.

The process by which we determine the probability that there is a significant difference between two samples is called significance testing or hypothesis testing. Before we discuss specific examples we will first establish a general approach to conducting and interpreting a significance test.

Constructing a Significance Test

The purpose of a ***significance test*** is to determine whether the difference between two or more results is sufficiently large that it cannot be explained by indeterminate errors. The first step in constructing a significance test is to state the problem as a yes or no question, such as "Is this medication effective at lowering a patient's blood glucose levels?" A null hypothesis and an alternative hypothesis define the two possible answers to our yes or no question. The ***null hypothesis***, H_0, is that indeterminate errors are sufficient to explain any differences between our results. The ***alternative hypothesis***, H_A, is that the differences in our results are too great to be explained by random error and that they must be determinate in nature. We test the null hypothesis, which we either retain or reject. If we reject the null hypothesis, then we must accept the alternative hypothesis and conclude that the difference is significant.

Failing to reject a null hypothesis is not the same as accepting it. We retain a null hypothesis because we have insufficient evidence to prove it incorrect. It is impossible to prove that a null hypothesis is true. This is an important point and one that is easy to forget. To appreciate this point let's return to our sample of 100 pennies in Table 4.4.3. After looking at the data we might propose the following null and alternative hypotheses.

H_0: The mass of a circulating U.S. penny is between 2.900 g and 3.200 g

H_A: The mass of a circulating U.S. penny may be less than 2.900 g or more than 3.200 g

To test the null hypothesis we find a penny and determine its mass. If the penny's mass is 2.512 g then we can reject the null hypothesis and accept the alternative hypothesis. Suppose that the penny's mass is 3.162 g. Although this result increases our confidence in the null hypothesis, it does not prove that the null hypothesis is correct because the next penny we sample might weigh less than 2.900 g or more than 3.200 g.

After we state the null and the alternative hypotheses, the second step is to choose a confidence level for the analysis. The confidence level defines the probability that we will reject the null hypothesis when it is, in fact, true. We can express this as our confidence that we are correct in rejecting the null hypothesis (e.g. 95%), or as the probability that we are incorrect in rejecting the null hypothesis. For the latter, the confidence level is given as α, where

$$\alpha = 1 - \frac{\text{confidence interval (}}{100} \tag{3.5.1}$$

For a 95% confidence level, α is 0.05.

> In this textbook we use α to represent the probability that we incorrectly reject the null hypothesis. In other textbooks this probability is given as p (often read as "p- value"). Although the symbols differ, the meaning is the same.

The third step is to calculate an appropriate test statistic and to compare it to a critical value. The test statistic's critical value defines a breakpoint between values that lead us to reject or to retain the null hypothesis, which is the fourth, and final, step of a significance test. How we calculate the test statistic depends on what we are comparing, a topic we cover in Section 4.6. The last step is to either retain the null hypothesis, or to reject it and accept the alternative hypothesis.

> The four steps for a statistical analysis of data using a significance test:
>
> 1. Pose a question, and state the null hypothesis, H_0, and the alternative hypothesis, H_A.
> 2. Choose a confidence level for the statistical analysis.
> 3. Calculate an appropriate test statistic and compare it to a critical value.
> 4. Either retain the null hypothesis, or reject it and accept the alternative hypothesis.

One-Tailed and Two-tailed Significance Tests

Suppose we want to evaluate the accuracy of a new analytical method. We might use the method to analyze a Standard Reference Material that contains a known concentration of analyte, μ. We analyze the standard several times, obtaining a mean value, $\overline{X}$, for the analyte's concentration. Our null hypothesis is that there is no difference between $\overline{X}$ and μ

$$H_0: \overline{X} = \mu$$

If we conduct the significance test at $\alpha = 0.05$, then we retain the null hypothesis if a 95% confidence interval around $\overline{X}$ contains μ. If the alternative hypothesis is

$$H_A: \overline{X} \neq \mu$$

then we reject the null hypothesis and accept the alternative hypothesis if μ lies in the shaded areas at either end of the sample's probability distribution curve (Figure 4.5.2 a). Each of the shaded areas accounts for 2.5% of the area under the probability distribution curve, for a total of 5%. This is a ***two-tailed significance test*** because we reject the null hypothesis for values of μ at either extreme of the sample's probability distribution curve.

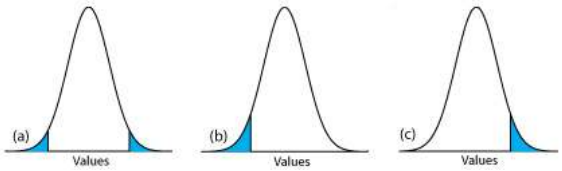


Figure 4.5.2 : Examples of (a) two-tailed, and (b, c) one-tailed, significance test of $\overline{X}$ and μ. The probability distribution curves, which are normal distributions, are based on the sample's mean and standard deviation. For α = 0.05, the blue areas account for 5% of the area under the curve. If the value of μ falls within the blue areas, then we reject the null hypothesis and accept the alternative hypothesis. We retain the null hypothesis if the value of μ falls within the unshaded area of the curve.

We also can write the alternative hypothesis in two additional ways

$$H_A: \overline{X} > \mu$$

$$H_A: \overline{X} < \mu$$

rejecting the null hypothesis if n falls within the shaded areas shown in Figure 4.5.2 b or Figure 4.5.2 c, respectively. In each case the shaded area represents 5% of the area under the probability distribution curve. These are examples of a ***one-tailed significance test***.

For a fixed confidence level, a two-tailed significance test is the more conservative test because rejecting the null hypothesis requires a larger difference between the parameters we are comparing. In most situations we have no particular reason to expect that one parameter must be larger (or must be smaller) than the other parameter. This is the case, for example, when we evaluate the accuracy of a new analytical method. A two-tailed significance test, therefore, usually is the appropriate choice.

We reserve a one-tailed significance test for a situation where we specifically are interested in whether one parameter is larger (or smaller) than the other parameter. For example, a one-tailed significance test is appropriate if we are evaluating a medication's ability to lower blood glucose levels. In this case we are interested only in whether the glucose levels after we administer the medication are less than the glucose levels before we initiated treatment. If a patient's blood glucose level is greater after we administer the medication, then we know the answer—the medication did not work—and do not need to conduct a statistical analysis.

Error in Significance Testing

Because a significance test relies on probability, its interpretation is subject to error. In a significance test, a defines the probability of rejecting a null hypothesis that is true. When we conduct a significance test at $\alpha = 0.05$, there is a 5% probability that we will incorrectly reject the null hypothesis. This is known as a ***type 1 error***, and its risk is always equivalent to α. A type 1 error in a two-tailed or a one-tailed significance tests corresponds to the shaded areas under the probability distribution curves in Figure 4.5.2 .

A second type of error occurs when we retain a null hypothesis even though it is false. This is as a ***type 2 error***, and the probability of its occurrence is β. Unfortunately, in most cases we cannot calculate or estimate the value for β. The probability of a type 2 error, however, is inversely proportional to the probability of a type 1 error.

Minimizing a type 1 error by decreasing α increases the likelihood of a type 2 error. When we choose a value for α we must compromise between these two types of error. Most of the examples in this text use a 95% confidence level ($\alpha = 0.05$) because this usually is a reasonable compromise between type 1 and type 2 errors for analytical work. It is not unusual, however, to use a more stringent (e.g. $\alpha = 0.01$) or a more lenient (e.g. $\alpha = 0.10$) confidence level when the situation calls for it.

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3.6: Statistical Methods for Normal Distributions

The most common distribution for our results is a normal distribution. Because the area between any two limits of a normal distribution curve is well defined, constructing and evaluating significance tests is straightforward.

Comparing $\overline{X}$ to μ

One way to validate a new analytical method is to analyze a sample that contains a known amount of analyte, μ. To judge the method's accuracy we analyze several portions of the sample, determine the average amount of analyte in the sample, $\overline{X}$, and use a significance test to compare $\overline{X}$ to μ. Our null hypothesis is that the difference between $\overline{X}$ and μ is explained by indeterminate errors that affect the determination of $\overline{X}$. The alternative hypothesis is that the difference between $\overline{X}$ and μ is too large to be explained by indeterminate error.

$$H_0: \overline{X} = \mu$$

$$H_A: \overline{X} \neq \mu$$

The test statistic is t_{exp}, which we substitute into the confidence interval for μ given by Equation 4.4.5

$$\mu = \overline{X} \pm \frac{t_{exp} s}{\sqrt{n}} \tag{3.6.1}$$

Rearranging this equation and solving for t_{exp}

$$t_{exp} = \frac{|\mu - \overline{X}|\sqrt{n}}{s} \tag{3.6.2}$$

gives the value for t_{exp} when μ is at either the right edge or the left edge of the sample's confidence interval (Figure 3.6.1a)

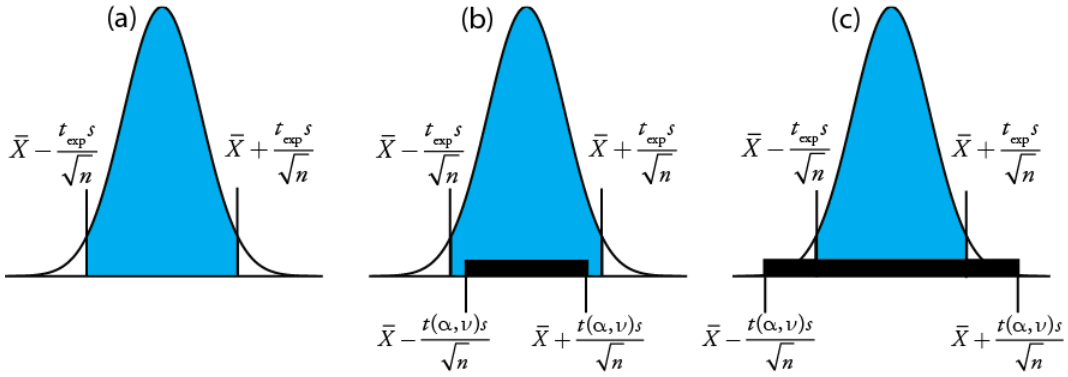


Figure 3.6.1: Relationship between a confidence interval and the result of a significance test. (a) The shaded area under the normal distribution curve shows the sample's confidence interval for μ based on t_{exp}. The solid bars in (b) and (c) show the expected confidence intervals for μ explained by indeterminate error given the choice of α and the available degrees of freedom, ν. For (b) we reject the null hypothesis because portions of the sample's confidence interval fall outside the confidence interval explained by indeterminate error. In the case of (c) we retain the null hypothesis because the confidence interval explained by indeterminate error completely encompasses the sample's confidence interval.

To determine if we should retain or reject the null hypothesis, we compare the value of t_{exp} to a critical value, $t(\alpha, \nu)$, where α is the confidence level and ν is the degrees of freedom for the sample. The critical value $t(\alpha, \nu)$ defines the largest confidence interval explained by indeterminate error. If $t_{exp} > t(\alpha, \nu)$, then our sample's confidence interval is greater than that explained by indeterminate errors (Figure 3.6.1b). In this case, we reject the null hypothesis and accept the alternative hypothesis. If $t_{exp} \leq t(\alpha, \nu)$, then our sample's confidence interval is smaller than that explained by indeterminate error, and we retain the null hypothesis (Figure 3.6.1c). Example 3.6.1 provides a typical application of this significance test, which is known as a **t-test** of $\overline{X}$ to μ.

You will find values for $t(\alpha, \nu)$ in Appendix 4.

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Another name for the *t*-test is Student's *t*-test. Student was the pen name for William Gossett (1876-1927) who developed the *t*-test while working as a statistician for the Guiness Brewery in Dublin, Ireland. He published under the name Student because the brewery did not want its competitors to know they were using statistics to help improve the quality of their products.

Example 3.6.1

Before determining the amount of Na_2CO_3 in a sample, you decide to check your procedure by analyzing a standard sample that is 98.76% w/w Na_2CO_3. Five replicate determinations of the %w/w Na_2CO_3 in the standard gave the following results

$$98.71\% \quad 98.59\% \quad 98.62\% \quad 98.44\% \quad 98.58\%$$

Using $\alpha = 0.05$, is there any evidence that the analysis is giving inaccurate results?

Solution

The mean and standard deviation for the five trials are

$$\overline{X} = 98.59 \qquad s = 0.0973$$

Because there is no reason to believe that the results for the standard must be larger or smaller than μ, a two-tailed *t*-test is appropriate. The null hypothesis and alternative hypothesis are

$$H_0\text{: } \overline{X} = \mu \qquad H_A\text{: } \overline{X} \neq \mu$$

The test statistic, t_{exp}, is

$$t_{exp} = \frac{|\mu - \overline{X}|\sqrt{n}}{2} = \frac{|98.76 - 98.59|\sqrt{5}}{0.0973} = 3.91$$

The critical value for *t*(0.05, 4) from Appendix 4 is 2.78. Since t_{exp} is greater than *t*(0.05, 4), we reject the null hypothesis and accept the alternative hypothesis. At the 95% confidence level the difference between $\overline{X}$ and μ is too large to be explained by indeterminate sources of error, which suggests there is a determinate source of error that affects the analysis.

There is another way to interpret the result of this *t*-test. Knowing that t_{exp} is 3.91 and that there are 4 degrees of freedom, we use Appendix 4 to estimate the α value corresponding to a *t*(α, 4) of 3.91. From Appendix 4, *t*(0.02, 4) is 3.75 and *t*(0.01, 4) is 4.60. Although we can reject the null hypothesis at the 98% confidence level, we cannot reject it at the 99% confidence level. For a discussion of the advantages of this approach, see J. A. C. Sterne and G. D. Smith "Sifting the evidence—what's wrong with significance tests?" *BMJ* **2001**, *322*, 226–231.

Exercise 3.6.1

To evaluate the accuracy of a new analytical method, an analyst determines the purity of a standard for which μ is 100.0%, obtaining the following results.

$$99.28\% \quad 103.93\% \quad 99.43\% \quad 99.84\% \quad 97.60\% \quad 96.70\% \quad 98.02\%$$

Is there any evidence at $\alpha = 0.05$ that there is a determinate error affecting the results?

Answer

The null hypothesis is $H_0\text{: } \overline{X} = \mu$ and the alternative hypothesis is $H_A\text{: } \overline{X} \neq \mu$. The mean and the standard deviation for the data are 99.26% and 2.35%, respectively. The value for t_{exp} is

$$t_{exp} = \frac{|100.0 - 99.26|\sqrt{7}}{2.35} = 0.833$$

and the critical value for *t*(0.05, 6) is 2.477. Because t_{exp} is less than *t*(0.05, 6) we retain the null hypothesis and have no evidence for a significant difference between $\overline{X}$ and μ.

Earlier we made the point that we must exercise caution when we interpret the result of a statistical analysis. We will keep returning to this point because it is an important one. Having determined that a result is inaccurate, as we did in Example 3.6.1, the next step

is to identify and to correct the error. Before we expend time and money on this, however, we first should examine critically our data. For example, the smaller the value of s, the larger the value of t_{exp}. If the standard deviation for our analysis is unrealistically small, then the probability of a type 2 error increases. Including a few additional replicate analyses of the standard and reevaluating the t-test may strengthen our evidence for a determinate error, or it may show us that there is no evidence for a determinate error.

Comparing s^2 to σ^2

If we analyze regularly a particular sample, we may be able to establish an expected variance, σ^2, for the analysis. This often is the case, for example, in a clinical lab that analyze hundreds of blood samples each day. A few replicate analyses of a single sample gives a sample variance, s^2, whose value may or may not differ significantly from σ^2.

We can use an F-test to evaluate whether a difference between s^2 and σ^2 is significant. The null hypothesis is H_0: $s^2 = \sigma^2$ and the alternative hypothesis is H_A: $s^2 \neq \sigma^2$. The test statistic for evaluating the null hypothesis is F_{exp}, which is given as either

$$F_{exp} = \frac{s^2}{\sigma^2} \text{ if } s^2 > \sigma^2 \text{ or } F_{exp} = \frac{\sigma^2}{s^2} \text{ if } \sigma^2 > s^2 \tag{3.6.3}$$

depending on whether s^2 is larger or smaller than σ^2. This way of defining F_{exp} ensures that its value is always greater than or equal to one.

If the null hypothesis is true, then F_{exp} should equal one; however, because of indeterminate errors F_{exp} usually is greater than one. A critical value, $F(\alpha, \nu_{num}, \nu_{den})$, is the largest value of F_{exp} that we can attribute to indeterminate error given the specified significance level, α, and the degrees of freedom for the variance in the numerator, ν_{num}, and the variance in the denominator, ν_{den}. The degrees of freedom for s^2 is $n - 1$, where n is the number of replicates used to determine the sample's variance, and the degrees of freedom for σ^2 is defined as infinity, ∞. Critical values of F for $\alpha = 0.05$ are listed in Appendix 5 for both one-tailed and two-tailed F-tests.

Example 3.6.2

A manufacturer's process for analyzing aspirin tablets has a known variance of 25. A sample of 10 aspirin tablets is selected and analyzed for the amount of aspirin, yielding the following results in mg aspirin/tablet.

254 249 252 252 249 249 250 247 251 252

Determine whether there is evidence of a significant difference between the sample's variance and the expected variance at $\alpha = 0.05$.

Solution

The variance for the sample of 10 tablets is 4.3. The null hypothesis and alternative hypotheses are

$$H_0: s^2 = \sigma^2 \qquad H_A: s^2 \neq \sigma^2$$

and the value for F_{exp} is

$$F_{exp} = \frac{\sigma^2}{s^2} = \frac{25}{4.3} = 5.8$$

The critical value for $F(0.05, \infty, 9)$ from Appendix 5 is 3.333. Since F_{exp} is greater than $F(0.05, \infty, 9)$, we reject the null hypothesis and accept the alternative hypothesis that there is a significant difference between the sample's variance and the expected variance. One explanation for the difference might be that the aspirin tablets were not selected randomly.

Comparing Variances for Two Samples

We can extend the F-test to compare the variances for two samples, A and B, by rewriting Equation 3.6.3 as

$$F_{exp} = \frac{s_A^2}{s_B^2}$$

defining A and B so that the value of Fexp is greater than or equal to 1.

Example 3.6.3

Table 4.4.1 shows results for two experiments to determine the mass of a circulating U.S. penny. Determine whether there is a difference in the variances of these analyses at $\alpha = 0.05$.

Solution

The standard deviations for the two experiments are 0.051 for the first experiment (*A*) and 0.037 for the second experiment (*B*). The null and alternative hypotheses are

$$H_0\text{: } s_A^2 = s_B^2 \qquad H_\text{A}\text{: } s_A^2 \neq s_B^2$$

and the value of F_{exp} is

$$F_{\text{exp}} = \frac{s_A^2}{s_B^2} = \frac{(0.051)^2}{(0.037)^2} = \frac{0.00260}{0.00137} = 1.90$$

From Appendix 5, the critical value for $F(0.05, 6, 4)$ is 9.197. Because $F_{\text{exp}} < F(0.05, 6, 4)$, we retain the null hypothesis. There is no evidence at $\alpha = 0.05$ to suggest that the difference in variances is significant.

Exercise 3.6.2

To compare two production lots of aspirin tablets, we collect an analyze samples from each, obtaining the following results (in mg aspirin/tablet).

Lot 1: **256 248 245 245 244 248 261**

Lot 2: **241 258 241 244 256 254**

Is there any evidence at $\alpha = 0.05$ that there is a significant difference in the variances for these two samples?

Answer

The standard deviations are 6.451 mg for Lot 1 and 7.849 mg for Lot 2. The null and alternative hypotheses are

$$H_0\text{: } s_{\text{Lot 1}}^2 = s_{\text{Lot 2}}^2 \qquad H_\text{A}\text{: } s_{\text{Lot 1}}^2 \neq s_{\text{Lot 2}}^2$$

and the value of F_{exp} is

$$F_{\text{exp}} = \frac{(7.849)^2}{(6.451)^2} = 1.480$$

The critical value for $F(0.05, 5, 6)$ is 5.988. Because $F_{\text{exp}} < F(0.05, 5, 6)$, we retain the null hypothesis. There is no evidence at $\alpha = 0.05$ to suggest that the difference in the variances is significant.

Comparing Means for Two Samples

Three factors influence the result of an analysis: the method, the sample, and the analyst. We can study the influence of these factors by conducting experiments in which we change one factor while holding constant the other factors. For example, to compare two analytical methods we can have the same analyst apply each method to the same sample and then examine the resulting means. In a similar fashion, we can design experiments to compare two analysts or to compare two samples.

It also is possible to design experiments in which we vary more than one of these factors. We will return to this point in Chapter 14.

Before we consider the significance tests for comparing the means of two samples, we need to make a distinction between ***unpaired data*** and ***paired data***. This is a critical distinction and learning to distinguish between these two types of data is important. Here are two simple examples that highlight the difference between unpaired data and paired data. In each example the goal is to compare two balances by weighing pennies.

- Example 1: We collect 10 pennies and weigh each penny on each balance. This is an example of paired data because we use the same 10 pennies to evaluate each balance.

- Example 2: We collect 10 pennies and divide them into two groups of five pennies each. We weigh the pennies in the first group on one balance and we weigh the second group of pennies on the other balance. Note that no penny is weighed on both balances. This is an example of unpaired data because we evaluate each balance using a different sample of pennies.

In both examples the samples of 10 pennies were drawn from the same population; the difference is how we sampled that population. We will learn why this distinction is important when we review the significance test for paired data; first, however, we present the significance test for unpaired data.

> One simple test for determining whether data are paired or unpaired is to look at the size of each sample. If the samples are of different size, then the data must be unpaired. The converse is not true. If two samples are of equal size, they may be paired or unpaired.

Unpaired Data

Consider two analyses, *A* and *B* with means of $\overline{X}_A$ and $\overline{X}_B$, and standard deviations of s_A and s_B. The confidence intervals for μ_A and for μ_B are

$$\mu_A = \overline{X}_A \pm \frac{ts_A}{\sqrt{n_A}} \tag{3.6.4}$$

$$\mu_B = \overline{X}_B \pm \frac{ts_B}{\sqrt{n_B}} \tag{3.6.5}$$

where n_A and n_B are the sample sizes for *A* and for *B*. Our null hypothesis, H_0: $\mu_A = \mu_B$, is that and any difference between μ_A and μ_B is the result of indeterminate errors that affect the analyses. The alternative hypothesis, H_A: $\mu_A \neq \mu_B$, is that the difference between $\mu_A$and μ_B is too large to be explained by indeterminate error.

To derive an equation for t_{exp}, we assume that μ_A equals μ_B, and combine Equation 3.6.4 and Equation 3.6.5

$$\overline{X}_A \pm \frac{t_{exp}s_A}{\sqrt{n_A}} = \overline{X}_B \pm \frac{t_{exp}s_B}{\sqrt{n_B}}$$

Solving for $|\overline{X}_A - \overline{X}_B|$ and using a propagation of uncertainty, gives

$$|\overline{X}_A - \overline{X}_B| = t_{exp} \times \sqrt{\frac{s_A^2}{n_A} + \frac{s_B^2}{n_B}} \tag{3.6.6}$$

Finally, we solve for t_{exp}

$$t_{exp} = \frac{|\overline{X}_A - \overline{X}_B|}{\sqrt{\frac{s_A^2}{n_A} + \frac{s_B^2}{n_B}}} \tag{3.6.7}$$

and compare it to a critical value, $t(\alpha, \nu)$, where α is the probability of a type 1 error, and ν is the degrees of freedom.

> Problem 9 asks you to use a propagation of uncertainty to show that Equation 3.6.6 is correct.

Thus far our development of this *t*-test is similar to that for comparing $\overline{X}$ to μ, and yet we do not have enough information to evaluate the *t*-test. Do you see the problem? With two independent sets of data it is unclear how many degrees of freedom we have.

Suppose that the variances s_A^2 and s_B^2 provide estimates of the same σ^2. In this case we can replace s_A^2 and s_B^2 with a pooled variance, s_{pool}^2, that is a better estimate for the variance. Thus, Equation 3.6.7 becomes

$$t_{exp} = \frac{|\overline{X}_A - \overline{X}_B|}{s_{pool} \times \sqrt{\frac{1}{n_A} + \frac{1}{n_B}}} = \frac{|\overline{X}_A - \overline{X}_B|}{s_{pool}} \times \sqrt{\frac{n_A n_B}{n_A + n_B}} \tag{3.6.8}$$

where s_{pool}, the pooled standard deviation, is

$$s_{pool} = \sqrt{\frac{(n_A - 1)s_A^2 + (n_B - 1)s_B^2}{n_A + n_B - 2}} \tag{3.6.9}$$

The denominator of Equation 3.6.9 shows us that the degrees of freedom for a pooled standard deviation is $n_A + n_B - 2$, which also is the degrees of freedom for the *t*-test. Note that we lose two degrees of freedom because the calculations for s_A^2 and s_B^2 require the prior calculation of $\overline{X}_A$ amd $\overline{X}_B$.

So how do you determine if it is okay to pool the variances? Use an *F*-test.

If s_A^2 and s_B^2 are significantly different, then we calculate t_{exp} using Equation 3.6.7. In this case, we find the degrees of freedom using the following imposing equation.

$$\nu = \frac{\left(\frac{s_A^2}{n_A} + \frac{s_B^2}{n_B}\right)^2}{\frac{\left(\frac{s_A^2}{n_A}\right)^2}{n_A+1} + \frac{\left(\frac{s_B^2}{n_B}\right)^2}{n_B+1}} - 2 \tag{3.6.10}$$

Because the degrees of freedom must be an integer, we round to the nearest integer the value of ν obtained using Equation 3.6.10.

Equation 3.6.10, which is from Miller, J.C.; Miller, J.N. *Statistics for Analytical Chemistry*, 2nd Ed., Ellis-Horward: Chichester, UK, 1988. In the 6th Edition, the authors note that several different equations have been suggested for the number of degrees of freedom for *t* when s_A and s_B differ, reflecting the fact that the determination of degrees of freedom an approximation. An alternative equation—which is used by statistical software packages, such as R, Minitab, Excel—is

$$\nu = \frac{\left(\frac{s_A^2}{n_A} + \frac{s_B^2}{n_B}\right)^2}{\frac{\left(\frac{s_A^2}{n_A}\right)^2}{n_A-1} + \frac{\left(\frac{s_B^2}{n_B}\right)^2}{n_B-1}} = \frac{\left(\frac{s_A^2}{n_A} + \frac{s_B^2}{n_B}\right)^2}{\frac{s_A^4}{n_A^2(n_A-1)} + \frac{s_B^4}{n_B^2(n_B-1)}}$$

For typical problems in analytical chemistry, the calculated degrees of freedom is reasonably insensitive to the choice of equation.

Regardless of whether we calculate t_{exp} using Equation 3.6.7 or Equation 3.6.8, we reject the null hypothesis if t_{exp} is greater than $t(\alpha, \nu)$ and retain the null hypothesis if t_{exp} is less than or equal to $t(\alpha, \nu)$.

Example 3.6.4

Table 4.4.1 provides results for two experiments to determine the mass of a circulating U.S. penny. Determine whether there is a difference in the means of these analyses at $\alpha = 0.05$.

Solution

First we use an *F*-test to determine whether we can pool the variances. We completed this analysis in Example 3.6.3, finding no evidence of a significant difference, which means we can pool the standard deviations, obtaining

$$s_{pool} = \sqrt{\frac{(7-1)(0.051)^2 + (5-1)(0.037)^2}{7+5-2}} = 0.0459$$

with 10 degrees of freedom. To compare the means we use the following null hypothesis and alternative hypotheses

$$H_0\text{: } \mu_A = \mu_B \qquad H_A\text{: } \mu_A \neq \mu_B$$

Because we are using the pooled standard deviation, we calculate t_{exp} using Equation 3.6.8.

$$t_{exp} = \frac{|3.117 - 3.081|}{0.0459} \times \sqrt{\frac{7 \times 5}{7+5}} = 1.34$$

The critical value for *t*(0.05, 10), from Appendix 4, is 2.23. Because t_{exp} is less than *t*(0.05, 10) we retain the null hypothesis. For $\alpha = 0.05$ we do not have evidence that the two sets of pennies are significantly different.

Example 3.6.5

One method for determining the %w/w Na_2CO_3 in soda ash is to use an acid–base titration. When two analysts analyze the same sample of soda ash they obtain the results shown here.

Analyst A: 86.82% 87.04% 86.93% 87.01% 86.20% 87.00%

Analyst B: 81.01% 86.15% 81.73% 83.19% 80.27% 83.93%

Determine whether the difference in the mean values is significant at $\alpha = 0.05$.

Solution

We begin by reporting the mean and standard deviation for each analyst.

$$\overline{X}_A = 86.83\% \qquad s_A = 0.32\%$$

$$\overline{X}_B = 82.71\% \qquad s_B = 2.16\%$$

To determine whether we can use a pooled standard deviation, we first complete an *F*-test using the following null and alternative hypotheses.

$$H_0\text{: } s_A^2 = s_B^2 \qquad H_A\text{: } s_A^2 \neq s_B^2$$

Calculating F_{exp}, we obtain a value of

$$F_{\text{exp}} = \frac{(2.16)^2}{(0.32)^2} = 45.6$$

Because F_{exp} is larger than the critical value of 7.15 for $F(0.05, 5, 5)$ from Appendix 5, we reject the null hypothesis and accept the alternative hypothesis that there is a significant difference between the variances; thus, we cannot calculate a pooled standard deviation.

To compare the means for the two analysts we use the following null and alternative hypotheses.

$$H_0\text{: } \overline{X}_A = \overline{X}_B \qquad H_A\text{: } \overline{X}_A \neq \overline{X}_B$$

Because we cannot pool the standard deviations, we calculate t_{exp} using Equation 3.6.7 instead of Equation 3.6.8

$$t_{\text{exp}} = \frac{|86.83 - 82.71|}{\sqrt{\frac{(0.32)^2}{6} + \frac{(2.16)^2}{6}}} = 4.62$$

and calculate the degrees of freedom using Equation 3.6.10.

$$\nu = \frac{\left(\frac{(0.32)^2}{6} + \frac{(2.16)^2}{6}\right)^2}{\frac{\left(\frac{(0.32)^2}{6}\right)^2}{6+1} + \frac{\left(\frac{(2.16)^2}{6}\right)^2}{6+1}} - 2 = 5.3 \approx 5$$

From Appendix 4, the critical value for $t(0.05, 5)$ is 2.57. Because t_{exp} is greater than $t(0.05, 5)$ we reject the null hypothesis and accept the alternative hypothesis that the means for the two analysts are significantly different at $\alpha = 0.05$.

Exercise 3.6.3

To compare two production lots of aspirin tablets, you collect samples from each and analyze them, obtaining the following results (in mg aspirin/tablet).

Lot 1: 256 248 245 245 244 248 261

Lot 2: 241 258 241 244 256 254

Is there any evidence at $\alpha = 0.05$ that there is a significant difference in the variance between the results for these two samples? This is the same data from Exercise 3.6.2.

Answer

To compare the means for the two lots, we use an unpaired *t*-test of the null hypothesis $H_0\text{: } \overline{X}_{\text{Lot 1}} = \overline{X}_{\text{Lot 2}}$ and the alternative hypothesis $H_A\text{: } \overline{X}_{\text{Lot 1}} \neq \overline{X}_{\text{Lot 2}}$. Because there is no evidence to suggest a difference in the variances (see Exercise 3.6.2) we pool the standard deviations, obtaining an s_{pool} of

$$s_{pool} = \sqrt{\frac{(7-1)(6.451)^2 + (6-1)(7.849)^2}{7+6-2}} = 7.121$$

The means for the two samples are 249.57 mg for Lot 1 and 249.00 mg for Lot 2. The value for t_{exp} is

$$t_{exp} = \frac{|249.57 - 249.00|}{7.121} \times \sqrt{\frac{7 \times 6}{7+6}} = 0.1439$$

The critical value for $t(0.05, 11)$ is 2.204. Because t_{exp} is less than $t(0.05, 11)$, we retain the null hypothesis and find no evidence at $\alpha = 0.05$ that there is a significant difference between the means for the two lots of aspirin tablets.

Paired Data

Suppose we are evaluating a new method for monitoring blood glucose concentrations in patients. An important part of evaluating a new method is to compare it to an established method. What is the best way to gather data for this study? Because the variation in the blood glucose levels amongst patients is large we may be unable to detect a small, but significant difference between the methods if we use different patients to gather data for each method. Using paired data, in which the we analyze each patient's blood using both methods, prevents a large variance within a population from adversely affecting a *t*-test of means.

Typical blood glucose levels for most non-diabetic individuals ranges between 80–120 mg/dL (4.4–6.7 mM), rising to as high as 140 mg/dL (7.8 mM) shortly after eating. Higher levels are common for individuals who are pre-diabetic or diabetic.

When we use paired data we first calculate the difference, d_i, between the paired values for each sample. Using these difference values, we then calculate the average difference, $\bar{d}$, and the standard deviation of the differences, s_d. The null hypothesis, H_0: $d = 0$, is that there is no difference between the two samples, and the alternative hypothesis, H_A: $d \neq 0$, is that the difference between the two samples is significant.

The test statistic, t_{exp}, is derived from a confidence interval around $\bar{d}$

$$t_{exp} = \frac{|\bar{d}|\sqrt{n}}{s_d}$$

where n is the number of paired samples. As is true for other forms of the *t*-test, we compare t_{exp} to $t(\alpha, \nu)$, where the degrees of freedom, ν, is n – 1. If t_{exp} is greater than $t(\alpha, \nu)$, then we reject the null hypothesis and accept the alternative hypothesis. We retain the null hypothesis if t_{exp} is less than or equal to t(a, o). This is known as a ***paired t-test.***

Example 3.6.6

Marecek et. al. developed a new electrochemical method for the rapid determination of the concentration of the antibiotic monensin in fermentation vats [Marecek, V.; Janchenova, H.; Brezina, M.; Betti, M. *Anal. Chim. Acta* **1991**, *244*, 15–19]. The standard method for the analysis is a test for microbiological activity, which is both difficult to complete and time-consuming. Samples were collected from the fermentation vats at various times during production and analyzed for the concentration of monensin using both methods. The results, in parts per thousand (ppt), are reported in the following table.

Sample	Microbiological	Electrochemical
1	129.5	132.3
2	89.6	91.0
3	76.6	73.6
4	52.2	58.2
5	110.8	104.2
6	50.4	49.9
7	72.4	82.1
8	141.4	154.1
9	75.0	73.4
10	34.1	38.1
11	60.3	60.1

Is there a significant difference between the methods at $\alpha = 0.05$?

Solution

Acquiring samples over an extended period of time introduces a substantial time-dependent change in the concentration of monensin. Because the variation in concentration between samples is so large, we use a paired *t*-test with the following null and alternative hypotheses.

$$H_0\text{: } \bar{d} = 0 \qquad H_A\text{: } \bar{d} \neq 0$$

Defining the difference between the methods as

$$d_i = (X_{\text{elect}})_i - (X_{\text{micro}})_i$$

we calculate the difference for each sample.

sample	1	2	3	4	5	6	7	8	9	10	11
d_i	2.8	1.4	–3.0	6.0	–6.6	–0.5	9.7	12.7	–1.6	4.0	–0.2

The mean and the standard deviation for the differences are, respectively, 2.25 ppt and 5.63 ppt. The value of t_{exp} is

$$t_{\text{exp}} = \frac{|2.25|\sqrt{11}}{5.63} = 1.33$$

which is smaller than the critical value of 2.23 for $t(0.05, 10)$ from Appendix 4. We retain the null hypothesis and find no evidence for a significant difference in the methods at $\alpha = 0.05$.

Exercise 3.6.4

Suppose you are studying the distribution of zinc in a lake and want to know if there is a significant difference between the concentration of Zn^{2+} at the sediment-water interface and its concentration at the air-water interface. You collect samples from six locations—near the lake's center, near its drainage outlet, etc.—obtaining the results (in mg/L) shown in the table. Using this

data, determine if there is a significant difference between the concentration of Zn^{2+} at the two interfaces at $\alpha = 0.05$. Complete this analysis treating the data as (a) unpaired and as (b) paired. Briefly comment on your results.

Location	Air-Water Interface	Sediment-Water Interface
1	0.430	0.415
2	0.266	0.238
3	0.457	0.390
4	0.531	0.410
5	0.707	0.605
6	0.716	0.609

Complete this analysis treating the data as (a) unpaired and as (b) paired. Briefly comment on your results.

Answer

Treating as Unpaired Data: The mean and the standard deviation for the concentration of Zn^{2+} at the air-water interface are 0.5178 mg/L and 0.1732 mg/L, respectively, and the values for the sediment-water interface are 0.4445 mg/L and 0.1418 mg/L, respectively. An *F*-test of the variances gives an F_{exp} of 1.493 and an $F(0.05, 5, 5)$ of 7.146. Because F_{exp} is smaller than $F(0.05, 5, 5)$, we have no evidence at $\alpha = 0.05$ to suggest that the difference in variances is significant. Pooling the standard deviations gives an s_{pool} of 0.1582 mg/L. An unpaired *t*-test gives t_{exp} as 0.8025. Because t_{exp} is smaller than $t(0.05, 11)$, which is 2.204, we have no evidence that there is a difference in the concentration of Zn^{2+} between the two interfaces.

Treating as Paired Data: To treat as paired data we need to calculate the difference, d_i, between the concentration of Zn^{2+} at the air-water interface and at the sediment-water interface for each location, where

$$d_i = \left([Zn^{2+}]_{\text{air-water}}\right)_i - \left([Zn^{2+}]_{\text{sed-water}}\right)_i$$

The mean difference is 0.07333 mg/L with a standard deviation of 0.0441 mg/L. The null hypothesis and the alternative hypothesis are

$$H_0: \bar{d} = 0 \qquad H_A: \bar{d} \neq 0$$

and the value of t_{exp} is

$$t_{exp} = \frac{|0.07333|\sqrt{6}}{0.0441} = 4.073$$

Because t_{exp} is greater than $t(0.05, 5)$, which is 2.571, we reject the null hypothesis and accept the alternative hypothesis that there is a significant difference in the concentration of Zn^{2+} between the air-water interface and the sediment-water interface.

The difference in the concentration of Zn^{2+} between locations is much larger than the difference in the concentration of Zn^{2+} between the interfaces. Because out interest is in studying the difference between the interfaces, the larger standard deviation when treating the data as unpaired increases the probability of incorrectly retaining the null hypothesis, a type 2 error.

One important requirement for a paired *t*-test is that the determinate and the indeterminate errors that affect the analysis must be independent of the analyte's concentration. If this is not the case, then a sample with an unusually high concentration of analyte will have an unusually large d_i. Including this sample in the calculation of $\bar{d}$ and s_d gives a biased estimate for the expected mean and standard deviation. This rarely is a problem for samples that span a limited range of analyte concentrations, such as those in Example **3.6.6** or Exercise **3.6.4**. When paired data span a wide range of concentrations, however, the magnitude of the determinate and indeterminate sources of error may not be independent of the analyte's concentration; when true, a paired *t*-test may give misleading results because the paired data with the largest absolute determinate and indeterminate errors will dominate $\bar{d}$. In this situation a regression analysis, which is the subject of the next chapter, is more appropriate method for comparing the data.

Outliers

Earlier in the chapter we examined several data sets consisting of the mass of a circulating United States penny. Table **3.6.1** provides one more data set. Do you notice anything unusual in this data? Of the 112 pennies included in Table 4.4.1 and Table 4.4.3, no penny weighed less than 3 g. In Table 4.6.1, however, the mass of one penny is less than 3 g. We might ask whether this penny's mass is so different from the other pennies that it is in error.

Table **3.6.1**: Mass (g) for Additional Sample of Circulating U. S. Pennies

3.067	2.514	3.094
3.049	3.048	3.109
3.039	3.079	3.102

A measurement that is not consistent with other measurements is called outlier. An ***outlier*** might exist for many reasons: the outlier might belong to a different population (Is this a Canadian penny?); the outlier might be a contaminated or otherwise altered sample (Is the penny damaged or unusually dirty?); or the outlier may result from an error in the analysis (Did we forget to tare the balance?). Regardless of its source, the presence of an outlier compromises any meaningful analysis of our data. There are many significance tests that we can use to identify a potential outlier, three of which we present here.

Dixon's *Q*-Test

One of the most common significance tests for identifying an outlier is ***Dixon's Q-test***. The null hypothesis is that there are no outliers, and the alternative hypothesis is that there is an outlier. The *Q*-test compares the gap between the suspected outlier and its nearest numerical neighbor to the range of the entire data set (Figure **3.6.2**).

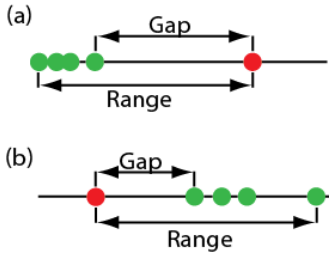


Figure **3.6.2**: Dotplots showing the distribution of two data sets containing a possible outlier. In (a) the possible outlier's value is larger than the remaining data, and in (b) the possible outlier's value is smaller than the remaining data.

The test statistic, Q_{exp}, is

$$Q_{\text{exp}} = \frac{\text{gap}}{\text{range}} = \frac{|\text{outlier's value} - \text{nearest value}|}{\text{largest value} - \text{smallest value}}$$

This equation is appropriate for evaluating a single outlier. Other forms of Dixon's *Q*-test allow its extension to detecting multiple outliers [Rorabacher, D. B. *Anal. Chem.* **1991**, *63*, 139–146].

The value of Q_{exp} is compared to a critical value, $Q(\alpha, n)$, where α is the probability that we will reject a valid data point (a type 1 error) and n is the total number of data points. To protect against rejecting a valid data point, usually we apply the more conservative two-tailed *Q*-test, even though the possible outlier is the smallest or the largest value in the data set. If Q_{exp} is greater than $Q(\alpha, n)$, then we reject the null hypothesis and may exclude the outlier. We retain the possible outlier when Q_{exp} is less than or equal to $Q(\alpha, n)$. Table **3.6.2** provides values for $Q(\alpha, n)$ for a data set that has 3–10 values. A more extensive table is in Appendix 6. Values for $Q(\alpha, n)$ assume an underlying normal distribution.

Table **3.6.2**: Dixon's Q-Test

n	$Q(0.05, n)$
3	0.970
4	0.829
5	0.710
6	0.625
7	0.568
8	0.526
9	0.493
10	0.466

Grubb's Test

Although Dixon's *Q*-test is a common method for evaluating outliers, it is no longer favored by the International Standards Organization (ISO), which recommends the ***Grubb's test***. There are several versions of Grubb's test depending on the number of potential outliers. Here we will consider the case where there is a single suspected outlier.

> For details on this recommendation, see International Standards ISO Guide 5752-2 "Accuracy (trueness and precision) of measurement methods and results–Part 2: basic methods for the determination of repeatability and reproducibility of a standard measurement method," 1994.

The test statistic for Grubb's test, G_{exp}, is the distance between the sample's mean, $\overline{X}$, and the potential outlier, X_{out}, in terms of the sample's standard deviation, *s*.

$$G_{exp} = \frac{|X_{out} - \overline{X}|}{s}$$

We compare the value of G_{exp} to a critical value $G(\alpha, n)$, where α is the probability that we will reject a valid data point and *n* is the number of data points in the sample. If G_{exp} is greater than $G(\alpha, n)$, then we may reject the data point as an outlier, otherwise we retain the data point as part of the sample. Table **3.6.3** provides values for $G(0.05, n)$ for a sample containing 3–10 values. A more extensive table is in Appendix 7. Values for $G(\alpha, n)$ assume an underlying normal distribution.

Table **3.6.3**: Grubb's Test

n	$G(0.05, n)$
3	1.115
4	1.481
5	1.715
6	1.887
7	2.020
8	2.126
9	2.215
10	2.290

Chauvenet's Criterion

Our final method for identifying an outlier is ***Chauvenet's criterion***. Unlike Dixon's *Q*-Test and Grubb's test, you can apply this method to any distribution as long as you know how to calculate the probability for a particular outcome. Chauvenet's criterion

states that we can reject a data point if the probability of obtaining the data point's value is less than $(2n)^{-1}$, where n is the size of the sample. For example, if n = 10, a result with a probability of less than $(2 \times 10)^{-1}$, or 0.05, is considered an outlier.

To calculate a potential outlier's probability we first calculate its standardized deviation, z

$$z = \frac{|X_{out} - \overline{X}|}{s}$$

where X_{out} is the potential outlier, $\overline{X}$ is the sample's mean and s is the sample's standard deviation. Note that this equation is identical to the equation for G_{exp} in the Grubb's test. For a normal distribution, we can find the probability of obtaining a value of z using the probability table in Appendix 3.

Example 3.6.7

Table 3.6.1 contains the masses for nine circulating United States pennies. One entry, 2.514 g, appears to be an outlier. Determine if this penny is an outlier using a Q-test, Grubb's test, and Chauvenet's criterion. For the Q-test and Grubb's test, let $\alpha = 0.05$.

Solution

For the Q-test the value for Qexp is

$$Q_{exp} = \frac{|2.514 - 3.039|}{3.109 - 2.514} = 0.882$$

From Table 3.6.2, the critical value for Q(0.05, 9) is 0.493. Because Q_{exp} is greater than Q(0.05, 9), we can assume the penny with a mass of 2.514 g likely is an outlier.

For Grubb's test we first need the mean and the standard deviation, which are 3.011 g and 0.188 g, respectively. The value for G_{exp} is

$$G_{exp} = \frac{|2.514 - 3.011}{0.188} = 2.64$$

Using Table 3.6.3, we find that the critical value for G(0.05, 9) is 2.215. Because G_{exp} is greater than G(0.05, 9), we can assume that the penny with a mass of 2.514 g likely is an outlier.

For Chauvenet's criterion, the critical probability is $(2 \times 9)^{-1}$, or 0.0556. The value of z is the same as G_{exp}, or 2.64. Using Appendix 3, the probability for z = 2.64 is 0.00415. Because the probability of obtaining a mass of 0.2514 g is less than the critical probability, we can assume the penny with a mass of 2.514 g likely is an outlier.

You should exercise caution when using a significance test for outliers because there is a chance you will reject a valid result. In addition, you should avoid rejecting an outlier if it leads to a precision that is much better than expected based on a propagation of uncertainty. Given these concerns it is not surprising that some statisticians caution against the removal of outliers [Deming, W. E. *Statistical Analysis of Data*; Wiley: New York, 1943 (republished by Dover: New York, 1961); p. 171].

The Median/MAD method

The Median/MAD method is the simplest of the robust methods for dealing with possible outliers. As a "robust" method none of the datum will be discarded.

The Median/Mad methods is as follows:

(1) Take the median as the estimate of the mean.

(2) To estimate the standard deviation

(a) calculate the deviations from the median for all data: $(x_i - x_{med})$

(b) arrange the differences in order of increasing magnitude (i.e. without regard to the sign)

(c) find the median absolute deviation (MAD)

(d) multiply the MAD by a factor that happens to have a value close to 1.5 (at the 95% CL)

Worked Example Using the Penny Data from Above

The following replicate observations were obtained during a measurement of the penny mass and they are arranged in descending order:

3.109, 3.102, 3.094, 3.079, 3.067, 3.049, 3.048, 3.039, 2.514 and the suspect point is 2.514

Mean of all masses: 3.011 g *Standard deviation of all masses: 0.188 g*

Reporting based on all the masses at the 95% CL would give 3.0 +/- 0.1 g

If the suspect point is discarded and the data set is eight masses

Mean of eight masses : 3.073 g *Standard deviation eight masses : 0.026 g*

Reporting based on all the data at the 95% CL would give 3.07 +/- 0.02 g

Following the Mdian/MAD methods

The estimate of the mean is the median = 3.067 g

The deviations from the median: 0.042, 0.035, 0.027, 0.012, 0, -0.018, -0.019, -0.028, -0.553

The magnitudes of deviations arranged in ascending order: 0, 0.012, 0.018, 0.019, 0.027, 0.028, 0.035, 0.042, 0.553

The MAD = 0.027

Then the estimate of the standard deviation at the 95% CL: 0.027 x 1.5 = 0.040 g

Median/MAD estimate of the mean of all data: 3.067 g

Median/Mad estimate of the standard deviation of all data: 0.040 g

And you would report your result at the 95% CL as 3.07 +/- 0.04 g

Overall recommendations for the treatment of outliers

1. re-examine data
2. what is the expected precision?
3. Repeat analysis
4. use Dixon's Q-test
5. If must retain the datum, consider reporting the median instead of mean as the median is not effected by severe outliers and use the Median/Mad method to estimate the standard deviation.

On the other hand, testing for outliers can provide useful information if we try to understand the source of the suspected outlier. For example, the outlier in Table 3.6.1 represents a significant change in the mass of a penny (an approximately 17% decrease in mass), which is the result of a change in the composition of the U.S. penny. In 1982 the composition of a U.S. penny changed from a brass alloy that was 95% w/w Cu and 5% w/w Zn (with a nominal mass of 3.1 g), to a pure zinc core covered with copper (with a nominal mass of 2.5 g) [Richardson, T. H. *J. Chem. Educ.* **1991**, *68*, 310–311]. The pennies in Table 3.6.1, therefore, were drawn from different populations.

A note about Dixon's Q, Grubbs and the Median MAD method

From the Stack Exchange (http://stats.stackexchange.com) and a Q&A for statisticians, data analysts, data miners and data visualization experts

Why would I want to use Grubbs's instead of Dixon's?

Answer: Really, these methods have been discredited long ago. For univariate outliers, the optimal (most efficient) filter is median/MAD method.

Response to comment:

Two levels.

A) Philosophical.

Both the Dixon and Grubb tests are only able to detect a particular type of (isolated) outliers. For the last 20-30 years the concept of outliers has involved unto "any observation that departs from the main body of the data". Without further specification of what the particular departure is. This characterization-free approach renders the idea of building tests to detect outliers void. The emphasize shifted to the concept of estimators (a classical example of which is the median) that retain all the values (i.e. are insensitive) even for large rate of contamination by outliers -such estimator is then said to be robust- and the question of detecting outliers becomes void.

B) Weakness,

You can see that the Grubb and Dixon tests easily break down: one can easily generate contaminated data that would pass either test like a bliss (i.e. without breaking the null). This is particularly obvious in the Grubb test, because outliers will break down the mean and s.d. used in the construction of the test stat. It's less obvious in the Dixon, until one learns that order statistics are not robust to outliers either.

If you consult any recent book/intro to robust data analysis, you will notice that neither the Grubbs test nor Dixon's Q are mentioned.

1.

> You also can adopt a more stringent requirement for rejecting data. When using the Grubb's test, for example, the ISO 5752 guidelines suggests retaining a value if the probability for rejecting it is greater than $\alpha = 0.05$, and flagging a value as a "straggler" if the probability for rejecting it is between $\alpha = 0.05$ and $\alpha = 0.01$. A "straggler" is retained unless there is compelling reason for its rejection. The guidelines recommend using $\alpha = 0.01$ as the minimum criterion for rejecting a possible outlier.

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3.7: Detection Limits

The International Union of Pure and Applied Chemistry (IUPAC) defines a method's detection limit as the smallest concentration or absolute amount of analyte that has a signal significantly larger than the signal from a suitable blank [IUPAC Compendium of Chemical Technology, Electronic Version]. Although our interest is in the amount of analyte, in this section we will define the detection limit in terms of the analyte's signal. Knowing the signal you can calculate the analyte's concentration, C_A, or the moles of analyte, n_A, using the equations

$$S_A = k_A C_A \text{ or } S_A = k_A n_A$$

where k is the method's sensitivity.

> See Chapter 3 for a review of these equations.

Let's translate the IUPAC definition of the detection limit into a mathematical form by letting S_{mb} represent the average signal for a method blank, and letting σ_{mb} represent the method blank's standard deviation. The null hypothesis is that the analyte is not present in the sample, and the alternative hypothesis is that the analyte is present in the sample. To detect the analyte, its signal must exceed S_{mb} by a suitable amount; thus,

$$(S_A)_{DL} = S_{mb} \pm z\sigma_{mb} \tag{3.7.1}$$

where $(S_A)_{DL}$ is the analyte's detection limit.

> If σ_{mb} is not known, we can replace it with s_{mb}; Equation 3.7.1 then becomes
>
> $$(S_A)_{DL} = S_{mb} \pm ts_{mb}$$
>
> You can make similar adjustments to other equations in this section. See, for example, Kirchner, C. J. "Estimation of Detection Limits for Environme tal Analytical Procedures," in Currie, L. A. (ed) *Detection in Analytical Chemistry: Importance, Theory, and Practice*; American Chemical Society: Washington, D. C., 1988.

The value we choose for z depends on our tolerance for reporting the analyte's concentration even if it is absent from the sample (a type 1 error). Typically, z is set to three, which, from Appendix 3, corresponds to a probability, α, of 0.00135. As shown in Figure 4.7.1 a, there is only a 0.135% probability of detecting the analyte in a sample that actually is analyte-free.

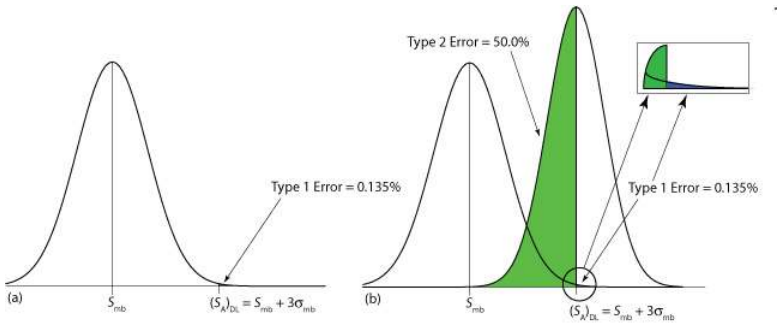


Figure 4.7.1 : Normal distribution curves showing the probability of type 1 and type 2 errors for the IUPAC detection limit. (a) The normal distribution curve for the method blank, with S_{mb} = 0 and σ_{mb} = 1. The minimum detectable signal for the analyte, $(S_A)_{DL}$, has a type 1 error of 0.135%. (b) The normal distribution curve for the analyte at its detection limit, $(S_A)_{DL}$= 3, is superimposed on the normal distribution curve for the method blank. The standard deviation for the analyte's signal, σ_A, is 0.8, The area in green represents the probability of a type 2 error, which is 50%. The inset shows, in blue, the probability of a type 1 error, which is 0.135%.

A detection limit also is subject to a type 2 error in which we fail to find evidence for the analyte even though it is present in the sample. Consider, for example, the situation shown in Figure 4.7.1 b where the signal for a sample that contains the analyte is exactly equal to $(S_A)_{DL}$. In this case the probability of a type 2 error is 50% because half of the sample's possible signals are below the detection limit. We correctly detect the analyte at the IUPAC detection limit only half the time. The IUPAC definition for the

detection limit is the smallest signal for which we can say, at a significance level of α, that an analyte is present in the sample; however, failing to detect the analyte does not mean it is not present in the sample.

The detection limit often is represented, particularly when discussing public policy issues, as a distinct line that separates detectable concentrations of analytes from concentrations we cannot detect. This use of a detection limit is incorrect [Rogers, L. B. *J. Chem. Educ.* **1986**, *63*, 3–6]. As suggested by Figure 4.7.1 , for an analyte whose concentration is near the detection limit there is a high probability that we will fail to detect the analyte.

An alternative expression for the detection limit, the ***limit of identification***, minimizes both type 1 and type 2 errors [Long, G. L.; Winefordner, J. D. *Anal. Chem.* **1983**, *55*, 712A–724A]. The analyte's signal at the limit of identification, $(S_A)_{\text{LOI}}$, includes an additional term, $z\sigma_A$, to account for the distribution of the analyte's signal.

$$(S_A)_{\text{LOI}} = (S_A)_{\text{DL}} + z\sigma_A = S_{mb} + z\sigma_{mb} + z\sigma_A$$

As shown in Figure 4.7.2 , the limit of identification provides an equal probability of a type 1 and a type 2 error at the detection limit. When the analyte's concentration is at its limit of identification, there is only a 0.135% probability that its signal is indistinguishable from that of the method blank.

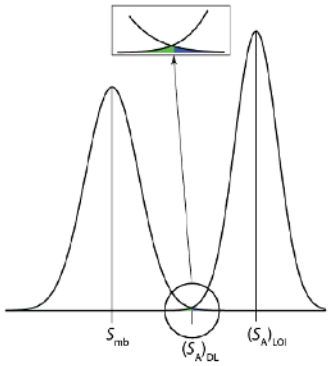


Figure 4.7.2 : Normal distribution curves for a method blank and for a sample at the limit of identification: S_{mb} = 0; $\sigma_{mb} = 1$; $\sigma_A = 0.8$; and $(S_A)_{\text{LOI}}$ = 0 + 3 × 1 + 3 × 0.8 = 5.4. The inset shows that the probability of a type 1 error (0.135%) is the same as the probability of a type 2 error (0.135%).

The ability to detect the analyte with confidence is not the same as the ability to report with confidence its concentration, or to distinguish between its concentration in two samples. For this reason the American Chemical Society's Committee on Environmental Analytical Chemistry recommends the ***limit of quantitation***, $(S_A)_{\text{LOQ}}$ ["Guidelines for Data Acquisition and Data Quality Evaluation in Environmental Chemistry," *Anal. Chem.* **1980**, *52*, 2242–2249].

$$(S_A)_{\text{LOQ}} = S_{mb} + 10\sigma_{mb}$$

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3.8: Using Excel and R to Analyze Data

Although the calculations in this chapter are relatively straightforward, it can be tedious to work problems using nothing more than a calculator. Both Excel and R include functions for many common statistical calculations. In addition, R provides useful functions for visualizing your data.

Excel

Excel has built-in functions that we can use to complete many of the statistical calculations covered in this chapter, including reporting descriptive statistics, such as means and variances, predicting the probability of obtaining a given outcome from a binomial distribution or a normal distribution, and carrying out significance tests. Table 4.8.1 provides the syntax for many of these functions; you can information on functions not included here by using Excel's Help menu.

Table 4.8.1 : Excel Functions for Statistics Calculations

Parameter	Excel Function
Descriptive Statistics	
mean	= average(data)
median	= median(data)
standard deviation for sample	= stdev.s(data)
standard deviation for populations	= stdev.p(data)
variance for sample	= var.s(data)
variance for population	= var.p(data)
maximum value	= max(data)
minimum value	= min(data)
Probability Distributions	
binomial distribution	= binom.dist(*X*, *N*, *p*, TRUE or FALSE)
normal distribution	= norm.dist(*x*, μ σ, TRUE or FALSE)
Significance Tests	
F-test	= f.test(data set 1, data set 2)
t-test	= t.test(data set 1, data set 2, tails = 1 or 2, type of *t*-test: 1 = paired; 2 = unpaired with equal variances; or 3 = unpaired with unequal variances)

Descriptive Statistics

Let's use Excel to provide a statistical summary of the data in Table 4.1.1. Enter the data into a spreadsheet, as shown in Figure 4.8.1 . To calculate the sample's mean, for example, click on any empty cell, enter the formula

= **average**(b2:b8)

and press Return or Enter to replace the cell's content with Excel's calculation of the mean (3.117285714), which we round to 3.117. Excel does not have a function for the range, but we can use the functions that report the maximum value and the minimum value to calculate the range; thus

= **max**(b2:b8) – **min**(b2:b8)

returns 0.142 as an answer.

	A	B
1		mass (g)
2		3.080
3		3.094
4		3.107
5		3.056
6		3.112
7		3.174
8		3.198

Figure 4.8.1 : Portion of a spreadsheet containing data from Table 4.1.1.

Probability Distributions

In Example 4.4.2 we showed that 91.10% of a manufacturer's analgesic tablets contained between 243 and 262 mg of aspirin. We arrived at this result by calculating the deviation, *z*, of each limit from the population's expected mean, μ, of 250 mg in terms of the population's expected standard deviation, σ, of 5 mg. After we calculated values for *z*, we used the table in Appendix 3 to find the area under the normal distribution curve between these two limits.

We can complete this calculation in Excel using the **norm.dist** function As shown in Figure 4.8.2 , the function calculates the probability of obtaining a result less than *x* from a normal distribution with a mean of μ and a standard deviation of σ. To solve Example 4.4.2 using Excel enter the following formulas into separate cells

$$= \textbf{norm.dist}(243, 250, 5, \text{TRUE})$$

$$= \textbf{norm.dist}(262, 250, 5, \text{TRUE})$$

obtaining results of 0.080756659 and 0.991802464. Subtracting the smaller value from the larger value and adjusting to the correct number of significant figures gives the probability as 0.9910, or 99.10%.

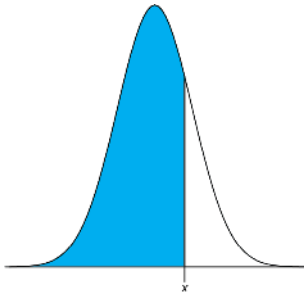


Figure 4.8.2 : Shown in blue is the area returned by the function norm.dist(*x*, μ σ, TRUE). The last parameter—TRUE—returns the cumulative distribution from $-\infty$ to x; entering FALSE gives the probability of obtaining a result greater than *x*. For our purposes, we want to use TRUE.

Excel also includes a function for working with binomial distributions. The function's syntax is

$$= \textbf{binom.dist}(X, N, p, \text{TRUE or FALSE})$$

where *X* is the number of times a particular outcome occurs in *N* trials, and *p* is the probability that *X* occurs in a single trial. Setting the function's last term to TRUE gives the total probability for any result up to *X* and setting it to FALSE gives the probability for *X*. Using Example 4.4.1 to test this function, we use the formula

$$= \textbf{binom.dist}(0, 27, 0.0111, \text{FALSE})$$

to find the probability of finding no atoms of ^{13}C atoms in a molecule of cholesterol, $C_{27}H_{44}O$, which returns a value of 0.740 after adjusting for significant figures. Using the formula

$$= \textbf{binom.dist}(2, 27, 0.0111, \text{TRUE})$$

we find that 99.7% of cholesterol molecules contain two or fewer atoms of ^{13}C.

Significance Tests

As shown in Table 4.8.1 , Excel includes functions for the following significance tests covered in this chapter:

- an *F*-test of variances
- an unpaired *t*-test of sample means assuming equal variances
- an unpaired *t*-test of sample means assuming unequal variances
- a paired *t*-test for of sample means

Let's use these functions to complete a *t*-test on the data in Table 4.4.1, which contains results for two experiments to determine the mass of a circulating U. S. penny. Enter the data from Table 4.4.1 into a spreadsheet as shown in Figure 4.8.3 .

	A	B	C
1		Set 1	Set 2
2		3.080	3.052
3		3.094	3.141
4		3.107	3.083
5		3.056	3.083
6		3.112	3.048
7		3.174	
8		3.198	

Figure 4.8.3 : Portion of a spreadsheet containing the data in Table 4.4.1.

Because the data in this case are unpaired, we will use Excel to complete an unpaired *t*-test. Before we can complete the *t*-test, we use an *F*-test to determine whether the variances for the two data sets are equal or unequal.

To complete the *F*-test, we click on any empty cell, enter the formula

$$= \textbf{f.test}(\text{b2:b8, c2:c6})$$

and press Return or Enter, which replaces the cell's content with the value of α for which we can reject the null hypothesis of equal variances. In this case, Excel returns an α of 0.566 105 03; because this value is not less than 0.05, we retain the null hypothesis that the variances are equal. Excel's *F*-test is two-tailed; for a one-tailed *F*-test, we use the same function, but divide the result by two; thus

$$= \textbf{f.test}(\text{b2:b8, c2:c6})/2$$

Having found no evidence to suggest unequal variances, we next complete an unpaired *t*-test assuming equal variances, entering into any empty cell the formula

$$= \textbf{t.test}(\text{b2:b8, c2:c6, 2, 2})$$

where the first 2 indicates that this is a two-tailed *t*-test, and the second 2 indicates that this is an unpaired *t*-test with equal variances. Pressing Return or Enter replaces the cell's content with the value of α for which we can reject the null hypothesis of equal means. In this case, Excel returns an α of 0.211 627 646; because this value is not less than 0.05, we retain the null hypothesis that the means are equal.

> See Example 4.6.3 and Example 4.6.4 for our earlier solutions to this problem.

The other significance tests in Excel work in the same format. The following practice exercise provides you with an opportunity to test yourself.

? Exercise 4.8.1

Rework Example 4.6.5 and Example 4.6.6 using Excel.

Answer

You will find small differences between the values you obtain using Excel's built in functions and the worked solutions in the chapter. These differences arise because Excel does not round off the results of intermediate calculations.

R

R is a programming environment that provides powerful capabilities for analyzing data. There are many functions built into R's standard installation and additional packages of functions are available from the R web site (www.r-project.org). Commands in R are not available from pull down menus. Instead, you interact with R by typing in commands.

> You can download the current version of R from www.r-project.org. Click on the link for Download: CRAN and find a local mirror site. Click on the link for the mirror site and then use the link for Linux, MacOS X, or Windows under the heading "Download and Install R."

Descriptive Statistics

Let's use R to provide a statistical summary of the data in Table 4.1.1. To do this we first need to create an object that contains the data, which we do by typing in the following command.

```
> penny1 = c(3.080, 3.094, 3.107, 3.056, 3.112, 3.174, 3.198)
```

> In R, the symbol '>' is a prompt, which indicates that the program is waiting for you to enter a command. When you press 'Return' or 'Enter,' R executes the command, displays the result (if there is a result to return), and returns the > prompt.

Table 4.8.2 lists some of the commands in R for calculating basic descriptive statistics. As is the case for Excel, R does not include stand alone commands for all descriptive statistics of interest to us, but we can calculate them using other commands. Using a command is easy—simply enter the appropriate code at the prompt; for example, to find the sample's variance we enter

```
> var(penny1)
[1] 0.002221918
```

Table 4.8.2 : R Functions for Descriptive Statistics

Parameter	Excel Function
mean	mean(object)
median	median(object)
standard deviation for sample	sd(object)
standard deviation for populations	sd(object) * ((length(object) – 1)/length(object))^0.5
variance for sample	var(object)
variance for population	var(object) * ((length(object) – 1)/length(object))
range	max(object) – min(object)

Probability Distributions

In Example 4.4.2 we showed that 91.10% of a manufacturer's analgesic tablets contained between 243 and 262 mg of aspirin. We arrived at this result by calculating the deviation, z, of each limit from the population's expected mean, μ, of 250 mg in terms of the population's expected standard deviation, σ, of 5 mg. After we calculated values for z, we used the table in Appendix 3 to find the area under the normal distribution curve between these two limits.

We can complete this calculation in R using the function **pnorm**. The function's general format is

$$\textbf{pnorm}(x, \mu, \sigma)$$

where x is the limit of interest, μ is the distribution's expected mean, and σ is the distribution's expected standard deviation. The function returns the probability of obtaining a result of less than x (Figure 4.8.4).

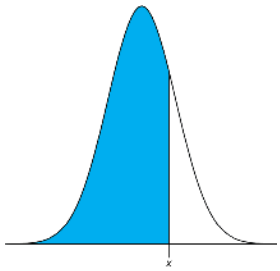


Figure 4.8.4 : Shown in blue is the area returned by the function **pnorm**(x, μ, σ).

Here is the output of an R session for solving Example 4.4.2.

> pnorm(243, 250, 5)

[1] 0.08075666

> pnorm(262, 250, 5)

[1] 0.9918025

Subtracting the smaller value from the larger value and adjusting to the correct number of significant figures gives the probability as 0.9910, or 99.10%.

R also includes functions for binomial distributions. To find the probability of obtaining a particular outcome, *X*, in *N* trials we use the **dbinom** function.

dbinom(*X*, *N*, *p*)

where *X* is the number of times a particular outcome occurs in *N* trials, and *p* is the probability that *X* occurs in a single trial. Using Example 4.4.1 to test this function, we find that the probability of finding no atoms of ^{13}C atoms in a molecule of cholesterol, $C_{27}H_{44}O$ is

> dbinom(0, 27, 0.0111)

[1] 0.7397997

0.740 after adjusting the significant figures. To find the probability of obtaining any outcome up to a maximum value of *X*, we use the **pbinom** function.

pbinom(*X*, *N*, *p*)

To find the percentage of cholesterol molecules that contain 0, 1, or 2 atoms of ^{13}C, we enter

> pbinom(2, 27, 0.0111)

[1] 0.9967226

and find that the answer is 99.7% of cholesterol molecules.

Significance Tests

R includes commands for the following significance tests covered in this chapter:

- *F*-test of variances
- unpaired *t*-test of sample means assuming equal variances
- unpaired *t*-test of sample means assuming unequal variances
- paired *t*-test for of sample means
- Dixon's *Q*-test for outliers
- Grubb's test for outliers

Let's use these functions to complete a *t*-test on the data in Table 4.4.1, which contains results for two experiments to determine the mass of a circulating U. S. penny. First, enter the data from Table 4.4.1 into two objects.

> penny1 = c(3.080, 3.094, 3.107, 3.056, 3.112, 3.174, 3.198)

> penny2 = c(3.052, 3.141, 3.083, 3.083, 3.048)

Because the data in this case are unpaired, we will use R to complete an unpaired *t*-test. Before we can complete a *t*-test we use an *F*-test to determine whether the variances for the two data sets are equal or unequal.

To complete a two-tailed *F*-test in R we use the command

var.test(*X*, *Y*)

where *X* and *Y* are the objects that contain the two data sets. Figure 4.8.5 shows the output from an R session to solve this problem.

```
> var.test(penny1, penny2)

  F test to compare two variances

data:  penny1 and penny2
F = 1.8726, num df = 6, denom df = 4, p-value = 0.5661
alternative hypothesis: true ratio of variances is not equal to 1
95 percent confidence interval:
 0.2036028 11.6609726
sample estimates:
ratio of variances
          1.872598
```

Figure 4.8.5 : Output of an R session for an *F*-test of variances. The *p*-value of 0.5661 is the probability of incorrectly rejecting the null hypothesis that the variances are equal (*note: R identifies* α *as a p-value*). The 95% confidence interval is the range of values for F_{exp} that are explained by random error. If this range includes the expected value for *F*, in this case 1.00, then there is insufficient evidence to reject the null hypothesis. Note that R does not adjust for significant figures.

Note that R does not provide the critical value for $F(0.05, 6, 4)$; instead it reports the 95% confidence interval for F_{exp}. Because this confidence interval of 0.204 to 11.661 includes the expected value for *F* of 1.00, we retain the null hypothesis and have no evidence for a difference between the variances. R also provides the probability of incorrectly rejecting the null hypothesis, which in this case is 0.5561.

For a one-tailed *F*-test the command is one of the following

var.test(*X*, *Y*, alternative = “greater”)

var.test(*X*, *Y*, alternative = “less”)

where “greater” is used when the alternative hypothesis is $s_X^2 > s_Y^2$, and “less” is used when the alternative hypothesis is $s_X^2 < s_Y^2$.

Having found no evidence suggesting unequal variances, we now complete an unpaired *t*-test assuming equal variances. The basic syntax for a two-tailed *t*-test is

t.test(*X*, *Y*, mu = 0, paired = FALSE, var.equal = FALSE)

where *X* and *Y* are the objects that contain the data sets. You can change the underlined terms to alter the nature of the *t*-test. Replacing “var.equal = FALSE” with “var.equal = TRUE” makes this a two-tailed *t*-test with equal variances, and replacing “paired = FALSE” with “paired = TRUE” makes this a paired *t*-test. The term “mu = 0” is the expected difference between the means, which for this problem is 0. You can, of course, change this to suit your needs. The underlined terms are default values; if you omit them, then R assumes you intend an unpaired two-tailed *t*-test of the null hypothesis that *X* = *Y* with unequal variances. Figure 4.8.6 shows the output of an R session for this problem.

```
> t.test(penny1, penny2, var.equal=TRUE)

  Two Sample t-test

data:  penny1 and penny2
t = 1.3345, df = 10, p-value = 0.2116
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -0.02403040  0.09580182
sample estimates:
mean of x mean of y
 3.117286  3.081400
```

Figure 4.8.6 : Output of an R session for an unpaired *t*-test with equal variances. The *p*-value of 0.2116 is the probability of incorrectly rejecting the null hypothesis that the means are equal (*note: R identifies* α *as a p-value*). The 95% confidence interval is the range of values for the difference between the means that is explained by random error. If this range includes the expected value for the difference, in this case zero, then there is insufficient evidence to reject the null hypothesis. Note that R does not adjust for significant figures.

We can interpret the results of this *t*-test in two ways. First, the *p*-value of 0.2116 means there is a 21.16% probability of incorrectly rejecting the null hypothesis. Second, the 95% confidence interval of –0.024 to 0.0958 for the difference between the sample means includes the expected value of zero. Both ways of looking at the results provide no evidence for rejecting the null hypothesis; thus, we retain the null hypothesis and find no evidence for a difference between the two samples.

The other significance tests in R work in the same format. The following practice exercise provides you with an opportunity to test yourself.

? Exercise 4.8.2

Rework Example 4.6.5 and Example 4.6.6 using R.

Answer

Shown here are copies of R sessions for each problem. You will find small differences between the values given here for t_{exp} and for F_{exp} and those values shown with the worked solutions in the chapter. These differences arise because R does not round off the results of intermediate calculations.

Example 4.6.5

> AnalystA = c(86.82, 87.04, 86.93, 87.01, 86.20, 87.00)

> AnalystB = c(81.01, 86.15, 81.73, 83.19, 80.27, 83.94)

> var.test(AnalystB, AnalystA)

F test to compare two variances

data: AnalystB and AnalystA

F = 45.6358, num df = 5, denom df = 5, p-value = 0.0007148

alternative hypothesis: true ratio of variances is not equal to 1

95 percent confidence interval:

6.385863 326.130970

sample estimates:

ratio of variances

45.63582

> t.test(AnalystA, AnalystB, var.equal=FALSE)

Welch Two Sample t-test

data: AnalystA and AnalystB

t = 4.6147, df = 5.219, p-value = 0.005177

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval: 1.852919 6.383748

sample estimates: mean of x mean of y

86.83333 82.71500

Example 4.21

> micro = c(129.5, 89.6, 76.6, 52.2, 110.8, 50.4, 72.4, 141.4, 75.0, 34.1, 60.3)
> elect = c(132.3, 91.0, 73.6, 58.2, 104.2, 49.9, 82.1, 154.1, 73.4, 38.1, 60.1)

> t.test(micro,elect,paired=TRUE)

Paired t-test

data: micro and elect

t = -1.3225, df = 10, p-value = 0.2155

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-6.028684 1.537775

sample estimates:

mean of the differences

–2.245455

Unlike Excel, R also includes functions for evaluating outliers. These functions are not part of R's standard installation. To install them enter the following command within R (*note: you will need an internet connection to download the package of functions*).

> install.packages("outliers")

After you install the package, you must load the functions into R by using the following command (*note: you need to do this step each time you begin a new R session as the package does not automatically load when you start R*).

> library("outliers")

You need to install a package once, but you need to load the package each time you plan to use it. There are ways to configure R so that it automatically loads certain packages; see *An Introduction to R* for more information (click here to view a PDF version of this document).

Let's use this package to find the outlier in Table 4.6.1 using both Dixon's *Q*-test and Grubb's test. The commands for these tests are

dixon.test(*X*, type = 10, two.sided = TRUE)

grubbs.test(*X*, type = 10, two.sided = TRUE)

where *X* is the object that contains the data, "type = 10" specifies that we are looking for one outlier, and "two.sided = TRUE" indicates that we are using the more conservative two-tailed test. Both tests have other variants that allow for the testing of outliers on both ends of the data set ("type = 11") or for more than one outlier ("type = 20"), but we will not consider these here. Figure 4.8.7 shows the output of a session for this problem. For both tests the very small *p*-value indicates that we can treat as an outlier the penny with a mass of 2.514 g.

```
> penny3=c(3.067,3.049, 3.039, 2.514, 3.048, 3.079, 3.094, 3.109, 3.102)
> dixon.test(penny3, type=10, two.sided=TRUE)

  Dixon test for outliers

data:  penny3
Q = 0.8824, p-value < 2.2e-16
alternative hypothesis: lowest value 2.514 is an outlier

> grubbs.test(penny3, type=10, two.sided=TRUE)

  Grubbs test for one outlier

data:  penny3
G = 2.6430, U = 0.0177, p-value = 1.938e-06
alternative hypothesis: lowest value 2.514 is an outlier
```

Figure 4.8.7 : Output of an R session for Dixon's *Q*-test and Grubb's test for outliers. The *p*-values for both tests show that we can treat as an outlier the penny with a mass of 2.514 g.

Visualizing Data

One of R's more useful features is the ability to visualize data. Visualizing data is important because it provides us with an intuitive feel for our data that can help us in applying and evaluating statistical tests. It is tempting to believe that a statistical analysis is foolproof, particularly if the probability for incorrectly rejecting the null hypothesis is small. Looking at a visual display of our data, however, can help us determine whether our data is normally distributed—a requirement for most of the significance tests in this chapter—and can help us identify potential outliers. There are many useful ways to look at data, four of which we consider here.

> Visualizing data is important, a point we will return to in Chapter 5 when we consider the mathematical modeling of data.

To plot data in R, we will use the package "lattice," which you will need to load using the following command.

> library("lattice")

To demonstrate the types of plots we can generate, we will use the object "penny," which contains the masses of the 100 pennies in Table 4.4.3.

> You do not need to use the command install.package this time because lattice was automatically installed on your computer when you downloaded R.

Our first visualization is a histogram. To construct the histogram we use mass to divide the pennies into bins and plot the number of pennies or the percent of pennies in each bin on the *y*-axis as a function of mass on the *x*-axis. Figure 4.8.8 shows the result of entering the command

> **histogram**(penny, type = "percent", xlab = "Mass (g)", ylab = "Percent of Pennies", main = "Histogram of Data in Table 4.4.3")

A histogram allows us to visualize the data's distribution. In this example the data appear to follow a normal distribution, although the largest bin does not include the mean of 3.095 g and the distribution is not perfectly symmetric. One limitation of a histogram is that its appearance depends on how we choose to bin the data. Increasing the number of bins and centering the bins around the data's mean gives a histogram that more closely approximates a normal distribution (Figure 4.4.5).

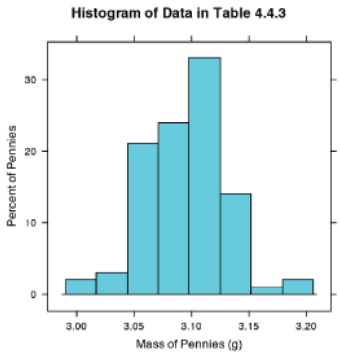


Figure 4.8.8 : Histogram of the data from Table 4.4.3.

An alternative to the histogram is a kernel density plot, which basically is a smoothed histogram. In this plot each value in the data set is replaced with a normal distribution curve whose width is a function of the data set's standard deviation and size. The resulting curve is a summation of the individual distributions. Figure 4.8.9 shows the result of entering the command

> **densityplot**(penny, xlab = "Mass of Pennies (g)", main = "Kernel Density Plot of Data in Table 4.4.3")

The circles at the bottom of the plot show the mass of each penny in the data set. This display provides a more convincing picture that the data in Table 4.4.3 are normally distributed, although we see evidence of a small clustering of pennies with a mass of approximately 3.06 g.

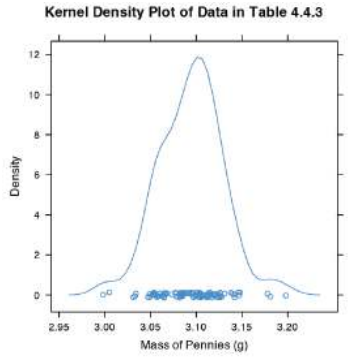


Figure 4.8.9 : Kernal plot of the data from Table 4.4.3.

We analyze samples to characterize the parent population. To reach a meaningful conclusion about a population, the samples must be representative of the population. One important requirement is that the samples are random. A dot chart provides a simple visual display that allows us to examine the data for non-random trends. Figure 4.8.10 shows the result of entering

> **dotchart**(penny, xlab = "Mass of Pennies (g)", ylab = "Penny Number", main = "Dotchart of Data in Table 4.4.3")

In this plot the masses of the 100 pennies are arranged along the *y*-axis in the order in which they were sampled. If we see a pattern in the data along the *y*-axis, such as a trend toward smaller masses as we move from the first penny to the last penny, then we have clear evidence of non-random sampling. Because our data do not show a pattern, we have more confidence in the quality of our data.

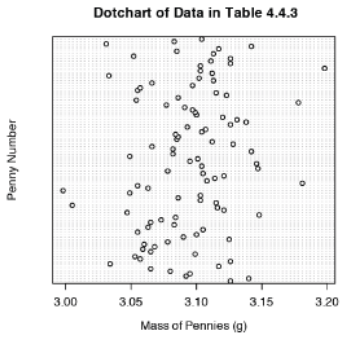


Figure 4.8.10 : Dot chart of the data from Table 4.4.3. Note that the dispersion of points along the *x*-axis is not uniform, with more points occurring near the center of the *x*-axis than at either end. This pattern is as expected for a normal distribution.

The last plot we will consider is a box plot, which is a useful way to identify potential outliers without making any assumptions about the data's distribution. A box plot contains four pieces of information about a data set: the median, the middle 50% of the data, the smallest value and the largest value within a set distance of the middle 50% of the data, and possible outliers. Figure 4.8.11 shows the result of entering

> **bwplot**(penny, xlab = "Mass of Pennies (g)", main = "Boxplot of Data in Table 4.4.3)"

The black dot (•) is the data set's median. The rectangular box shows the range of masses spanning the middle 50% of the pennies. This also is known as the interquartile range, or IQR. The dashed lines, which are called "whiskers," extend to the smallest value and the largest value that are within $\pm 1.5 \times \text{IQR}$ of the rectangular box. Potential outliers are shown as open circles (o). For normally distributed data the median is near the center of the box and the whiskers will be equidistant from the box. As is often the case in statistics, the converse is not true—finding that a boxplot is perfectly symmetric does not prove that the data are normally distributed.

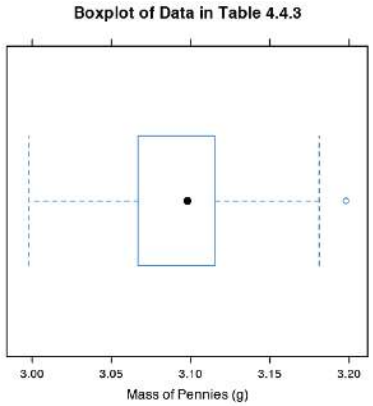


Figure 4.8.11 : Box plot of the data from Table 4.4.3.

To find the interquartile range you first find the median, which divides the data in half. The median of each half provides the limits for the box. The IQR is the median of the upper half of the data minus the median for the lower half of the data. For the data in Table 4.4.3 the median is 3.098. The median for the lower half of the data is 3.068 and the median for the upper half of the data is 3.115. The IQR is 3.115 – 3.068 = 0.047. You can use the command "summary(penny)" in R to obtain these values.

The lower "whisker" extend to the first data point with a mass larger than

$$3.068 - 1.5 \times \text{IQR} = 3.068 - 1.5 \times 0.047 = 2.9975$$

which for this data is 2.998 g. The upper "whisker" extends to the last data point with a mass smaller than

$$3.115 + 1.5 \times \text{IQR} = 3.115 + 1.5 \times 0.047 = 3.1855$$

which for this data is 3.181 g.

The box plot in Figure 4.8.11 is consistent with the histogram (Figure 4.8.8) and the kernel density plot (Figure 4.8.9). Together, the three plots provide evidence that the data in Table 4.4.3 are normally distributed. The potential outlier, whose mass of 3.198 g, is not sufficiently far away from the upper whisker to be of concern, particularly as the size of the data set (n = 100) is so large. A Grubb's test on the potential outlier does not provide evidence for treating it as an outlier.

? Exercise 4.8.3

Use R to create a data set consisting of 100 values from a uniform distribution by entering the command

> data = runif(100, min = 0, max = 100)

A uniform distribution is one in which every value between the minimum and the maximum is equally probable. Examine the data set by creating a histogram, a kernel density plot, a dot chart, and a box plot. Briefly comment on what the plots tell you about the your sample and its parent population.

Answer

Because we are selecting a random sample of 100 members from a uniform distribution, you will see subtle differences between your plots and the plots shown as part of this answer. Here is a record of my R session and the resulting plots.

> data = runif(100, min = 0, max = 0)

> data

[1] 18.928795 80.423589 39.399693 23.757624 30.088554

[6] 76.622174 36.487084 62.186771 81.115515 15.726404

[11] 85.765317 53.994179 7.919424 10.125832 93.153308

[16] 38.079322 70.268597 49.879331 73.115203 99.329723

[21] 48.203305 33.093579 73.410984 75.128703 98.682127

[26] 11.433861 53.337359 81.705906 95.444703 96.843476

[31] 68.251721 40.567993 32.761695 74.635385 70.914957

[36] 96.054750 28.448719 88.580214 95.059215 20.316015

[41] 9.828515 44.172774 99.648405 85.593858 82.745774

[46] 54.963426 65.563743 87.820985 17.791443 26.417481

[51] 72.832037 5.518637 58.231329 10.213343 40.581266

[56] 6.584000 81.261052 48.534478 51.830513 17.214508

[61] 31.232099 60.545307 19.197450 60.485374 50.414960

[66] 88.908862 68.939084 92.515781 72.414388 83.195206

[71] 74.783176 10.643619 41.775788 20.464247 14.547841

[76] 89.887518 56.217573 77.606742 26.956787 29.641171

[81] 97.624246 46.406271 15.906540 23.007485 17.715668

[86] 84.652814 29.379712 4.093279 46.213753 57.963604

[91] 91.160366 34.278918 88.352789 93.004412 31.055807

[96] 47.822329 24.052306 95.498610 21.089686 2.629948

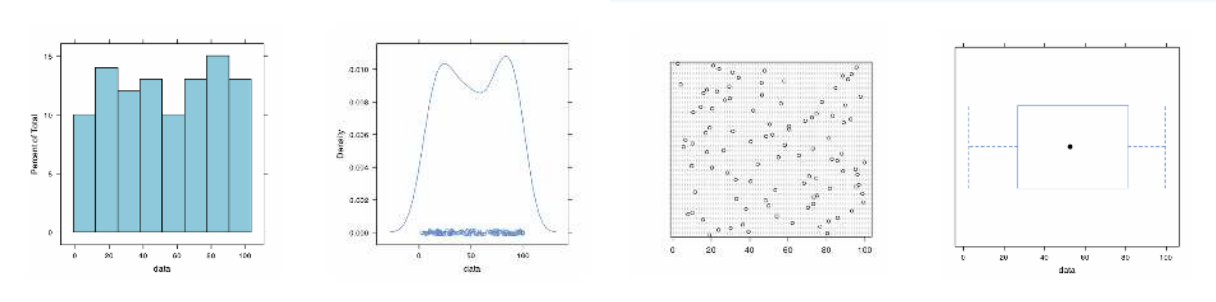
> histogram(data, type = "percent")

> densityplot(data)

> dotchart(data)

> bwplot(data)

The histogram (far left) divides the data into eight bins, each of which contains between 10 and 15 members. As we expect for a uniform distribution, the histogram's overall pattern suggests that each outcome is equally probable. In interpreting the kernel density plot (second from left), it is important to remember that it treats each data point as if it is from a normally distributed population (even though, in this case, the underlying population is uniform). Although the plot appears to suggest that there are two normally distributed populations, the individual results shown at the bottom of the plot provide further evidence for a uniform distribution. The dot chart (second from right) shows no trend along the *y*-axis, which indicates that the individual members of this sample were drawn at random from the population. The distribution along the *x*-axis also shows no pattern, as expected for a uniform distribution, Finally, the box plot (far right) shows no evidence of outliers.



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3.9: Problems

1. The following masses were recorded for 12 different U.S. quarters (all given in grams):

5.683	5.549	5.548	5.552
5.620	5.536	5.539	5.684
5.551	5.552	5.554	5.632

Report the mean, median, range, standard deviation and variance for this data.

2. A determination of acetaminophen in 10 separate tablets of Excedrin Extra Strength Pain Reliever gives the following results (in mg)

224.3	240.4	246.3	239.4	253.1
261.7	229.4	255.5	235.5	249.7

(a) Report the mean, median, range, standard deviation and variance for this data.

(b) Assuming that $\overline{X}$ and s^2 are good approximations for μ and for σ^2, and that the population is normally distributed, what percentage of tablets contain more than the standard amount of 250 mg acetaminophen per tablet?

The data in this problem are from Simonian, M. H.; Dinh, S.; Fray, L. A. *Spectroscopy* **1993**, *8(6)*, 37–47.

3. Salem and Galan developed a new method to determine the amount of morphine hydrochloride in tablets. An analysis of tablets with different nominal dosages gave the following results (in mg/tablet).

100-mg tablets	60-mg tablets	30-mg tablets	10-mg tablets
99.17	54.21	28.51	9.06
94.31	55.62	26.25	8.83
95.92	57.40	25.92	9.08
94.55	57.51	28.62	
93.83	52.59	24.93	

(a) For each dosage, calculate the mean and the standard deviation for the mg of morphine hydrochloride per tablet.

(b) For each dosage level, and assuming that $\overline{X}$ and s^2 are good approximations for μ and for σ^2, and that the population is normally distributed, what percentage of tablets contain more than the nominal amount of morphine hydro- chloride per tablet?

The data in this problem are from Salem, I. I.; Galan, A. C. Anal. Chim. Acta 1993, 283, 334–337.

4. Daskalakis and co-workers evaluated several procedures for digesting oyster and mussel tissue prior to analyzing them for silver. To evaluate the procedures they spiked samples with known amounts of silver and analyzed the samples to determine the amount of silver, reporting results as the percentage of added silver found in the analysis. A procedure was judged acceptable if its spike recoveries fell within the range 100±15%. The spike recoveries for one method are shown here.

105%	108%	92%	99%
101%	93%	93%	104%

Assuming a normal distribution for the spike recoveries, what is the probability that any single spike recovery is within the accepted range?

The data in this problem are from Daskalakis, K. D.; O'Connor, T. P.; Crecelius, E. A. *Environ. Sci. Technol.* **1997**, *31*, 2303– 2306. See Chapter 15 to learn more about using a spike recovery to evaluate an analytical method.

5. The formula weight (*FW*) of a gas can be determined using the following form of the ideal gas law

$$FW = \frac{gRT}{PV}$$

where g is the mass in grams, R is the gas constant, T is the temperature in Kelvin, P is the pressure in atmospheres, and V is the volume in liters. In a typical analysis the following data are obtained (with estimated uncertainties in parentheses)

g = 0.118 g (± 0.002 g)

R = 0.082056 L atm mol^{-1} K^{-1} (± 0.000001 L atm mol^{-1} K^{-1})

T = 298.2 K (± 0.1 K)

P = 0.724 atm (± 0.005 atm)

V = 0.250 L (± 0.005 L)

(a) What is the compound's formula weight and its estimated uncertainty?

(b) To which variable(s) should you direct your attention if you wish to improve the uncertainty in the compound's molecular weight?

6. To prepare a standard solution of Mn^{2+}, a 0.250 g sample of Mn is dissolved in 10 mL of concentrated HNO_3 (measured with a graduated cylinder). The resulting solution is quantitatively transferred to a 100-mL volumetric flask and diluted to volume with distilled water. A 10-mL aliquot of the solution is pipeted into a 500-mL volumetric flask and diluted to volume.

(a) Express the concentration of Mn in mg/L, and estimate its uncertainty using a propagation of uncertainty.

(b) Can you improve the concentration's uncertainty by using a pipet to measure the HNO_3, instead of a graduated cylinder?

7. The mass of a hygroscopic compound is measured using the technique of weighing by difference. In this technique the compound is placed in a sealed container and weighed. A portion of the compound is removed and the container and the remaining material are reweighed. The difference between the two masses gives the sample's mass. A solution of a hygroscopic compound with a gram formula weight of 121.34 g/mol (±0.01 g/mol) is prepared in the following manner. A sample of the compound and its container has a mass of 23.5811 g. A portion of the compound is transferred to a 100-mL volumetric flask and diluted to volume. The mass of the compound and container after the transfer is 22.1559 g. Calculate the compound's molarity and estimate its uncertainty by a propagation of uncertainty.

8. Use a propagation of uncertainty to show that the standard error of the mean for n determinations is $\sigma/\sqrt{n}$.

9. Beginning with Equation 4.6.4 and Equation 4.6.5, use a propagation of uncertainty to derive Equation 4.6.6.

10. What is the smallest mass you can measure on an analytical balance that has a tolerance of ±0.1 mg, if the relative error must be less than 0.1%?

11. Which of the following is the best way to dispense 100.0 mL if we wish to minimize the uncertainty: (a) use a 50-mL pipet twice; (b) use a 25-mL pipet four times; or (c) use a 10-mL pipet ten times?

12. You can dilute a solution by a factor of 200 using readily available pipets (1-mL to 100-mL) and volumetric flasks (10-mL to 1000-mL) in either one step, two steps, or three steps. Limiting yourself to the glassware in Table 4.2.1, determine the proper combination of glassware to accomplish each dilution, and rank them in order of their most probable uncertainties.

13. Explain why changing all values in a data set by a constant amount will change $\overline{X}$ but has no effect on the standard deviation, s.

14. Obtain a sample of a metal, or other material, from your instructor and determine its density by one or both of the following methods:

> **Method A**: Determine the sample's mass with a balance. Calculate the sample's volume using appropriate linear dimensions.
>
> **Method B**: Determine the sample's mass with a balance. Calculate the sample's volume by measuring the amount of water it displaces by adding water to a graduated cylinder, reading the volume, adding the sample, and reading the new volume. The

difference in volumes is equal to the sample's volume.

Determine the density at least five times.

(a) Report the mean, the standard deviation, and the 95% confidence interval for your results.

(b) Find the accepted value for the metal's density and determine the absolute and relative error for your determination of the metal's density.

(c) Use a propagation of uncertainty to determine the uncertainty for your method of analysis. Is the result of this calculation consistent with your experimental results? If not, suggest some possible reasons for this disagreement.

15. How many carbon atoms must a molecule have if the mean number of ^{13}C atoms per molecule is at least one? What percentage of such molecules will have no atoms of ^{13}C?

16. In Example 4.4.1 we determined the probability that a molecule of cholesterol, $C_{27}H_{44}O$, had no atoms of ^{13}C.

(a) Calculate the probability that a molecule of cholesterol, has 1 atom of ^{13}C.

(b) What is the probability that a molecule of cholesterol has two or more atoms of ^{13}C?

17. Berglund and Wichardt investigated the quantitative determination of Cr in high-alloy steels using a potentiometric titration of Cr(VI). Before the titration, samples of the steel were dissolved in acid and the chromium oxidized to Cr(VI) using peroxydisulfate. Shown here are the results (as %w/w Cr) for the analysis of a reference steel.

16.968	16.922	16.840	16.883
16.887	16.977	16.857	16.728

Calculate the mean, the standard deviation, and the 95% confidence interval about the mean. What does this confidence interval mean?

The data in this problem are from Berglund, B.; Wichardt, C. *Anal. Chim. Acta* **1990**, *236*, 399–410.

18. Ketkar and co-workers developed an analytical method to determine trace levels of atmospheric gases. An analysis of a sample that is 40.0 parts per thousand (ppt) 2-chloroethylsulfide gave the following results

43.3	34.8	31.9
37.8	34.4	31.9
42.1	33.6	35.3

(a) Determine whether there is a significant difference between the experimental mean and the expected value at $\alpha = 0.05$.

(b) As part of this study, a reagent blank was analyzed 12 times giving a mean of 0.16 ppt and a standard deviation of 1.20 ppt. What are the IUPAC detection limit, the limit of identification, and limit of quantitation for this method assuming $\alpha = 0.05$?

The data in this problem are from Ketkar, S. N.; Dulak, J. G.; Dheandhanou, S.; Fite, W. L. *Anal. Chim. Acta* **1991**, *245*, 267–270.

19. To test a spectrophotometer's accuracy a solution of 60.06 ppm $K_2Cr_2O_7$ in 5.0 mM H_2SO_4 is prepared and analyzed. This solution has an expected absorbance of 0.640 at 350.0 nm in a 1.0-cm cell when using 5.0 mM H_2SO_4 as a reagent blank. Several aliquots of the solution produce the following absorbance values.

0.639	0.638	0.640	0.639	0.640	0.639	0.638

Determine whether there is a significant difference between the experimental mean and the expected value at $\alpha = 0.01$.

20. Monna and co-workers used radioactive isotopes to date sediments from lakes and estuaries. To verify this method they analyzed a ^{208}Po standard known to have an activity of 77.5 decays/min, obtaining the following results.

77.09	75.37	72.42	76.84	77.84	76.69
78.03	74.96	77.54	76.09	81.12	75.75

Determine whether there is a significant difference between the mean and the expected value at $\alpha = 0.05$.

The data in this problem are from Monna, F.; Mathieu, D.; Marques, A. N.; Lancelot, J.; Bernat, M. *Anal. Chim. Acta* **1996**, *330*, 107–116.

21. A 2.6540-g sample of an iron ore, which is 53.51% w/w Fe, is dissolved in a small portion of concentrated HCl and diluted to volume in a 250-mL volumetric flask. A spectrophotometric determination of the concentration of Fe in this solution yields results of 5840, 5770, 5650, and 5660 ppm. Determine whether there is a significant difference between the experimental mean and the expected value at $\alpha = 0.05$.

22. Horvat and co-workers used atomic absorption spectroscopy to determine the concentration of Hg in coal fly ash. Of particular interest to the authors was developing an appropriate procedure for digesting samples and releasing the Hg for analysis. As part of their study they tested several reagents for digesting samples. Their results using HNO_3 and using a 1 + 3 mixture of HNO_3 and HCl are shown here. All concentrations are given as ppb Hg sample.

HNO_3:	161	165	160	167	166	
1 + 3 HNO_3 – HCl:	159	145	154	147	143	156

Determine whether there is a significant difference between these methods at $\alpha = 0.05$.

The data in this problem are from Horvat, M.; Lupsina, V.; Pihlar, B. *Anal. Chim. Acta* **1991**, *243*, 71–79.

23, Lord Rayleigh, John William Strutt (1842-1919), was one of the most well known scientists of the late nineteenth and early twentieth centuries, publishing over 440 papers and receiving the Nobel Prize in 1904 for the discovery of argon. An important turning point in Rayleigh's discovery of Ar was his experimental measurements of the density of N_2. Rayleigh approached this experiment in two ways: first by taking atmospheric air and removing O_2 and H_2; and second, by chemically producing N_2 by decomposing nitrogen containing compounds (NO, N_2O, and NH_4NO_3) and again removing O_2 and H_2. The following table shows his results for the density of N_2, as published in *Proc. Roy. Soc.* **1894**, *LV*, 340 (publication 210); all values are the grams of gas at an equivalent volume, pressure, and temperature.

atmospheric origin	chemical origin
2.31017	2.30143
2.30986	2.29890
2.31010	2.29816
2.31001	2.30182
2.31024	2.29869
2.31010	2.29940
2.31028	2.29849
	2.29889

Explain why this data led Rayleigh to look for and to discover Ar. You can read more about this discovery here: Larsen, R. D. *J. Chem. Educ.* **1990**, *67*, 925–928.

24. Gács and Ferraroli reported a method for monitoring the concentration of SO_2 in air. They compared their method to the standard method by analyzing urban air samples collected from a single location. Samples were collected by drawing air through a collection solution for 6 min. Shown here is a summary of their results with SO_2 concentrations reported in $\mu L/m^3$.

standard method	new method
21.62	21.54
22.20	20.51
24.27	22.31
23.54	21.30
24.25	24.62
23.09	25.72
21.02	21.54

Using an appropriate statistical test, determine whether there is any significant difference between the standard method and the new method at $\alpha = 0.05$.

The data in this problem are from Gács, I.; Ferraroli, R. *Anal. Chim. Acta* **1992**, *269*, 177–185.

25. One way to check the accuracy of a spectrophotometer is to measure absorbances for a series of standard dichromate solutions obtained from the National Institute of Standards and Technology. Absorbances are measured at 257 nm and compared to the accepted values. The results obtained when testing a newly purchased spectrophotometer are shown here. Determine if the tested spectrophotometer is accurate at $\alpha = 0.05$.

standard	measured absorbance	expected absorbance
1	0.2872	0.2871
2	0.5773	0.5760
3	0.8674	0.8677
4	1.1623	1.1608
5	1.4559	1.4565

26. Maskarinec and co-workers investigated the stability of volatile organics in environmental water samples. Of particular interest was establishing the proper conditions to maintain the sample's integrity between its collection and its analysis. Two preservatives were investigated—ascorbic acid and sodium bisulfate—and maximum holding times were determined for a number of volatile organics and water matrices. The following table shows results for the holding time (in days) of nine organic compounds in surface water.

compound	Ascorbic Acid	Sodium Bisulfate
methylene chloride	77	62
carbon disulfide	23	54
trichloroethane	52	51
benzene	62	42
1,1,2-trichlorethane	57	53
1,1,2,2-tetrachloroethane	33	85
tetrachloroethene	32	94
chlorbenzene	36	86

Determine whether there is a significant difference in the effectiveness of the two preservatives at $\alpha = 0.10$.

The data in this problem are from Maxkarinec, M. P.; Johnson, L. H.; Holladay, S. K.; Moody, R. L.; Bayne, C. K.; Jenkins, R. A. *Environ. Sci. Technol.* **1990**, *24*, 1665–1670.

27. Using X-ray diffraction, Karstang and Kvalhein reported a new method to determine the weight percent of kaolinite in complex clay minerals using X-ray diffraction. To test the method, nine samples containing known amounts of kaolinite were prepared and analyzed. The results (as % w/w kaolinite) are shown here.

actual	5.0	10.0	20.0	40.0	50.0	60.0	80.0	90.0	95.0
found	6.8	11.7	19.8	40.5	53.6	61.7	78.9	91.7	94.7

Evaluate the accuracy of the method at $\alpha = 0.05$.

The data in this problem are from Karstang, T. V.; Kvalhein, O. M. *Anal. Chem.* **1991**, *63*, 767–772.

28. Mizutani, Yabuki and Asai developed an electrochemical method for analyzing *l*-malate. As part of their study they analyzed a series of beverages using both their method and a standard spectrophotometric procedure based on a clinical kit purchased from Boerhinger Scientific. The following table summarizes their results. All values are in ppm.

Sample	Electrode	Spectrophotometric
Apple Juice 1	34.0	33.4
Apple Juice 2	22.6	28.4
Apple Juice 3	29.7	29.5
Apple Juice 4	24.9	24.8
Grape Juice 1	17.8	18.3
Grape Juice 2	14.8	15.4
Mixed Fruit Juice 1	8.6	8.5
Mixed Fruit Juice 2	31.4	31.9
White Wine 1	10.8	11.5
White Wine 2	17.3	17.6
White Wine 3	15.7	15.4
White Wine 4	18.4	18.3

The data in this problem are from Mizutani, F.; Yabuki, S.; Asai, M. *Anal. Chim. Acta* **1991**, *245*,145–150.

29. Alexiev and colleagues describe an improved photometric method for determining Fe^{3+} based on its ability to catalyze the oxidation of sulphanilic acid by KIO_4. As part of their study, the concentration of Fe^{3+} in human serum samples was determined by the improved method and the standard method. The results, with concentrations in $\mu\text{mol/L}$, are shown in the following table.

Sample	Improved Method	Standard Method
1	8.25	8.06
2	9.75	8.84
3	9.75	8.36
4	9.75	8.73
5	10.75	13.13
6	11.25	13.65
7	13.88	13.85
8	14.25	13.43

Determine whether there is a significant difference between the two methods at $\alpha = 0.05$.

The data in this problem are from Alexiev, A.; Rubino, S.; Deyanova, M.; Stoyanova, A.; Sicilia, D.; Perez Bendito, D. *Anal. Chim. Acta*, **1994**, *295*, 211–219.

30. Ten laboratories were asked to determine an analyte's concentration of in three standard test samples. Following are the results, in $\mu\text{g/ml}$.

Laboratory	Sample 1	Sample 2	Sample 3
1	22.6	13.6	16.0
2	23.0	14.9	15.9
3	21.5	13.9	16.9
4	21.9	13.9	16.9
5	21.3	13.5	16.7
6	22.1	13.5	17.4
7	23.1	13.5	17.5
8	21.7	13.5	16.8
9	22.2	13.0	17.2
10	21.7	13.8	16.7

Determine if there are any potential outliers in Sample 1, Sample 2 or Sample 3. Use all three methods—Dixon's *Q*-test, Grubb's test, and Chauvenet's criterion—and compare the results to each other. For Dixon's *Q*-test and for the Grubb's test, use a significance level of $\alpha = 0.05$.

The data in this problem are adapted from Steiner, E. H. "Planning and Analysis of Results of Collaborative Tests," in *Statistical Manual of the Association of Official Analytical Chemists*, Association of Official Analytical Chemists: Washington, D. C., 1975.

31.When copper metal and powdered sulfur are placed in a crucible and ignited, the product is a sulfide with an empirical formula of Cu_xS. The value of *x* is determined by weighing the Cu and the S before ignition and finding the mass of Cu_xS when the reaction is complete (any excess sulfur leaves as SO_2). The following table shows the Cu/S ratios from 62 such experiments (note that the values are organized from smallest-to-largest by rows).

1.764	1.838	1.865	1.866	1.872	1.877
1.890	1.891	1.891	1.897	1.899	1.900
1.906	1.908	1.910	1.911	1.916	1.919
1.920	1.922	1.927	1.931	1.935	1.936
1.936	1.937	1.939	1.939	1.940	1.941
1.941	1.942	1.943	1.948	1.953	1.955
1.957	1.957	1.957	1.959	1.962	1.963
1.963	1.963	1.966	1.968	1.969	1.973
1.975	1.976	1.977	1.981	1.981	1.988
1.993	1.993	1.995	1.995	1.995	2.017
2.029	2.042				

(a) Calculate the mean, the median, and the standard deviation for this data.

(b) Construct a histogram for this data. From a visual inspection of your histogram, do the data appear normally distributed?

(c) In a normally distributed population 68.26% of all members lie within the range $\mu \pm 1\sigma$. What percentage of the data lies within the range $\overline{X} \pm 1\sigma$? Does this support your answer to the previous question?

(d) Assuming that $\overline{X}$ and s^2 are good approximations for μ and for σ^2, what percentage of all experimentally determined Cu/S ratios should be greater than 2? How does this compare with the experimental data? Does this support your conclusion about whether the data is normally distributed?

(e) It has been reported that this method of preparing copper sulfide results in a non-stoichiometric compound with a Cu/S ratio of less than 2. Determine if the mean value for this data is significantly less than 2 at a significance level of $\alpha = 0.01$.

See Blanchnik, R.; Müller, A. "The Formation of Cu_2S From the Elements I. Copper Used in Form of Powders," *Thermochim. Acta*, **2000**, *361*, 31-52 for a discussion of some of the factors affecting the formation of non-stoichiometric copper sulfide. The data in this problem were collected by students at DePauw University.

32. Real-time quantitative PCR is an analytical method for determining trace amounts of DNA. During the analysis, each cycle doubles the amount of DNA. A probe species that fluoresces in the presence of DNA is added to the reaction mixture and the increase in fluorescence is monitored during the cycling. The cycle threshold, C_t, is the cycle when the fluorescence exceeds a threshold value. The data in the following table shows C_t values for three samples using real-time quantitative PCR. Each sample was analyzed 18 times.

Sample X		Sample Y		Sample Z	
24.24	25.14	24.41	28.06	22.97	23.43
23.97	24.57	27.21	27.77	22.93	23.66
24.44	24.49	27.02	28.74	22.95	28.79
24.79	24.68	26.81	28.35	23.12	23.77
23.92	24.45	26.64	28.80	23.59	23.98
24.53	24,48	27.63	27.99	23.37	23.56
24.95	24.30	28.42	28.21	24.17	22.80
24.76	24.60	25.16	28.00	23.48	23.29
25.18	24.57	28.53	28.21	23.80	23.86

Examine this data and write a brief report on your conclusions. Issues you may wish to address include the presence of outliers in the samples, a summary of the descriptive statistics for each sample, and any evidence for a difference between the samples.

The data in this problem is from Burns, M. J.; Nixon, G. J.; Foy, C. A.; Harris, N. *BMC Biotechnol.* **2005**, *5:31* (open access publication).

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3.10: Additional Resources

The following experiments provide useful introductions to the statistical analysis of data in the analytical chemistry laboratory.

- Bularzik, J. “The Penny Experiment Revisited: An Illustration of Significant Figures, Accuracy, Precision, and Data Analysis,” *J. Chem. Educ.* **2007**, *84*, 1456–1458.
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- Cunningham, C. C.; Brown, G. R.; St Pierre, L. E. “Evaluation of Experimental Data,” *J. Chem. Educ.* **1981**, *58*, 509–511.
- Edminston, P. L.; Williams, T. R. “An Analytical Laboratory Experiment in Error Analysis: Repeated Determination of Glucose Using Commercial Glucometers,” *J. Chem. Educ.* **2000**, *77*, 377–379.
- Gordus, A. A. “Statistical Evaluation of Class Data for Two Buret Readings,” *J. Chem. Educ.* **1987**, *64*, 376–377.
- Harvey, D. T. “Statistical Evaluation of Acid/Base Indicators,” *J. Chem. Educ.* **1991**, *68*, 329–331.
- Hibbert, D. B. “Teaching modern data analysis with The Royal Austrian Chemical Institute’s titration competition,” *Aust. J. Ed. Chem.* **2006**, *66*, 5–11.
- Johll, M. E.; Poister, D.; Ferguson, J. “Statistical Comparison of Multiple Methods for the Determination of Dissolved Oxygen Levels in Natural Water,” *Chem. Educator* **2002**, *7*, 146–148.
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- Samide, M. J. “Statistical Comparison of Data in the Analytical Laboratory,” *J. Chem. Educ.* **2004**, *81*, 1641–1643.
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- Spencer, R. D. “The Dependence of Strength in Plastics upon Polymer Chain Length and Chain Orientation,” *J. Chem. Educ.* **1984**, *61*, 555–563.
- Stolzberg, R. J. “Do New Pennies Lose Their Shells? Hypothesis Testing in the Sophomore Analytical Chemistry Laboratory,” *J. Chem. Educ.* **1998**, *75*, 1453–1455.
- Stone, C. A.; Mumaw, L. D. “Practical Experiments in Statistics,” *J. Chem. Educ.* **1995**, *72*, 518– 524.
- Thomasson, K.; Lofthus-Merschman, S.; Humbert, M.; Kulevsky, N. “Applying Statistics in the Undergraduate Chemistry Laboratory: Experiments with Food Dyes,” *J. Chem. Educ.* **1998**, *75*, 231–233.
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A more comprehensive discussion of the analysis of data, which includes all topics considered in this chapter as well as additional material, are found in many textbook on statistics or data analysis; several such texts are listed here.

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3.11: Chapter Summary and Key Terms

Summary

The data we collect are characterized by their central tendency (where the values cluster), and their spread (the variation of individual values around the central value). We report our data's central tendency by stating the mean or median, and our data's spread using the range, standard deviation or variance. Our collection of data is subject to errors, including determinate errors that affect the data's accuracy and indeterminate errors that affect its precision. A propagation of uncertainty allows us to estimate how these determinate and indeterminate errors affect our results.

When we analyze a sample several times the distribution of the results is described by a probability distribution, two examples of which are the binomial distribution and the normal distribution. Knowing the type of distribution allows us to determine the probability of obtaining a particular range of results. For a normal distribution we express this range as a confidence interval.

A statistical analysis allows us to determine whether our results are significantly different from known values, or from values obtained by other analysts, by other methods of analysis, or for other samples. We can use a *t*-test to compare mean values and an *F*-test to compare variances. To compare two sets of data you first must determine whether the data is paired or unpaired. For unpaired data you also must decide if you can pool the standard deviations. A decision about whether to retain an outlying value can be made using Dixon's *Q*-test, Grubb's test, or Chauvenet's criterion.

You should be sure to exercise caution if you decide to reject an outlier. Finally, the detection limit is a statistical statement about the smallest amount of analyte we can detect with confidence. A detection limit is not exact since its value depends on how willing we are to falsely report the analyte's presence or absence in a sample. When reporting a detection limit you should clearly indicate how you arrived at its value.

Key Terms

alternative hypothesis	bias	binomial distribution
box plot	central limit theorem	Chauvenet's criterion
confidence interval	constant determinate error	degrees of freedom
detection limit	determinate error	Dixon's *Q*-test
dot chart	error	*F*-test
Grubb's test	histogram	indeterminate error
kernel density plot	limit of identification	limit of quantitation
mean	median	measurement error
method error	normal distribution	null hypothesis
one-tailed significance test	outlier	paired data
paired t-test	personal error	population
probability distribution	propagation of uncertainty	proportional determinate error
range	repeatability	reproducibility
sample	sampling error	significance test
standard deviation	standard error of the mean	Standard Reference Material
tolerance	*t*-test	two-tailed significance test
type 1 error	type 2 error	uncertainty
unpaired data	variance	

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CHAPTER OVERVIEW

4: The Vocabulary of Analytical Chemistry

If you browse through an issue of the journal *Analytical Chemistry*, you will discover that the authors and readers share a common vocabulary of analytical terms. You probably are familiar with some of these terms, such as accuracy and precision, but other terms, such as analyte and matrix, are perhaps less familiar to you. In order to participate in any community, one must first understand its vocabulary; the goal of this chapter, therefore, is to introduce some important analytical terms. Becoming comfortable with these terms will make the chapters that follow easier to read and to understand.

4.1: Analysis, Determination, and Measurement
4.2: Techniques, Methods, Procedures, and Protocols
4.3: Classifying Analytical Techniques
4.4: Selecting an Analytical Method
4.5: Developing the Procedure
4.6: Protocols
4.7: The Importance of Analytical Methodology
4.8: Problems
4.9: Additional Resources
4.10: Chapter Summary and Key Terms

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4.1: Analysis, Determination, and Measurement

The first important distinction we will make is among the terms analysis, determination, and measurement. An analysis provides chemical or physical information about a sample. The component in the sample of interest to us is called the ***analyte***, and the remainder of the sample is the matrix. In an analysis we determine the identity, the concentration, or the properties of an analyte. To make this determination we measure one or more of the analyte's chemical or physical properties.

An example will help clarify the difference between an ***analysis***, a ***determination*** and a ***measurement***. In 1974 the federal government enacted the Safe Drinking Water Act to ensure the safety of the nation's public drinking water supplies. To comply with this act, municipalities monitor their drinking water supply for potentially harmful substances, such as fecal coliform bacteria. Municipal water departments collect and analyze samples from their water supply. To determine the concentration of fecal coliform bacteria an analyst passes a portion of water through a membrane filter, places the filter in a dish that contains a nutrient broth, and incubates the sample for 22–24 hrs at 44.5 ^{o}C ± 0.2 ^{o}C. At the end of the incubation period the analyst counts the number of bacterial colonies in the dish and reports the result as the number of colonies per 100 mL (Figure 3.1.1). Thus, a municipal water department analyzes samples of water to determine the concentration of fecal coliform bacteria by measuring the number of bacterial colonies that form during a carefully defined incubation period

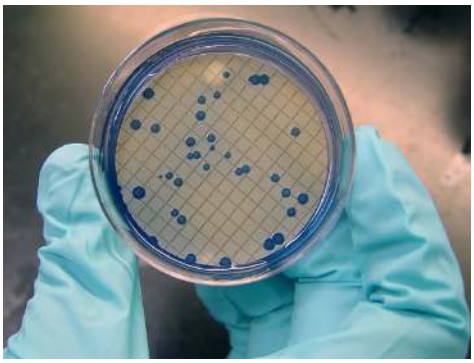


Figure 3.1.1 : Colonies of fecal coliform bacteria from a water supply. Source: Susan Boyer. Photo courtesy of ARS–USDA (www.ars.usda.gov).

> A fecal coliform count provides a general measure of the presence of pathogenic organisms in a water supply. For drinking water, the current maximum contaminant level (MCL) for total coliforms, including fecal coliforms is less than 1 colony/100 mL. Municipal water departments must regularly test the water supply and must take action if more than 5% of the samples in any month test positive for coliform bacteria.

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4.2: Techniques, Methods, Procedures, and Protocols

Suppose you are asked to develop an analytical method to determine the concentration of lead in drinking water. How would you approach this problem? To provide a structure for answering this question, it is helpful to consider four levels of analytical methodology: techniques, methods, procedures, and protocols [Taylor, J. K. *Anal. Chem.* **1983**, *55*, 600A–608A].

A ***technique*** is any chemical or physical principle that we can use to study an analyte. There are many techniques for that we can use to determine the concentration of lead in drinking water [Fitch, A.; Wang, Y.; Mellican, S.; Macha, S. *Anal. Chem.* **1996**, *68*, 727A–731A]. In graphite furnace atomic absorption spectroscopy (GFAAS), for example, we first convert aqueous lead ions into free atoms—a process we call atomization. We then measure the amount of light absorbed by the free atoms. Thus, GFAAS uses both a chemical principle (atomization) and a physical principle (absorption of light).

> See Chapter 10 for a discussion of graphite furnace atomic absorption spectroscopy.

A ***method*** is the application of a technique for a specific analyte in a specific matrix. As shown in Figure 3.2.1 , the GFAAS method for determining the concentration of lead in water is different from that for lead in soil or blood.

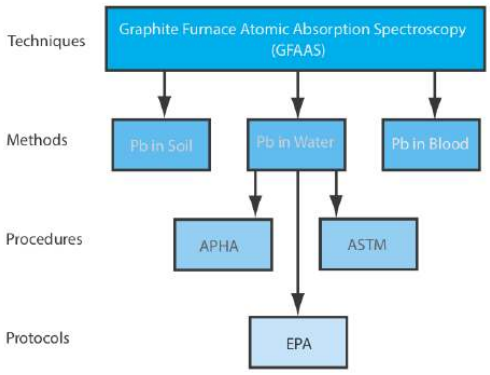


Figure 3.2.1 : Chart showing the hierarchical relationship between a technique, methods that use the technique, and procedures and protocols for a method. The abbreviations are APHA: American Public Health Association, ASTM: American Society for Testing Materials, EPA: Environmental Protection Agency.

A ***procedure*** is a set of written directions that tell us how to apply a method to a particular sample, including information on how to collect the sample, how to handle interferents, and how to validate results. A method may have several procedures as each analyst or agency adapts it to a specific need. As shown in Figure 3.2.1 , the American Public Health Agency and the American Society for Testing Materials publish separate procedures for determining the concentration of lead in water.

Finally, a ***protocol*** is a set of stringent guidelines that specify a procedure that an analyst must follow if an agency is to accept the results. Protocols are common when the result of an analysis supports or defines public policy. When determining the concentration of lead in water under the Safe Drinking Water Act, for example, the analyst must use a protocol specified by the Environmental Protection Agency.

There is an obvious order to these four levels of analytical methodology. Ideally, a protocol uses a previously validated procedure. Before developing and validating a procedure, a method of analysis must be selected. This requires, in turn, an initial screening of available techniques to determine those that have the potential for monitoring the analyte.

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4.3: Classifying Analytical Techniques

The analysis of a sample generates a chemical or physical signal that is proportional to the amount of analyte in the sample. This signal may be anything we can measure, such as volume or absorbance. It is convenient to divide analytical techniques into two general classes based on whether the signal is proportional to the mass or moles of analyte, or is proportional to the analyte's concentration

Consider the two graduated cylinders in Figure 3.3.1 , each of which contains a solution of 0.010 M $Cu(NO_3)_2$. Cylinder 1 contains 10 mL, or $\mathbf{1.0 \times 10^{-4}}$ moles of Cu^{2+}, and cylinder 2 contains 20 mL, or $\mathbf{2.0 \times 10^{-4}}$ moles of Cu^{2+}. If a technique responds to the absolute amount of analyte in the sample, then the signal due to the analyte S_A

$$S_A = k_A n_A \tag{4.3.1}$$

where n_A is the moles or grams of analyte in the sample, and k_A is a proportionality constant. Because cylinder 2 contains twice as many moles of Cu^{2+} as cylinder 1, analyzing the contents of cylinder 2 gives a signal twice as large as that for cylinder 1.



Figure 3.3.1 : Two graduated cylinders, each containing 0.10 M $Cu(NO_3)_2$. Although the cylinders contain the same concentration of Cu^{2+}, the cylinder on the left contains $\mathbf{1.0 \times 10^{-4}}$ mol Cu^{2+} and the cylinder on the right contains $\mathbf{2.0 \times 10^{-4}}$ mol Cu^{2+}.

A second class of analytical techniques are those that respond to the analyte's concentration, C_A

$$S_A = k_A C_A \tag{4.3.2}$$

Since the solutions in both cylinders have the same concentration of Cu^{2+}, their analysis yields identical signals.

A technique that responds to the absolute amount of analyte is a ***total analysis*** technique. Mass and volume are the most common signals for a total analysis technique, and the corresponding techniques are gravimetry (Chapter 8) and titrimetry (Chapter 9). With a few exceptions, the signal for a total analysis technique is the result of one or more chemical reactions, the stoichiometry of which determines the value of k_A in Equation 4.3.1.

> Historically, most early analytical methods used a total analysis technique. For this reason, total analysis techniques are often called "classical" techniques.

Spectroscopy (Chapter 10) and electrochemistry (Chapter 11), in which an optical or an electrical signal is proportional to the relative amount of analyte in a sample, are examples of concentration techniques. The relationship between the signal and the analyte's concentration is a theoretical function that depends on experimental conditions and the instrumentation used to measure the signal. For this reason the value of k_A in Equation 4.3.2 is determined experimentally.

> Since most concentration techniques rely on measuring an optical or electrical signal, they also are known as "instrumental" techniques.

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4.4: Selecting an Analytical Method

A method is the application of a technique to a specific analyte in a specific matrix. We can develop an analytical method to determine the concentration of lead in drinking water using any of the techniques mentioned in the previous section. A gravimetric method, for example, might precipitate the lead as $PbSO_4$ or as $PbCrO_4$, and use the precipitate's mass as the analytical signal. Lead forms several soluble complexes, which we can use to design a complexation titrimetric method. As shown in Figure 3.2.1, we can use graphite furnace atomic absorption spectroscopy to determine the concentration of lead in drinking water. Finally, lead's multiple oxidation states (Pb^0, Pb^{2+}, Pb^{4+}) makes feasible a variety of electrochemical methods.

Ultimately, the requirements of the analysis determine the best method. In choosing among the available methods, we give consideration to some or all the following design criteria or **Figures of Merit**: accuracy, precision, sensitivity, dynamic range, selectivity, robustness, ruggedness, scale of operation, analysis time, availability of equipment, and cost.

Accuracy

Accuracy is how closely the result of an experiment agrees with the "true" or expected result. We can express accuracy as an absolute error, e

$$e = \text{obtained result} - \text{expected result}$$

or as a percentage relative error, $\%e_r$

$$\%e_r = \frac{\text{obtained result} - \text{expected result}}{\text{expected result}} \times 100$$

A method's accuracy depends on many things, including the signal's source, the value of k_A in Equation 3.3.1 or Equation 3.3.2, and the ease of handling samples without loss or contamination. A total analysis technique, such as gravimetry and titrimetry, often produce more accurate results than does a concentration technique because we can measure mass and volume with high accuracy, and because the value of k_A is known exactly through stoichiometry.

> Because it is unlikely that we know the true result, we use an expected or accepted result to evaluate accuracy. For example, we might use a standard reference material, which has an accepted value, to establish an analytical method's accuracy. You will find a more detailed treatment of accuracy in Chapter 4, including a discussion of sources of errors.

Precision

When a sample is analyzed several times, the individual results vary from trial-to-trial. ***Precision*** is a measure of this variability. The closer the agreement between individual analyses, the more precise the results. For example, the results shown in the upper half of Figure 4.4.1 for the concentration of K^+ in a sample of serum are more precise than those in the lower half of Figure 4.4.1 . It is important to understand that precision does not imply accuracy. That the data in the upper half of Figure 4.4.1 are more precise does not mean that the first set of results is more accurate. In fact, neither set of results may be accurate.

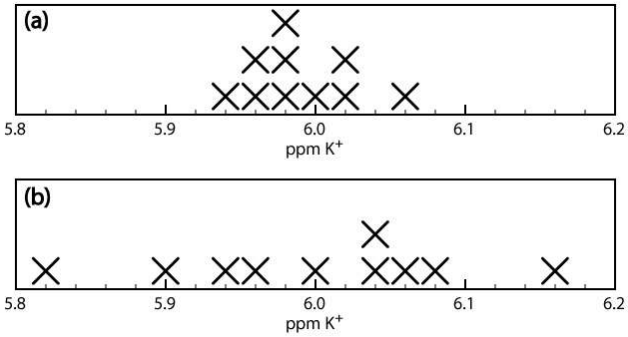


Figure 4.4.1 : Two determinations of the concentration of K^+ in serum, showing the effect of precision on the distribution of individual results. The data in (a) are less scattered and, therefore, more precise than the data in (b).

A method's precision depends on several factors, including the uncertainty in measuring the signal and the ease of handling samples reproducibly. In most cases we can measure the signal for a total analysis technique with a higher precision than is the case for a concentration method.

> Confusing accuracy and precision is a common mistake. See Ryder, J.; Clark, A. *U. Chem. Ed.* **2002**, *6*, 1–3, and Tomlinson, J.; Dyson, P. J.; Garratt, J. *U. Chem. Ed.* **2001**, *5*, 16–23 for discussions of this and other common misconceptions about the meaning of error. You will find a more detailed treatment of precision in Chapter 4, including a discussion of sources of errors.

Sensitivity

The ability to demonstrate that two samples have different amounts of analyte is an essential part of many analyses. A method's ***sensitivity*** is a measure of its ability to establish that such a difference is significant. Sensitivity is often confused with a method's ***detection limit***, which is the smallest amount of analyte we can determine with confidence.

> Confidence, as we will see in Chapter 4, is a statistical concept that builds on the idea of a population of results. For this reason, we will postpone our discussion of detection limits to Chapter 4. For now, the definition of a detection limit given here is sufficient.

Sensitivity is equivalent to the proportionality constant, k_A, in Equation 3.3.1 and Equation 3.3.2 [IUPAC Compendium of Chemical Terminology, Electronic version]. If ΔS_A is the smallest difference we can measure between two signals, then the smallest detectable difference in the absolute amount or the relative amount of analyte is

$$\Delta n_A = \frac{\Delta S_A}{k_A} \quad \text{or} \quad \Delta C_A = \frac{\Delta S_A}{k_A}$$

Suppose, for example, that our analytical signal is a measurement of mass using a balance whose smallest detectable increment is ±0.0001 g. If our method's sensitivity is 0.200, then our method can conceivably detect a difference in mass of as little as

$$\Delta n_A = \frac{\pm 0.0001 \text{ g}}{0.200} = \pm 0.0005 \text{ g}$$

For two methods with the same ΔS_A, the method with the greater sensitivity—that is, the method with the larger k_A—is better able to discriminate between smaller amounts of analyte.

Specificity and Selectivity

An analytical method is specific if its signal depends only on the analyte [Persson, B-A; Vessman, J. *Trends Anal. Chem.* **1998**, *17*, 117–119; Persson, B-A; Vessman, J. *Trends Anal. Chem.* **2001**, *20*, 526–532]. Although ***specificity*** is the ideal, few analytical methods are free from interferences. When an ***interferent*** contributes to the signal, we expand Equation 3.3.1 and Equation 3.3.2 to include its contribution to the sample's signal, S_{samp}

$$S_{samp} = S_A + S_I = k_A n_A + k_I n_I \tag{4.4.1}$$

$$S_{samp} = S_A + S_I = k_A C_A + k_I C_I \tag{4.4.2}$$

where S_I is the interferent's contribution to the signal, k_I is the interferent's sensitivity, and n_I and C_I are the moles (or grams) and the concentration of interferent in the sample, respectively.

Selectivity is a measure of a method's freedom from interferences [Valcárcel, M.; Gomez-Hens, A.; Rubio, S. *Trends Anal. Chem.* **2001**, *20*, 386–393]. A method's selectivity for an interferent relative to the analyte is defined by a ***selectivity coefficient***, $K_{A,I}$

$$K_{A,I} = \frac{k_I}{k_A} \tag{4.4.3}$$

which may be positive or negative depending on the signs of k_I and k_A. The selectivity coefficient is greater than +1 or less than –1 when the method is more selective for the interferent than for the analyte.

Although k_A and k_I usually are positive, they can be negative. For example, some analytical methods work by measuring the concentration of a species that remains after is reacts with the analyte. As the analyte's concentration increases, the concentration of the species that produces the signal decreases, and the signal becomes smaller. If the signal in the absence of analyte is assigned a value of zero, then the subsequent signals are negative.

Determining the selectivity coefficient's value is easy if we already know the values for k_A and k_I. As shown by Example 4.4.1 , we also can determine $K_{A,I}$ by measuring S_{samp} in the presence of and in the absence of the interferent.

Example 4.4.1

A method for the analysis of Ca^{2+} in water suffers from an interference in the presence of Zn^{2+}. When the concentration of Ca^{2+} is 100 times greater than that of Zn^{2+}, an analysis for Ca^{2+} has a relative error of +0.5%. What is the selectivity coefficient for this method?

Solution

Since only relative concentrations are reported, we can arbitrarily assign absolute concentrations. To make the calculations easy, we will let C_{Ca} = 100 (arbitrary units) and C_{Zn} = 1. A relative error of +0.5% means the signal in the presence of Zn^{2+} is 0.5% greater than the signal in the absence of Zn^{2+}. Again, we can assign values to make the calculation easier. If the signal for Cu^{2+} in the absence of Zn^{2+} is 100 (arbitrary units), then the signal in the presence of Zn^{2+} is 100.5.

The value of k_{Ca} is determined using Equation 3.3.2

$$k_{Ca} = \frac{S_{Ca}}{C_{Ca}} = \frac{100}{100} = 1$$

In the presence of Zn^{2+} the signal is given by Equation 3.4.2; thus

$$S_{samp} = 100.5 = k_{Ca}C_{Ca} + k_{Zn}C_{Zn} = (1 \times 100) + k_{Zn} \times 1$$

Solving for k_{Zn} gives its value as 0.5. The selectivity coefficient is

$$K_{Ca,Zn} = \frac{k_{Zn}}{k_{Ca}} = \frac{0.5}{1} = 0.5$$

If you are unsure why, in the above example, the signal in the presence of zinc is 100.5, note that the percentage relative error for this problem is given by

$$\frac{\text{obtained result} - 100}{100} \times 100 = +0.5\%$$

Solving gives an obtained result of 100.5.

Exercise 4.4.1

Wang and colleagues describe a fluorescence method for the analysis of Ag^+ in water. When analyzing a solution that contains 1.0×10^{-9} M Ag^+ and 1.1×10^{-7} M Ni^{2+}, the fluorescence intensity (the signal) was +4.9% greater than that obtained for a sample of 1.0×10^{-9} M Ag^+. What is $K_{Ag,Ni}$ for this analytical method? The full citation for the data in this exercise is Wang, L.; Liang, A. N.; Chen, H.; Liu, Y.; Qian, B.; Fu, J. *Anal. Chim. Acta* **2008**, *616*, 170-176.

Answer

Because the signal for Ag^+ in the presence of Ni^{2+} is reported as a relative error, we will assign a value of 100 as the signal for 1×10^{-9} M Ag^+. With a relative error of +4.9%, the signal for the solution of 1×10^{-9} M Ag^+ and 1.1×10^{-7} M Ni^{2+} is 104.9. The sensitivity for Ag^+ is determined using the solution that does not contain Ni^{2+}; thus

$$k_{Ag} = \frac{S_{Ag}}{C_{Ag}} = \frac{100}{1 \times 10^{-9}\ \text{M}} = 1.0 \times 10^{11}\ \text{M}^{-1}$$

Substituting into Equation 4.4.2 values for k_{Ag}, S_{samp} , and the concentrations of Ag^+ and Ni^{2+}

$$104.9 = (1.0 \times 10^{11}\ M^{-1}) \times (1 \times 10^{-9}\ M) + k_{Ni} \times (1.1 \times 10^{-7}\ M)$$

and solving gives k_{Ni} as $4.5 \times 10^7\ M^{-1}$. The selectivity coefficient is

$$K_{Ag,Ni} = \frac{k_{Ni}}{k_{Ag}} = \frac{4.5 \times 10^7\ M^{-1}}{1.0 \times 10^{11}\ M^{-1}} = 4.5 \times 10^{-4}$$

A selectivity coefficient provides us with a useful way to evaluate an interferent's potential effect on an analysis. Solving Equation 4.4.3 for k_I

$$k_I = K_{A,I} \times k_A \tag{4.4.4}$$

and substituting in Equation 4.4.1 and Equation 4.4.2, and simplifying gives

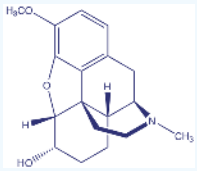
$$S_{samp} = k_A\{n_A + K_{A,I} \times n_I\} \tag{4.4.5}$$

$$S_{samp} = k_A\{C_A + K_{A,I} \times C_I\} \tag{4.4.6}$$

An interferent will not pose a problem as long as the term $K_{A,I} \times n_I$ in Equation 4.4.5 is significantly smaller than n_A, or if $K_{A,I} \times C_I$ in Equation 4.4.6 is significantly smaller than C_A.

Example 4.4.2

Barnett and colleagues developed a method to determine the concentration of codeine (structure shown below) in poppy plants [Barnett, N. W.; Bowser, T. A.; Geraldi, R. D.; Smith, B. *Anal. Chim. Acta* **1996**, *318*, 309– 317]. As part of their study they evaluated the effect of several interferents. For example, the authors found that equimolar solutions of codeine and the interferent 6-methoxycodeine gave signals, respectively of 40 and 6 (arbitrary units).



(a) What is the selectivity coefficient for the interferent, 6-methoxycodeine, relative to that for the analyte, codeine.

(b) If we need to know the concentration of codeine with an accuracy of ±0.50%, what is the maximum relative concentration of 6-methoxy-codeine that we can tolerate?

Solution

(a) The signals due to the analyte, S_A, and the interferent, S_I, are

$$S_A = k_A C_A \qquad S_I = k_I C_I$$

Solving these equations for k_A and for k_I, and substituting into Equation 4.4.4 gives

$$K_{A,I} = \frac{S_I / C_I}{S_A / C_I}$$

Because the concentrations of analyte and interferent are equimolar ($C_A = C_I$), the selectivity coefficient is

$$K_{A,I} = \frac{S_I}{S_A} = \frac{6}{40} = 0.15$$

(b) To achieve an accuracy of better than ±0.50% the term $K_{A,I} \times C_I$ in Equation 4.4.6 must be less than 0.50% of C_A; thus

$$K_{A,I} \times C_I \leq 0.0050 \times C_A$$

Solving this inequality for the ratio C_I/C_A and substituting in the value for $K_{A,I}$ from part (a) gives

$$\frac{C_I}{C_A} \leq \frac{0.0050}{K_{A,I}} = \frac{0.0050}{0.15} = 0.033$$

Therefore, the concentration of 6-methoxycodeine must be less than 3.3% of codeine's concentration.

When a method's signal is the result of a chemical reaction—for example, when the signal is the mass of a precipitate—there is a good chance that the method is not very selective and that it is susceptible to an interference.

? Exercise 4.4.2

Mercury (II) also is an interferent in the fluorescence method for Ag^+ developed by Wang and colleagues (see Practice Exercise 3.4.1). The selectivity coefficient, $K_{Ag,Hg}$ has a value of -1.0×10^{-3}.

(a) What is the significance of the selectivity coefficient's negative sign?

(b) Suppose you plan to use this method to analyze solutions with concentrations of Ag^+ no smaller than 1.0 nM. What is the maximum concentration of Hg^{2+} you can tolerate if your percentage relative errors must be less than ±1.0%?

Answer

(a) A negative value for $K_{Ag,Hg}$ means that the presence of Hg^{2+} decreases the signal from Ag^+.

(b) In this case we need to consider an error of –1%, since the effect of Hg^{2+} is to decrease the signal from Ag^+. To achieve this error, the term $K_{A,I} \times C_I$ in Equation 4.4.6 must be less than –1% of C_A; thus

$$K_{Ag,Hg} \times C_{Hg} = -0.01 \times C_{Ag}$$

Substituting in known values for $K_{Ag,Hg}$ and C_{Ag}, we find that the maximum concentration of Hg^{2+} is 1.0×10^{-8} M.

Problems with selectivity also are more likely when the analyte is present at a very low concentration [Rodgers, L. B. *J. Chem. Educ.* **1986**, *63*, 3–6].

Look back at Figure 1.1.1, which shows Fresenius' analytical method for the determination of nickel in ores. The reason there are so many steps in this procedure is that precipitation reactions generally are not very selective. The method in Figure 1.1.2 includes fewer steps because dimethylglyoxime is a more selective reagent. Even so, if an ore contains palladium, additional steps are needed to prevent the palladium from interfering.

Robustness and Ruggedness

For a method to be useful it must provide reliable results. Unfortunately, methods are subject to a variety of chemical and physical interferences that contribute uncertainty to the analysis. If a method is relatively free from chemical interferences, we can use it to analyze an analyte in a wide variety of sample matrices. Such methods are considered ***robust***.

Random variations in experimental conditions introduces uncertainty. If a method's sensitivity, *k*, is too dependent on experimental conditions, such as temperature, acidity, or reaction time, then a slight change in any of these conditions may give a significantly different result. A ***rugged*** method is relatively insensitive to changes in experimental conditions.

Scale of Operation

Another way to narrow the choice of methods is to consider three potential limitations: the amount of sample available for the analysis, the expected concentration of analyte in the samples, and the minimum amount of analyte that will produce a measurable signal. Collectively, these limitations define the analytical method's scale of operations.

We can display the scale of operations visually (Figure 4.4.2) by plotting the sample's size on the *x*-axis and the analyte's concentration on the *y*-axis. For convenience, we divide samples into macro (>0.1 g), meso (10 mg–100 mg), micro (0.1 mg–10 mg), and ultramicro (<0.1 mg) sizes, and we divide analytes into major (>1% w/w), minor (0.01% w/w–1% w/w), trace (10^{-7}% w/w–0.01% w/w), and ultratrace (<10^{-7}% w/w) components. Together, the analyte's concentration and the sample's size provide a

characteristic description for an analysis. For example, in a microtrace analysis the sample weighs between 0.1 mg and 10 mg and contains a concentration of analyte between 10^{-7}% w/w and 10^{-2}% w/w.

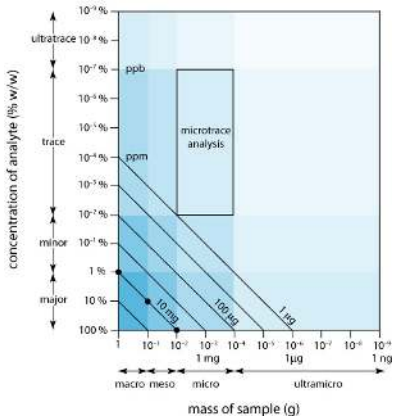


Figure 4.4.2 : Scale of operations for analytical methods. The shaded areas define different types of analyses. The boxed area, for example, represents a microtrace analysis. The diagonal lines show combinations of sample size and analyte concentration that contain the same mass of analyte. The three filled circles (•), for example, indicate analyses that use 10 mg of analyte. See Sandell, E. B.; Elving, P. J. in Kolthoff, I. M.; Elving, P. J., eds. *Treatise on Analytical Chem-istry*, Interscience: New York, Part I, Vol. 1, Chapter 1, pp. 3–6; (b) Potts, L. W. *Quantitative Analysis–Theory and Practice*, Harper and Row: New York, 1987, pp. 12 for more details.

The diagonal lines connecting the axes show combinations of sample size and analyte concentration that contain the same absolute mass of analyte. As shown in Figure 4.4.2 , for example, a 1-g sample that is 1% w/w analyte has the same amount of analyte (10 mg) as a 100-mg sample that is 10% w/w analyte, or a 10-mg sample that is 100% w/w analyte.

We can use Figure 4.4.2 to establish limits for analytical methods. If a method's minimum detectable signal is equivalent to 10 mg of analyte, then it is best suited to a major analyte in a macro or meso sample. Extending the method to an analyte with a concentration of 0.1% w/w requires a sample of 10 g, which rarely is practical due to the complications of carrying such a large amount of material through the analysis. On the other hand, a small sample that contains a trace amount of analyte places significant restrictions on an analysis. For example, a 1-mg sample that is 10^{-4}% w/w in analyte contains just 1 ng of analyte. If we isolate the analyte in 1 mL of solution, then we need an analytical method that reliably can detect it at a concentration of 1 ng/mL.

> It should not surprise you to learn that a total analysis technique typically requires a macro or a meso sample that contains a major analyte. A concentration technique is particularly useful for a minor, trace, or ultratrace analyte in a macro, meso, or micro sample.

Equipment, Time, and Cost

Finally, we can compare analytical methods with respect to their equipment needs, the time needed to complete an analysis, and the cost per sample. Methods that rely on instrumentation are equipment-intensive and may require significant operator training. For example, the graphite furnace atomic absorption spectroscopic method for determining lead in water requires a significant capital investment in the instrument and an experienced operator to obtain reliable results. Other methods, such as titrimetry, require less expensive equipment and less training.

The time to complete an analysis for one sample often is fairly similar from method-to-method. This is somewhat misleading, however, because much of this time is spent preparing samples, preparing reagents, and gathering together equipment. Once the samples, reagents, and equipment are in place, the sampling rate may differ substantially. For example, it takes just a few minutes to analyze a single sample for lead using graphite furnace atomic absorption spectroscopy, but several hours to analyze the same sample using gravimetry. This is a significant factor in selecting a method for a laboratory that handles a high volume of samples.

The cost of an analysis depends on many factors, including the cost of equipment and reagents, the cost of hiring analysts, and the number of samples that can be processed per hour. In general, methods that rely on instruments cost more per sample then other methods.

Making the Final Choice

Unfortunately, the design criteria discussed in this section are not mutually independent [Valcárcel, M.; Ríos, A. *Anal. Chem.* **1993**, *65*, 781A–787A]. Working with smaller samples or improving selectivity often comes at the expense of precision. Minimizing cost and analysis time may decrease accuracy. Selecting a method requires carefully balancing the various design criteria. Usually, the most important design criterion is accuracy, and the best method is the one that gives the most accurate result. When the need for a result is urgent, as is often the case in clinical labs, analysis time may become the critical factor.

In some cases it is the sample's properties that determine the best method. A sample with a complex matrix, for example, may require a method with excellent selectivity to avoid interferences. Samples in which the analyte is present at a trace or ultratrace concentration usually require a concentration method. If the quantity of sample is limited, then the method must not require a large amount of sample.

Determining the concentration of lead in drinking water requires a method that can detect lead at the parts per billion concentration level. Selectivity is important because other metal ions are present at significantly higher concentrations. A method that uses graphite furnace atomic absorption spectroscopy is a common choice for determining lead in drinking water because it meets these specifications. The same method is also useful for determining lead in blood where its ability to detect low concentrations of lead using a few microliters of sample is an important consideration.

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4.5: Developing the Procedure

After selecting a method, the next step is to develop a procedure that accomplish our goals for the analysis. In developing a procedure we give attention to compensating for interferences, to selecting and calibrating equipment, to acquiring a representative sample, and to validating the method.

Compensating for Interferences

A method's accuracy depends on its selectivity for the analyte. Even the best method, however, may not be free from interferents that contribute to the measured signal. Potential interferents may be present in the sample itself or in the reagents used during the analysis.

When the sample is free of interferents, the total signal, S_{total}, is a sum of the signal due to the analyte, S_A, and the signal due to interferents in the reagents, S_{reag},

$$S_{total} = S_A + S_{reag} = k_A n_A + S_{reag} \tag{4.5.1}$$

$$S_{total} = S_A + S_{reag} = k_A C_A + S_{reag} \tag{4.5.2}$$

Without an independent determination of S_{reag} we cannot solve Equation 4.5.1 or 4.5.2 for the moles or concentration of analyte.

To determine the contribution of S_{reag} in Equations 4.5.1 and 4.5.2 we measure the signal for a ***method blank***, a solution that does not contain the sample. Consider, for example, a procedure in which we dissolve a 0.1-g sample in a portion of solvent, add several reagents, and dilute to 100 mL with additional solvent. To prepare the method blank we omit the sample and dilute the reagents to 100 mL using the solvent. Because the analyte is absent, S_{total} for the method blank is equal to S_{reag}. Knowing the value for S_{reag} makes it is easy to correct S_{total} for the reagent's contribution to the total signal; thus

$$(S_{total} - S_{reag}) = S_A = k_A n_A$$

$$(S_{total} - S_{reag}) = S_A = k_A C_A$$

By itself, a method blank cannot compensate for an interferent that is part of the sample's matrix. If we happen to know the interferent's identity and concentration, then we can be add it to the method blank; however, this is not a common circumstance and we must, instead, find a method for separating the analyte and interferent before continuing the analysis.

> A method blank also is known as a reagent blank. When the sample is a liquid, or is in solution, we use an equivalent volume of an inert solvent as a substitute for the sample.

Calibration

A simple definition of a quantitative analytical method is that it is a mechanism for converting a measurement, the signal, into the amount of analyte in a sample. Assuming we can correct for interferents, a quantitative analysis is nothing more than solving Equation 3.3.1 or Equation 3.3.2 for n_A or for C_A.

To solve these equations we need the value of k_A. For a total analysis method usually we know the value of k_A because it is defined by the stoichiometry of the chemical reactions responsible for the signal. For a concentration method, however, the value of k_A usually is a complex function of experimental conditions. A ***calibration*** is the process of experimentally determining the value of k_A by measuring the signal for one or more standard samples, each of which contains a known concentration of analyte.

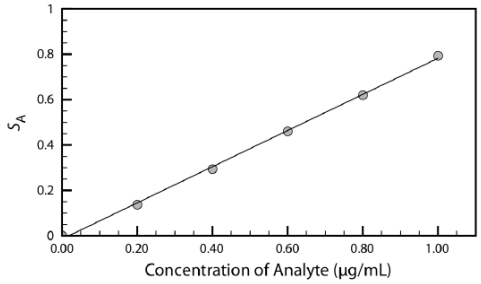


Figure 3.5.1 : Example of a calibration curve. The filled circles (•) are the results for five standard samples, each with a different concentration of analyte, and the line is the best fit to the data determined by a linear regression analysis. See Chapter 5 for a further discussion of calibration curves and an explanation of linear regression.

With a single standard we can calculate the value of k_A using Equation 3.3.1 or Equation 3.3.2. When using several standards with different concentrations of analyte, the result is best viewed visually by plotting S_A versus the concentration of analyte in the standards. Such a plot is known as a ***calibration curve***, an example of which is shown in Figure 3.5.1 .

Sampling

Selecting an appropriate method and executing it properly helps us ensure that our analysis is accurate. If we analyze the wrong sample, however, then the accuracy of our work is of little consequence.

A proper sampling strategy ensures that our samples are representative of the material from which they are taken. Biased or nonrepresentative sampling, and contaminating samples during or after their collection are two examples of sampling errors that can lead to a significant error in accuracy. It is important to realize that sampling errors are independent of errors in the analytical method. As a result, we cannot correct a sampling error in the laboratory by, for example, evaluating a reagent blank.

> Chapter 7 provides a more detailed discussion of sampling, including strategies for obtaining representative samples.

Validation

If we are to have confidence in our procedure we must demonstrate that it can provide acceptable results, a process we call ***validation***. Perhaps the most important part of validating a procedure is establishing that its precision and accuracy are appropriate for the problem we are trying to solve. We also ensure that the written procedure has sufficient detail so that different analysts or laboratories will obtain comparable results. Ideally, validation uses a standard sample whose composition closely matches the samples we will analyze. In the absence of appropriate standards, we can evaluate accuracy by comparing results to those obtained using a method of known accuracy.

> You will find more details about validating analytical methods in Chapter 14.

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4.6: Protocols

Earlier we defined a protocol as a set of stringent written guidelines that specify an exact procedure that we must follow if an agency is to accept the results of our analysis. In addition to the considerations that went into the procedure's design, a protocol also contains explicit instructions regarding internal and external quality assurance and quality control (***QA/QC***) procedures [Amore, F. *Anal. Chem.* **1979**, *51*, 1105A–1110A; Taylor, J. K. *Anal. Chem.* **1981**, *53*, 1588A–1593A]. The goal of internal QA/QC is to ensure that a laboratory's work is both accurate and precise. External QA/QC is a process in which an external agency certifies a laboratory.

As an example, let's outline a portion of the Environmental Protection Agency's protocol for determining trace metals in water by graphite furnace atomic absorption spectroscopy as part of its Contract Laboratory Program (CLP). The CLP protocol (see Figure 3.6.1) calls for an initial calibration using a method blank and three standards, one of which is at the detection limit. The resulting calibration curve is verified by analyzing initial calibration verification (ICV) and initial calibration blank (ICB) samples. The lab's result for the ICV sample must fall within ±10% of its expected concentration. If the result is outside this limit the analysis is stopped and the problem identified and corrected before continuing.

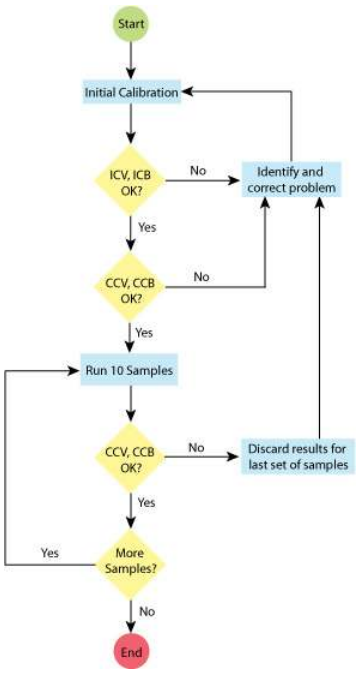


Figure 3.6.1 : Schematic diagram showing a portion of the EPA's protocol for determining trace metals in water using graphite furnace atomic absorption spectrometry. The abbreviations are ICV: initial calibration verification; ICB: initial calibration blank; CCV: continuing calibration verification; CCB: continuing calibration blank.

After a successful analysis of the ICV and ICB samples, the lab reverifies the calibration by analyzing a continuing calibration verification (CCV) sample and a continuing calibration blank (CCB). Results for the CCV also must be within ±10% of its expected concentration. Again, if the lab's result for the CCV is outside the established limits, the analysis is stopped, the problem identified and corrected, and the system recalibrated as described above. Additional CCV and the CCB samples are analyzed before the first sample and after the last sample, and between every set of ten samples. If the result for any CCV or CCB sample is unacceptable, the results for the last set of samples are discarded, the system is recalibrated, and the samples reanalyzed. By following this protocol, each result is bound by successful checks on the calibration. Although not shown in Figure 3.6.1 , the protocol also contains instructions for analyzing duplicate or split samples, and for using spike tests to verify accuracy.

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4.7: The Importance of Analytical Methodology

The importance of the issues raised in this chapter is evident if we examine environmental monitoring programs. The purpose of a monitoring program is to determine the present status of an environmental system, and to assess long term trends in the system's health. These are broad and poorly defined goals. In many cases, an environmental monitoring program begins before the essential questions are known. This is not surprising since it is difficult to formulate questions in the absence of results. Without careful planning, however, a poor experimental design may result in data that has little value.

These concerns are illustrated by the Chesapeake Bay Monitoring Program. This research program, designed to study nutrients and toxic pollutants in the Chesapeake Bay, was initiated in 1984 as a cooperative venture between the federal government, the state governments of Maryland, Virginia, and Pennsylvania, and the District of Columbia. A 1989 review of the program highlights the problems common to many monitoring programs [D'Elia, C. F.; Sanders, J. G.; Capone, D. G. *Envrion. Sci. Technol.* **1989**, *23*, 768–774].

At the beginning of the Chesapeake Bay monitoring program, little attention was given to selecting analytical methods, in large part because the eventual use of the data was not yet specified. The analytical methods initially chosen were standard methods already approved by the Environmental Protection Agency (EPA). In many cases these methods were not useful because they were designed to detect pollutants at their legally mandated maximum allowed concentrations. In unpolluted waters, however, the concentrations of these contaminants often are well below the detection limit of the EPA methods. For example, the detection limit for the EPA approved standard method for phosphate was 7.5 ppb. Since the actual phosphate concentrations in Chesapeake Bay were below the EPA method's detection limit, it provided no useful information. On the other hand, the detection limit for a non-approved variant of the EPA method, a method routinely used by chemical oceanographers, was 0.06 ppb, a more realistic detection limit for their samples. In other cases, such as the elemental analysis for particulate forms of carbon, nitrogen and phosphorous, EPA approved procedures provided poorer reproducibility than nonapproved methods.

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4.8: Problems

1. When working with a solid sample, often it is necessary to bring the analyte into solution by digesting the sample with a suitable solvent. Any remaining solid impurities are removed by filtration before continuing with the analysis. In a typical total analysis method, the procedure might read

   "After digesting the sample in a beaker using approximately 25 mL of solvent, remove any solid impurities that remain by passing the solution the analyte through filter paper, collecting the filtrate in a clean Erlenmeyer flask. Rinse the beaker with several small portions of solvent, passing these rinsings through the filter paper and collecting them in the same Erlenmeyer flask. Finally, rinse the filter paper with several portions of solvent, collecting the rinsings in the same Erlenmeyer flask."

   For a typical concentration method, however, the procedure might state

   "After digesting the sample in a beaker using 25.00 mL of solvent, remove any solid impurities by filtering a portion of the solution containing the analyte. Collect and discard the first several mL of filtrate before collecting a sample of 5.00 mL for further analysis."

   Explain why these two procedures are different.
2. A certain concentration method works best when the analyte's concentration is approximately 10 ppb.

   (a) If the method requires a sample of 0.5 mL, about what mass of analyte is being measured?

   (b) If the analyte is present at 10% w/v, how would you prepare the sample for analysis?

   (c) Repeat for the case where the analyte is present at 10% w/w.

   (d) Based on your answers to parts (a)–(c), comment on the method's suitability for the determination of a major analyte.
3. An analyst needs to evaluate the potential effect of an interferent, *I*, on the quantitative analysis for an analyte, *A*. She begins by measuring the signal for a sample in which the interferent is absent and the analyte is present with a concentration of 15 ppm, obtaining an average signal of 23.3 (arbitrary units). When she analyzes a sample in which the analyte is absent and the interferent is present with a concentration of 25 ppm, she obtains an average signal of 13.7.

   (a) What is the sensitivity for the analyte?

   (b) What is the sensitivity for the interferent?

   (c) What is the value of the selectivity coefficient?

   (d) Is the method more selective for the analyte or the interferent?

   (e) What is the maximum concentration of interferent relative to that of the analyte if the error in the analysis is to be less than 1%?
4. A sample is analyzed to determine the concentration of an analyte. Under the conditions of the analysis the sensitivity is $17.2\ \text{ppm}^{-1}$. What is the analyte's concentration if S_{total} is 35.2 and S_{reag} is 0.6?
5. A method for the analysis of Ca^{2+} in water suffers from an interference in the presence of Zn^{2+}. When the concentration of Ca^{2+} is 50 times greater than that of Zn^{2+}, an analysis for Ca^{2+} gives a relative error of –2.0%. What is the value of the selectivity coefficient for this method?
6. The quantitative analysis for reduced glutathione in blood is complicated by many potential interferents. In one study, when analyzing a solution of 10.0 ppb glutathione and 1.5 ppb ascorbic acid, the signal was 5.43 times greater than that obtained for the analysis of 10.0 ppb glutathione [Jiménez-Prieto, R.; Velasco, A.; Silva, M; Pérez-Bendito, D. *Anal. Chem. Acta* **1992**, *269*,

273– 279]. What is the selectivity coefficient for this analysis? The same study found that analyzing a solution of 3.5×10^2 ppb methionine and 10.0 ppb glutathione gives a signal that is 0.906 times less than that obtained for the analysis of 10.0 ppb glutathione. What is the selectivity coefficient for this analysis? In what ways do these interferents behave differently?

7. Oungpipat and Alexander described a method for determining the concentration of glycolic acid (GA) in a variety of samples, including physiological fluids such as urine [Oungpipat, W.; Alexander, P. W. *Anal. Chim. Acta* **1994**, *295*, 36–46]. In the presence of only GA, the signal is

$$S_{samp,1} = k_{\text{GA}} C_{\text{GA}}$$

and in the presence of both glycolic acid and ascorbic acid (AA), the signal is

$$S_{samp,2} = k_{\text{GA}} C_{\text{GA}} + k_{\text{AA}} C_{\text{AA}}$$

When the concentration of glycolic acid is 1.0×10^{-4} M and the concentration of ascorbic acid is 1.0×10^{-5} M, the ratio of their signals is

$$\frac{S_{samp,2}}{S_{samp,1}} = 1.44$$

(a) Using the ratio of the two signals, determine the value of the selectivity ratio $K_{\text{GA,AA}}$.

(b) Is the method more selective toward glycolic acid or ascorbic acid?

(c) If the concentration of ascorbic acid is 1.0×10^{-5} M, what is the smallest concentration of glycolic acid that can be determined such that the error introduced by failing to account for the signal from ascorbic acid is less than 1%?

8. Ibrahim and co-workers developed a new method for the quantitative analysis of hypoxanthine, a natural compound of some nucleic acids [Ibrahim, M. S.; Ahmad, M. E.; Temerk, Y. M.; Kaucke, A. M. *Anal. Chim. Acta* **1996**, *328*, 47–52]. As part of their study they evaluated the method's selectivity for hypoxanthine in the presence of several possible interferents, including ascorbic acid.

(a) When analyzing a solution of 1.12×10^{-6} M hypoxanthine the authors obtained a signal of 7.45×10^{-5} amps. What is the sensitivity for hypoxanthine? You may assume the signal has been corrected for the method blank.

(b) When a solution containing 1.12×10^{-6} M hypoxanthine and 6.5×10^{-5} M ascorbic acid is analyzed a signal of 4.04×10^{-5} amps is obtained. What is the selectivity coefficient for this method?

(c) Is the method more selective for hypoxanthine or for ascorbic acid?

(d) What is the largest concentration of ascorbic acid that may be present if a concentration of 1.12×10^{-6} M hypoxanthine is to be determined within 1.0%?

9. Examine a procedure from *Standard Methods for the Analysis of Waters and Wastewaters* (or another manual of standard analytical methods) and identify the steps taken to compensate for interferences, to calibrate equipment and instruments, to standardize the method, and to acquire a representative sample.

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4.9: Additional Resources

The International Union of Pure and Applied Chemistry (IUPAC) maintains a web-based compendium of analytical terminology. You can find it at the following web site.

- old.iupac.org/publications/an...al_compendium/

The following papers provide alternative schemes for classifying analytical methods.

- Booksh, K. S.; Kowalski, B. R. "Theory of Analytical Chemistry," *Anal. Chem.* **1994**, *66*, 782A– 791A.
- Phillips, J. B. "Classification of Analytical Methods," *Anal. Chem.* **1981**, *53*, 1463A–1470A.
- Valcárcel, M.; Luque de Castro, M. D. "A Hierarchical Approach to Analytical Chemistry," *Trends Anal. Chem.* **1995**, *14*, 242–250.
- Valcárcel, M.; Simonet, B. M. "Types of Analytical Information and Their Mutual Relationships," *Trends Anal. Chem.* **1995**, *14*, 490–495.

Further details on criteria for evaluating analytical methods are found in the following series of papers.

- Wilson, A. L. "The Performance-Characteristics of Analytical Methods", Part I-*Talanta*, **1970**, *17*, 21–29; Part II-*Talanta*, **1970**, *17*, 31–44; Part III-*Talanta*, **1973**, *20*, 725–732; Part IV-*Talanta*, **1974**, *21*, 1109–1121.

For a point/counterpoint debate on the meaning of sensitivity consult the following two papers and two letters of response.

- Ekins, R.; Edwards, P. "On the Meaning of 'Sensitivity'," *Clin. Chem.* **1997**, *43*, 1824–1831.
- Ekins, R.; Edwards, P. "On the Meaning of 'Sensitivity:' A Rejoinder," *Clin. Chem.* **1998**, *44*, 1773–1776.
- Pardue, H. L. "The Inseparable Triangle: Analytical Sensitivity, Measurement Uncertainty, and Quantitative Resolution," *Clin. Chem.* **1997**, *43*, 1831–1837.
- Pardue, H. L. "Reply to 'On the Meaning of 'Sensitivity:' A Rejoinder'," *Clin. Chem.* **1998**, *44*, 1776–1778.

Several texts provide analytical procedures for specific analytes in well-defined matrices.

- Basset, J.; Denney, R. C.; Jeffery, G. H.; Mendham, J. *Vogel's Textbook of Quantitative Inorganic Analysis*, 4th Edition; Longman: London, 1981.
- Csuros, M. *Environmental Sampling and Analysis for Technicians*, Lewis: Boca Raton, 1994.
- Keith, L. H. (ed) *Compilation of EPA's Sampling and Analysis Methods*, Lewis: Boca Raton, 1996
- Rump, H. H.; Krist, H. *Laboratory Methods for the Examination of Water, Wastewater and Soil*, VCH Publishers: NY, 1988.
- *Standard Methods for the Analysis of Waters and Wastewaters*, 21st Edition, American Public Health Association: Washington, D. C.; 2005.

For a review of the importance of analytical methodology in today's regulatory environment, consult the following text.

- Miller, J. M.; Crowther, J. B. (eds) *Analytical Chemistry in a GMP Environment*, John Wiley & Sons: New York, 2000.

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4.10: Chapter Summary and Key Terms

Chapter Summary

Every discipline has its own vocabulary and your success in studying ana- lytical chemistry will improve if you master this vocabulary. Be sure you understand the difference between an analyte and its matrix, between a technique and a method, between a procedure and a protocol, and between a total analysis technique and a concentration technique.

In selecting an analytical method we consider criteria such as accu- racy, precision, sensitivity, selectivity, robustness, ruggedness, the amount of available sample, the amount of analyte in the sample, time, cost, and the availability of equipment. These criteria are not mutually independent, and often it is necessary to find an acceptable balance between them.

In developing a procedure or protocol, we give consideration to compensating for interferences, calibrating the method, obtaining an appropriate sample, and validating the analysis. Poorly designed procedures and protocols produce results that are insufficient to meet the needs of the analysis.

Key Terms

accuracy	analysis	analyte
calibration	calibration curve	concentration technique
detection limit	determination	interferent
matrix	measurement	method
method blank	precision	procedure
protocol	QA/QC	robust
rugged	selectivity	selectivity coefficient
sensitivity	signal	specificity
technique	total analysis technique	validation

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CHAPTER OVERVIEW

5: Standardizing Analytical Methods

The American Chemical Society's Committee on Environmental Improvement defines standardization as the process of determining the relationship between the signal and the amount of analyte in a sample. In Chapter 3 we defined this relationship as

$$S_{total} = k_A n_A + S_{reag} \text{ or } S_{total} = k_A C_A + S_{reag}$$

where S_{total} is the signal, n_A is the moles of analyte, C_A is the analyte's concentration, k_A is the method's sensitivity for the analyte, and S_{reag} is the contribution to S_{total} from sources other than the sample. To standardize a method we must determine values for k_A and S_{reag}. Strategies for accomplishing this are the subject of this chapter.

5.1: Analytical Signals
5.2: Calibrating the Signal
5.3: Determining the Sensitivity
5.4: Linear Regression and Calibration Curves
5.5: Compensating for the Reagent Blank
5.6: Using Excel for a Linear Regression
5.7: Problems
5.8: Additional Resources
5.9: Chapter Summary and Key Terms

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5.1: Analytical Signals

To standardize an analytical method we use standards that contain known amounts of analyte. The accuracy of a standardization, therefore, depends on the quality of the reagents and the glassware we use to prepare these standards. For example, in an acid–base titration the stoichiometry of the acid–base reaction defines the relationship between the moles of analyte and the moles of titrant. In turn, the moles of titrant is the product of the titrant's concentration and the volume of titrant used to reach the equivalence point. The accuracy of a titrimetric analysis, therefore, is never better than the accuracy with which we know the titrant's concentration.

> See Chapter 9 for a thorough discussion of titrimetric methods of analysis.

Primary and Secondary Standards

There are two categories of analytical standards: primary standards and secondary standards. A ***primary standard*** is a reagent that we can use to dispense an accurately known amount of analyte. For example, a 0.1250-g sample of $K_2Cr_2O_7$ contains 4.249×10^{-4} moles of $K_2Cr_2O_7$. If we place this sample in a 250-mL volumetric flask and dilute to volume, the concentration of $K_2Cr_2O_7$ in the resulting solution is 1.700×10^{-3} M. A primary standard must have a known stoichiometry, a known purity (or assay), and it must be stable during long-term storage. Because it is difficult to establishing accurately the degree of hydration, even after drying, a hydrated reagent usually is not a primary standard.

Reagents that do not meet these criteria are secondary standards. The concentration of a ***secondary standard*** is determined relative to a primary standard. Lists of acceptable primary standards are available (see, for instance, Smith, B. W.; Parsons, M. L. *J. Chem. Educ.* **1973**, *50*, 679–681; or Moody, J. R.; Green- burg, P. R.; Pratt, K. W.; Rains, T. C. *Anal. Chem.* **1988**, *60*, 1203A–1218A). Appendix 8 provides examples of some common primary standards.

> NaOH is one example of a secondary standard. Commercially available NaOH contains impurities of NaCl, Na_2CO_3, and Na_2SO_4, and readily absorbs H_2O from the atmosphere. To determine the concentration of NaOH in a solution, we titrate it against a primary standard weak acid, such as potassium hydrogen phthalate, $KHC_8H_4O_4$.

Other Reagents

Preparing a standard often requires additional reagents that are not primary standards or secondary standards, such as a suitable solvent or reagents needed to adjust the standard's matrix. These solvents and reagents are potential sources of additional analyte, which, if not accounted for, produce a determinate error in the standardization. If available, reagent grade chemicals that conform to standards set by the American Chemical Society are used [Committee on Analytical Reagents, *Reagent Chemicals*, 8th ed., American Chemical Society: Washington, D. C., 1993]. The label on the bottle of a reagent grade chemical (Figure 5.1.1) lists either the limits for specific impurities or provides an assay for the impurities. We can improve the quality of a reagent grade chemical by purifying it, or by conducting a more accurate assay. As discussed later in the chapter, we can correct for contributions to S_{total} from reagents used in an analysis by including an appropriate blank determination in the analytical procedure.



Figure 5.1.1 : Two examples of packaging labels for reagent grade chemicals. The label on the bottle on the right provides the manufacturer's assay for the reagent, NaBr. Note that potassium is flagged with an asterisk (*) because its assay exceeds the limit established by the American Chemical Society (ACS). The label for the bottle on the left does not provide an assay for impurities; however it indicates that the reagent meets ACS specifications by providing the maximum limits for impurities. An assay for the reagent, $NaHCO_3$, is provided.

Preparing a Standard Solution

It often is necessary to prepare a series of standards, each with a different concentration of analyte. We can prepare these standards in two ways. If the range of concentrations is limited to one or two orders of magnitude, then each solution is best prepared by transferring a known mass or volume of the pure standard to a volumetric flask and diluting to volume.

When working with a larger range of concentrations, particularly a range that extends over more than three orders of magnitude, standards are best prepared by a serial dilution from a single stock solution. In a ***serial dilution*** we prepare the most concentrated standard and then dilute a portion of that solution to prepare the next most concentrated standard. Next, we dilute a portion of the second standard to prepare a third standard, continuing this process until we have prepared all of our standards. Serial dilutions must be prepared with extra care because an error in preparing one standard is passed on to all succeeding standards.

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5.2: Calibrating the Signal

The accuracy with which we determine k_A and S_{reag} depends on how accurately we can measure the signal, S_{total}. We measure signals using equipment, such as glassware and balances, and instrumentation, such as spectrophotometers and pH meters. To minimize determinate errors that might affect the signal, we first calibrate our equipment and instrumentation by measuring S_{total} for a standard with a known response of S_{std}, adjusting S_{total} until

$$S_{total} = S_{std}$$

Here are two examples of how we calibrate signals; other examples are provided in later chapters that focus on specific analytical methods.

When the signal is a measurement of mass, we determine S_{total} using an analytical balance. To calibrate the balance's signal we use a reference weight that meets standards established by a governing agency, such as the National Institute for Standards and Technology or the American Society for Testing and Materials. An electronic balance often includes an internal calibration weight for routine calibrations, as well as programs for calibrating with external weights. In either case, the balance automatically adjusts S_{total} to match S_{std}.

> See Chapter 2.4 to review how an electronic balance works. Calibrating a balance is important, but it does not eliminate all sources of determinate error when measuring mass. See Appendix 9 for a discussion of correcting for the buoyancy of air.

We also must calibrate our instruments. For example, we can evaluate a spectrophotometer's accuracy by measuring the absorbance of a carefully prepared solution of 60.06 mg/L $K_2Cr_2O_7$ in 0.0050 M H_2SO_4, using 0.0050 M H_2SO_4 as a reagent blank [Ebel, S. *Fresenius J. Anal. Chem.* **1992**, *342*, 769]. An absorbance of $\mathbf{0.640 \pm 0.010}$ absorbance units at a wavelength of 350.0 nm indicates that the spectrometer's signal is calibrated properly.

> Be sure to read and follow carefully the calibration instructions provided with any instrument you use.

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5.3: Determining the Sensitivity

To standardize an analytical method we also must determine the analyte's sensitivity, k_A, in Equation 5.3.1 or Equation 5.3.2 .

$$S_{total} = k_A n_A + S_{reag} \tag{5.3.1}$$

$$S_{total} = k_A C_A + S_{reag} \tag{5.3.2}$$

In principle, it is possible to derive the value of k_A for any analytical method if we understand fully all the chemical reactions and physical processes responsible for the signal. Unfortunately, such calculations are not feasible if we lack a sufficiently developed theoretical model of the physical processes or if the chemical reaction's evince non-ideal behavior. In such situations we must determine the value of k_A by analyzing one or more standard solutions, each of which contains a known amount of analyte. In this section we consider several approaches for determining the value of k_A. For simplicity we assume that S_{reag} is accounted for by a proper reagent blank, allowing us to replace S_{total} in Equation 5.3.1 and Equation 5.3.2 with the analyte's signal, S_A.

$$S_A = k_A n_A \tag{5.3.3}$$

$$S_A = k_A C_A \tag{5.3.4}$$

> Equation 5.3.3 and Equation 5.3.4 essentially are identical, differing only in whether we choose to express the amount of analyte in moles or as a concentration. For the remainder of this chapter we will limit our treatment to Equation 5.3.4. You can extend this treatment to Equation 5.3.3 by replacing C_A with n_A.

Single-Point versus Multiple-Point Standardizations

The simplest way to determine the value of k_A in Equation 5.3.4 is to use a single-point standardization in which we measure the signal for a standard, S_{std}, that contains a known concentration of analyte, C_{std}. Substituting these values into Equation 5.3.4

$$k_A = \frac{S_{std}}{C_{std}} \tag{5.3.5}$$

gives us the value for k_A. Having determined k_A, we can calculate the concentration of analyte in a sample by measuring its signal, S_{samp}, and calculating C_A using Equation 5.3.6.

$$C_A = \frac{S_{samp}}{k_A} \tag{5.3.6}$$

A single-point standardization is the least desirable method for standardizing a method. There are two reasons for this. First, any error in our determination of k_A carries over into our calculation of C_A. Second, our experimental value for k_A is based on a single concentration of analyte. To extend this value of k_A to other concentrations of analyte requires that we assume a linear relationship between the signal and the analyte's concentration, an assumption that often is not true [Cardone, M. J.; Palmero, P. J.; Sybrandt, L. B. *Anal. Chem.* **1980**, *52*, 1187–1191]. Figure 5.3.1 shows how assuming a constant value of k_A leads to a determinate error in C_A if k_A becomes smaller at higher concentrations of analyte. Despite these limitations, single-point standardizations find routine use when the expected range for the analyte's concentrations is small. Under these conditions it often is safe to assume that k_A is constant (although you should verify this assumption experimentally). This is the case, for example, in clinical labs where many automated analyzers use only a single standard.

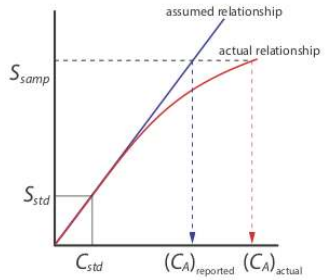


Figure 5.3.1 : Example showing how a single-point standardization leads to a determinate error in an analyte's reported concentration if we incorrectly assume that k_A is constant. The assumed relationship between S_{samp} and C_A is based on a single standard and is a straight-line; the actual relationship between S_{samp} and C_A becomes curved for larger concentrations of analyte.

The better way to standardize a method is to prepare a series of standards, each of which contains a different concentration of analyte. Standards are chosen such that they bracket the expected range for the analyte's concentration. A ***multiple-point standardization*** should include at least three standards, although more are preferable. A plot of S_{std} versus C_{std} is called a ***calibration curve***. The exact standardization, or calibration relationship, is determined by an appropriate curve-fitting algorithm.

> Linear regression, which also is known as the method of least squares, is one such algorithm. Its use is covered in Section 5.4.

There are two advantages to a multiple-point standardization. First, although a determinate error in one standard introduces a determinate error, its effect is minimized by the remaining standards. Second, because we measure the signal for several concentrations of analyte, we no longer must assume k_A is independent of the analyte's concentration. Instead, we can construct a calibration curve similar to the "actual relationship" in Figure 5.3.1 .

External Standards

The most common method of standardization uses one or more ***external standards***, each of which contains a known concentration of analyte. We call these standards "external" because they are prepared and analyzed separate from the samples.

> Appending the adjective "external" to the noun "standard" might strike you as odd at this point, as it seems reasonable to assume that standards and samples are analyzed separately. As we will soon learn, however, we can add standards to our samples and analyze both simultaneously.

Single External Standard

With a single external standard we determine k_A using EEquation 5.3.5 and then calculate the concentration of analyte, C_A, using Equation 5.3.6.

Example 5.3.1

A spectrophotometric method for the quantitative analysis of Pb^{2+} in blood yields an S_{std} of 0.474 for a single standard for which the concentration of lead is 1.75 ppb. What is the concentration of Pb^{2+} in a sample of blood for which S_{samp} is 0.361?

Solution

Equation 5.3.5 allows us to calculate the value of k_A using the data for the single external standard.

$$k_A = \frac{S_{std}}{C_{std}} = \frac{0.474}{1.75 \text{ ppb}} = 0.2709 \text{ ppb}^{-1}$$

Having determined the value of k_A, we calculate the concentration of Pb^{2+} in the sample of blood is calculated using Equation 5.3.6.

$$C_A = \frac{S_{samp}}{k_A} = \frac{0.361}{0.2709\ \text{ppb}^{-1}} = 1.33\ \text{ppb}$$

Multiple External Standards

Figure 5.3.2 shows a typical multiple-point external standardization. The volumetric flask on the left contains a reagent blank and the remaining volumetric flasks contain increasing concentrations of Cu^{2+}. Shown below the volumetric flasks is the resulting calibration curve. Because this is the most common method of standardization, the resulting relationship is called a ***normal calibration curve***.

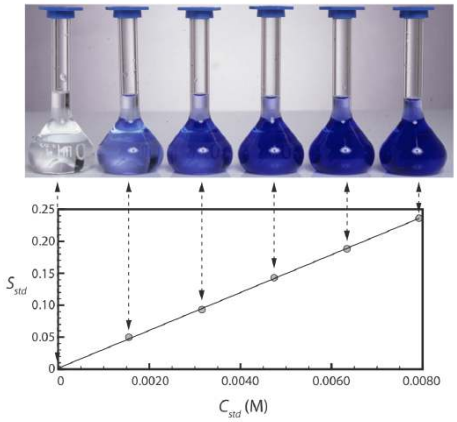


Figure 5.3.2 : The photo at the top of the figure shows a reagent blank (far left) and a set of five external standards for Cu^{2+} with concentrations that increase from left-to-right. Shown below the external standards is the resulting normal calibration curve. The absorbance of each standard, S_{std}, is shown by the filled circles.

When a calibration curve is a straight-line, as it is in Figure 5.3.2 , the slope of the line gives the value of k_A. This is the most desirable situation because the method's sensitivity remains constant throughout the analyte's concentration range. When the calibration curve is not a straight-line, the method's sensitivity is a function of the analyte's concentration. In Figure 5.3.1 , for example, the value of k_A is greatest when the analyte's concentration is small and it decreases continuously for higher concentrations of analyte. The value of k_A at any point along the calibration curve in Figure 5.3.1 is the slope at that point. In either case, a calibration curve allows to relate S_{samp} to the analyte's concentration.

✔ Example 5.3.2

A second spectrophotometric method for the quantitative analysis of Pb^{2+} in blood has a normal calibration curve for which

$$S_{std} = (0.296\ \text{ppb}^{-1} \times C_{std}) + 0.003$$

What is the concentration of Pb^{2+} in a sample of blood if S_{samp} is 0.397?

Solution

To determine the concentration of Pb^{2+} in the sample of blood, we replace S_{std} in the calibration equation with S_{samp} and solve for C_A.

$$C_A = \frac{S_{samp} - 0.003}{0.296\ \text{ppb}^{-1}} = \frac{0.397 - 0.003}{0.296\ \text{ppb}^{-1}} = 1.33\ \text{ppb}$$

It is worth noting that the calibration equation in this problem includes an extra term that does not appear in Equation 5.3.6. Ideally we expect our calibration curve to have a signal of zero when C_A is zero. This is the purpose of using a reagent blank to correct the measured signal. The extra term of +0.003 in our calibration equation results from the uncertainty in measuring the signal for the reagent blank and the standards.

? Exercise 5.3.1

Figure 5.3.2 shows a normal calibration curve for the quantitative analysis of Cu^{2+}. The equation for the calibration curve is

$$S_{std} = 29.59\ \text{M}^{-1} \times C_{std} + 0.015$$

What is the concentration of Cu^{2+} in a sample whose absorbance, S_{samp}, is 0.114? Compare your answer to a one-point standardization where a standard of 3.16×10^{-3} M Cu^{2+} gives a signal of 0.0931.

Answer

Substituting the sample's absorbance into the calibration equation and solving for C_A give

$$S_{samp} = 0.114 = 29.59\ \text{M}^{-1} \times C_A + 0.015$$

$$C_A = 3.35 \times 10^{-3}\ \text{M}$$

For the one-point standardization, we first solve for k_A

$$k_A = \frac{S_{std}}{C_{std}} = \frac{0.0931}{3.16 \times 10^{-3}\ \text{M}} = 29.46\ \text{M}^{-1}$$

and then use this value of k_A to solve for C_A.

$$C_A = \frac{S_{samp}}{k_A} = \frac{0.114}{29.46\ \text{M}^{-1}} = 3.87 \times 10^{-3}\ \text{M}$$

When using multiple standards, the indeterminate errors that affect the signal for one standard are partially compensated for by the indeterminate errors that affect the other standards. The standard selected for the one-point standardization has a signal that is smaller than that predicted by the regression equation, which underestimates k_A and overestimates C_A.

An external standardization allows us to analyze a series of samples using a single calibration curve. This is an important advantage when we have many samples to analyze. Not surprisingly, many of the most common quantitative analytical methods use an external standardization.

There is a serious limitation, however, to an external standardization. When we determine the value of k_A using Equation 5.3.5, the analyte is present in the external standard's matrix, which usually is a much simpler matrix than that of our samples. When we use an external standardization we assume the matrix does not affect the value of k_A. If this is not true, then we introduce a proportional determinate error into our analysis. This is not the case in Figure 5.3.3 , for instance, where we show calibration curves for an analyte in the sample's matrix and in the standard's matrix. In this case, using the calibration curve for the external standards leads to a negative determinate error in analyte's reported concentration. If we expect that matrix effects are important, then we try to match the standard's matrix to that of the sample, a process known as ***matrix matching***. If we are unsure of the sample's matrix, then we must show that matrix effects are negligible or use an alternative method of standardization. Both approaches are discussed in the following section.

The matrix for the external standards in Figure 5.3.2 , for example, is dilute ammonia. Because the $Cu(NH_3)_4^{2+}$ complex absorbs more strongly than Cu^{2+}, adding ammonia increases the signal's magnitude. If we fail to add the same amount of ammonia to our samples, then we will introduce a proportional determinate error into our analysis.

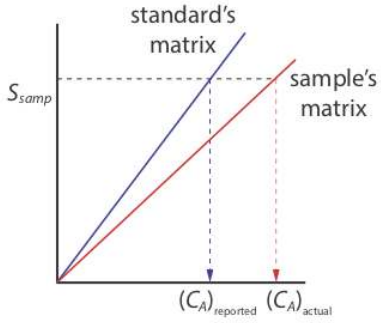


Figure 5.3.3 : Calibration curves for an analyte in the standard's matrix and in the sample's matrix. If the matrix affects the value of k_A, as is the case here, then we introduce a proportional determinate error into our analysis if we use a normal calibration curve.

Standard Additions

We can avoid the complication of matching the matrix of the standards to the matrix of the sample if we carry out the standardization in the sample. This is known as the ***method of standard additions***.

Single Standard Addition

The simplest version of a standard addition is shown in Figure 5.3.4 . First we add a portion of the sample, V_o, to a volumetric flask, dilute it to volume, V_f, and measure its signal, S_{samp}. Next, we add a second identical portion of sample to an equivalent volumetric flask along with a spike, V_{std}, of an external standard whose concentration is C_{std}. After we dilute the spiked sample to the same final volume, we measure its signal, S_{spike}.

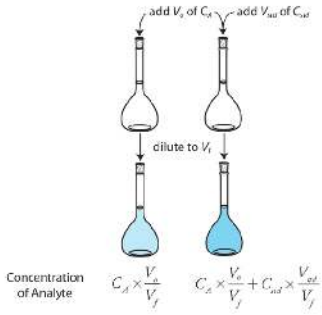


Figure 5.3.4 : Illustration showing the method of standard additions. The volumetric flask on the left contains a portion of the sample, V_o, and the volumetric flask on the right contains an identical portion of the sample and a spike, V_{std}, of a standard solution of the analyte. Both flasks are diluted to the same final volume, V_f. The concentration of analyte in each flask is shown at the bottom of the figure where C_A is the analyte's concentration in the original sample and C_{std} is the concentration of analyte in the external standard.

The following two equations relate S_{samp} and S_{spike} to the concentration of analyte, C_A, in the original sample.

$$S_{samp} = k_A C_A \frac{V_o}{V_f} \tag{5.3.7}$$

$$S_{spike} = k_A \left(C_A \frac{V_o}{V_f} + C_{std} \frac{V_{std}}{V_f} \right) \tag{5.3.8}$$

As long as V_{std} is small relative to V_o, the effect of the standard's matrix on the sample's matrix is insignificant. Under these conditions the value of k_A is the same in Equation 5.3.7 and Equation 5.3.8. Solving both equations for k_A and equating gives

$$\frac{S_{samp}}{C_A \frac{V_o}{V_f}} = \frac{S_{spike}}{C_A \frac{V_o}{V_f} + C_{std} \frac{V_{std}}{V_f}} \tag{5.3.9}$$

which we can solve for the concentration of analyte, C_A, in the original sample.

Example 5.3.3

A third spectrophotometric method for the quantitative analysis of Pb^{2+} in blood yields an S_{samp} of 0.193 when a 1.00 mL sample of blood is diluted to 5.00 mL. A second 1.00 mL sample of blood is spiked with 1.00 microliter of a 1560-ppb Pb^{2+} external standard and diluted to 5.00 mL, yielding an S_{spike} of 0.419. What is the concentration of Pb^{2+} in the original sample of blood?

Solution

We begin by making appropriate substitutions into Equation 5.3.9 and solving for C_A. Note that all volumes must be in the same units; thus, we first convert V_{std} from 1.00 mL to 1.00×10^{-3} mL.

$$\frac{0.193}{C_A \frac{1.00\text{ mL}}{5.00\text{ mL}}} = \frac{0.419}{C_A \frac{1.00\text{ mL}}{5.00\text{ mL}} + 1560\text{ ppb} \frac{1.00\times10^{-3}\text{ mL}}{5.00\text{ mL}}}$$

$$\frac{0.193}{0.200C_A} = \frac{0.419}{0.200C_A + 0.3120\text{ ppb}}$$

$$0.0386C_A + 0.0602\text{ ppb} = 0.0838C_A$$

$$0.0452C_A = 0.0602\text{ ppb}$$

$$C_A = 1.33\text{ ppb}$$

The concentration of Pb^{2+} in the original sample of blood is 1.33 ppb.

It also is possible to add the standard addition directly to the sample, measuring the signal both before and after the spike (Figure 5.3.5). In this case the final volume after the standard addition is $V_o + V_{std}$ and Equation 5.3.7, Equation 5.3.8, and Equation 5.3.9 become

$$S_{samp} = k_A C_A$$

$$S_{spike} = k_A \left(C_A \frac{V_o}{V_o + V_{std}} + C_{std} \frac{V_{std}}{V_o + V_{std}} \right) \tag{5.3.10}$$

$$\frac{S_{samp}}{C_A} = \frac{S_{spike}}{C_A \frac{V_o}{V_o + V_{std}} + C_{std} \frac{V_{std}}{V_o + V_{std}}} \tag{5.3.11}$$

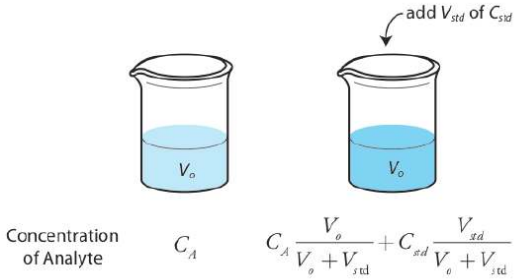


Figure 5.3.5 : Illustration showing an alternative form of the method of standard additions. In this case we add the spike of external standard directly to the sample without any further adjust in the volume.

Example 5.3.4

A fourth spectrophotometric method for the quantitative analysis of Pb^{2+} in blood yields an S_{samp} of 0.712 for a 5.00 mL sample of blood. After spiking the blood sample with 5.00 microliters of a 1560-ppb Pb^{2+} external standard, an S_{spike} of 1.546 is measured. What is the concentration of Pb^{2+} in the original sample of blood?

Solution

$$\frac{0.712}{C_A} = \frac{1.546}{C_A \frac{5.00\text{ mL}}{5.005\text{ mL}} + 1560\text{ ppb} \frac{5.00\times10^{-3}\text{ mL}}{5.005\text{ mL}}}$$

$$\frac{0.712}{C_A} = \frac{1.546}{0.9990C_A + 1.558 \text{ ppb}}$$

$$0.7113C_A + 1.109 \text{ ppb} = 1.546C_A$$

$$C_A = 1.33 \text{ ppb}$$

The concentration of Pb^{2+} in the original sample of blood is 1.33 ppb.

Multiple Standard Additions

We can adapt a single-point standard addition into a multiple-point standard addition by preparing a series of samples that contain increasing amounts of the external standard. Figure 5.3.6 shows two ways to plot a standard addition calibration curve based on Equation 5.3.8. In Figure 5.3.6 a we plot S_{spike} against the volume of the spikes, V_{std}. If k_A is constant, then the calibration curve is a straight-line. It is easy to show that the *x*-intercept is equivalent to $-C_AV_o/C_{std}$.

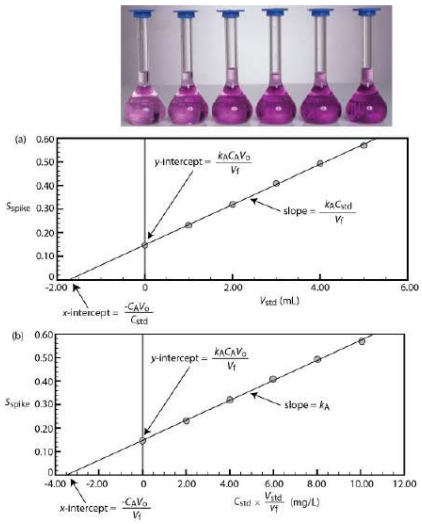


Figure 5.3.6 : Shown at the top of the figure is a set of six standard additions for the determination of Mn^{2+}. The flask on the left is a 25.00 mL sample diluted to 50.00 mL with water. The remaining flasks contain 25.00 mL of sample and, from left-to-right, 1.00, 2.00, 3.00, 4.00, and 5.00 mL spikes of an external standard that is 100.6 mg/L Mn^{2+}. Shown below are two ways to plot the standard additions calibration curve. The absorbance for each standard addition, S_{spike}, is shown by the filled circles.

Example 5.3.5

Beginning with Equation 5.3.8 show that the equations in Figure 5.3.6 a for the slope, the *y*-intercept, and the *x*-intercept are correct.

Solution

We begin by rewriting Equation 5.3.8 as

$$S_{spike} = \frac{k_AC_AV_o}{V_f} + \frac{k_AC_{std}}{V_f} \times V_{std}$$

which is in the form of the equation for a straight-line

$$y = y\text{-intercept} + \text{slope} \times x\text{-intercept}$$

where *y* is S_{spike} and *x* is V_{std}. The slope of the line, therefore, is k_AC_{std}/V_f and the *y*-intercept is $k_AC_AV_o/V_f$. The *x*-intercept is the value of *x* when *y* is zero, or

$$0 = \frac{k_AC_AV_o}{V_f} + \frac{k_AC_{std}}{V_f} \times x\text{-intercept}$$

$$x\text{-intercept} = -\frac{k_AC_AV_o/V_f}{K_AC_{std}/V_f} = -\frac{C_AV_o}{C_{std}}$$

Exercise 5.3.2

Beginning with Equation 5.3.8 show that the Equations in Figure 5.3.6 b for the slope, the *y*-intercept, and the *x*-intercept are correct.

Answer

We begin with Equation 5.3.8

$$S_{spike} = k_A \left(C_A \frac{V_o}{V_f} + C_{std} \frac{V_{std}}{V_f} \right)$$

rewriting it as

$$S_{spike} = \frac{k_A C_A V_o}{V_f} + k_A \left(C_{std} \frac{V_{std}}{V_f} \right)$$

which is in the form of the linear equation

$$y = y\text{-intercept} + \text{slope} \times x\text{-intercept}$$

where y is S_{spike} and x is $C_{std} \times V_{std}/V_f$. The slope of the line, therefore, is k_A, and the y-intercept is $k_A C_A V_o / V_f$. The x-intercept is the value of x when y is zero, or

$$x\text{-intercept} = -\frac{k_A C_A V_o / V_F}{k_A} = -\frac{C_A V_o}{V_f}$$

Because we know the volume of the original sample, V_o, and the concentration of the external standard, C_{std}, we can calculate the analyte's concentrations from the *x*-intercept of a multiple-point standard additions.

Example 5.3.6

A fifth spectrophotometric method for the quantitative analysis of Pb^{2+} in blood uses a multiple-point standard addition based on Equation 5.3.8. The original blood sample has a volume of 1.00 mL and the standard used for spiking the sample has a concentration of 1560 ppb Pb^{2+}. All samples were diluted to 5.00 mL before measuring the signal. A calibration curve of S_{spike} versus V_{std} has the following equation

$$S_{spike} = 0.266 + 312\ \text{mL}^{-1} \times V_{std}$$

What is the concentration of Pb^{2+} in the original sample of blood?

Solution

To find the *x*-intercept we set S_{spike} equal to zero.

$$S_{spike} = 0.266 + 312\ \text{mL}^{-1} \times V_{std}$$

Solving for V_{std}, we obtain a value of -8.526×10^{-4} mL for the *x*-intercept. Substituting the *x*-intercept's value into the equation from Figure 5.3.6 a

$$-8.526 \times 10^{-4}\ \text{mL} = -\frac{C_A V_o}{C_{std}} = -\frac{C_A \times 1.00\ \text{mL}}{1560\ \text{ppb}}$$

and solving for C_A gives the concentration of Pb^{2+} in the blood sample as 1.33 ppb.

Exercise 5.3.3

Figure 5.3.6 shows a standard additions calibration curve for the quantitative analysis of Mn^{2+}. Each solution contains 25.00 mL of the original sample and either 0, 1.00, 2.00, 3.00, 4.00, or 5.00 mL of a 100.6 mg/L external standard of Mn^{2+}. All

standard addition samples were diluted to 50.00 mL with water before reading the absorbance. The equation for the calibration curve in Figure 5.3.6 a is

$$S_{std} = 0.0854 \times V_{std} + 0.1478$$

What is the concentration of Mn^{2+} in this sample? Compare your answer to the data in Figure 5.3.6 b, for which the calibration curve is

$$S_{std} = 0.425 \times C_{std}(V_{std}/V_f) + 0.1478$$

Answer

Using the calibration equation from Figure 5.3.6 a, we find that the *x*-intercept is

$$x\text{-intercept} = -\frac{0.1478}{0.0854\ \text{mL}^{-1}} = -1.731\ \text{mL}$$

If we plug this result into the equation for the *x*-intercept and solve for C_A, we find that the concentration of Mn^{2+} is

$$C_A = -\frac{x\text{-intercept} \times C_{std}}{V_o} = -\frac{-1.731\ \text{mL} \times 100.6\ \text{mg/L}}{25.00\ \text{mL}} = 6.96\ \text{mg/L}$$

For Figure 5.3.6 b, the *x*-intercept is

$$x\text{-intercept} = -\frac{0.1478}{0.0425\ \text{mL/mg}} = -3.478\ \text{mg/mL}$$

and the concentration of Mn^{2+} is

$$C_A = -\frac{x\text{-intercept} \times V_f}{V_o} = -\frac{-3.478\ \text{mg/mL} \times 50.00\ \text{mL}}{25.00\ \text{mL}} = 6.96\ \text{mg/L}$$

Since we construct a standard additions calibration curve in the sample, we can not use the calibration equation for other samples. Each sample, therefore, requires its own standard additions calibration curve. This is a serious drawback if you have many samples. For example, suppose you need to analyze 10 samples using a five-point calibration curve. For a normal calibration curve you need to analyze only 15 solutions (five standards and ten samples). If you use the method of standard additions, however, you must analyze 50 solutions (each of the ten samples is analyzed five times, once before spiking and after each of four spikes).

Using a Standard Addition to Identify Matrix Effects

We can use the method of standard additions to validate an external standardization when matrix matching is not feasible. First, we prepare a normal calibration curve of S_{std} versus C_{std} and determine the value of k_A from its slope. Next, we prepare a standard additions calibration curve using Equation **5.3.8**, plotting the data as shown in Figure 5.3.6 b. The slope of this standard additions calibration curve provides an independent determination of k_A. If there is no significant difference between the two values of k_A, then we can ignore the difference between the sample's matrix and that of the external standards. When the values of k_A are significantly different, then using a normal calibration curve introduces a proportional determinate error.

Internal Standards

To use an external standardization or the method of standard additions, we must be able to treat identically all samples and standards. When this is not possible, the accuracy and precision of our standardization may suffer. For example, if our analyte is in a volatile solvent, then its concentration will increase if we lose solvent to evaporation. Suppose we have a sample and a standard with identical concentrations of analyte and identical signals. If both experience the same proportional loss of solvent, then their respective concentrations of analyte and signals remain identical. In effect, we can ignore evaporation if the samples and the standards experience an equivalent loss of solvent. If an identical standard and sample lose different amounts of solvent, however, then their respective concentrations and signals are no longer equal. In this case a simple external standardization or standard addition is not possible.

We can still complete a standardization if we reference the analyte's signal to a signal from another species that we add to all samples and standards. The species, which we call an ***internal standard***, must be different than the analyte.

Because the analyte and the internal standard receive the same treatment, the ratio of their signals is unaffected by any lack of reproducibility in the procedure. If a solution contains an analyte of concentration C_A and an internal standard of concentration C_{IS}, then the signals due to the analyte, S_A, and the internal standard, S_{IS}, are

$$S_A = k_A C_A$$

$$S_{IS} = k_{SI} C_{IS}$$

where k_A and k_{IS} are the sensitivities for the analyte and the internal standard, respectively. Taking the ratio of the two signals gives the fundamental equation for an internal standardization.

$$\frac{S_A}{S_{IS}} = \frac{k_A C_A}{k_{IS} C_{IS}} = K \times \frac{C_A}{C_{IS}} \tag{5.3.12}$$

Because K is a ratio of the analyte's sensitivity and the internal standard's sensitivity, it is not necessary to determine independently values for either k_A or k_{IS}.

Single Internal Standard

In a single-point internal standardization, we prepare a single standard that contains the analyte and the internal standard, and use it to determine the value of K in Equation 5.3.12.

$$K = \left(\frac{C_{IS}}{C_A}\right)_{std} \times \left(\frac{S_A}{S_{IS}}\right)_{std} \tag{5.3.13}$$

Having standardized the method, the analyte's concentration is given by

$$C_A = \frac{C_{IS}}{K} \times \left(\frac{S_A}{S_{IS}}\right)_{samp}$$

Example 5.3.7

A sixth spectrophotometric method for the quantitative analysis of Pb^{2+} in blood uses Cu^{2+} as an internal standard. A standard that is 1.75 ppb Pb^{2+} and 2.25 ppb Cu^{2+} yields a ratio of $(S_A/S_{IS})_{std}$ of 2.37. A sample of blood spiked with the same concentration of Cu^{2+} gives a signal ratio, $(S_A/S_{IS})_{samp}$, of 1.80. What is the concentration of Pb^{2+} in the sample of blood?

Solution

Equation 5.3.13 allows us to calculate the value of K using the data for the standard

$$K = \left(\frac{C_{IS}}{C_A}\right)_{std} \times \left(\frac{S_A}{S_{IS}}\right)_{std} = \frac{2.25 \text{ ppb } Cu^{2+}}{1.75 \text{ ppb } Pb^{2+}} \times 2.37 = 3.05 \frac{\text{ppb } Cu^{2+}}{\text{ppb } Pb^{2+}}$$

The concentration of Pb^{2+}, therefore, is

$$C_A = \frac{C_{IS}}{K} \times \left(\frac{S_A}{S_{IS}}\right)_{samp} = \frac{2.25 \text{ ppb } Cu^{2+}}{3.05 \frac{\text{ppb } Cu^{2+}}{\text{ppb } Pb^{2+}}} \times 1.80 = 1.33 \text{ ppb } Pb^{2+}$$

Multiple Internal Standards

A single-point internal standardization has the same limitations as a single-point normal calibration. To construct an internal standard calibration curve we prepare a series of standards, each of which contains the same concentration of internal standard and a different concentrations of analyte. Under these conditions a calibration curve of $(S_A/S_{IS})_{std}$ versus C_A is linear with a slope of K/C_{IS}.

> Although the usual practice is to prepare the standards so that each contains an identical amount of the internal standard, this is not a requirement.

Example 5.3.8

A seventh spectrophotometric method for the quantitative analysis of Pb^{2+} in blood gives a linear internal standards calibration curve for which

$$\left(\frac{S_A}{S_{IS}}\right)_{std} = (2.11\ \text{ppb}^{-1} \times C_A) - 0.006$$

What is the ppb Pb^{2+} in a sample of blood if $(S_A/S_{IS})_{samp}$ is 2.80?

Solution

To determine the concentration of Pb^{2+} in the sample of blood we replace $(S_A/S_{IS})_{std}$ in the calibration equation with $(S_A/S_{IS})_{samp}$ and solve for C_A.

$$C_A = \frac{\left(\frac{S_A}{S_{IS}}\right)_{samp} + 0.006}{2.11\ \text{ppb}^{-1}} = \frac{2.80 + 0.006}{2.11\ \text{ppb}^{-1}} = 1.33\ \text{ppb Pb}^{2+}$$

The concentration of Pb^{2+} in the sample of blood is 1.33 ppb.

In some circumstances it is not possible to prepare the standards so that each contains the same concentration of internal standard. This is the case, for example, when we prepare samples by mass instead of volume. We can still prepare a calibration curve, however, by plotting $(S_A/S_{IS})_{std}$ versus C_A/C_{IS}, giving a linear calibration curve with a slope of K.

You might wonder if it is possible to include an internal standard in the method of standard additions to correct for both matrix effects and uncontrolled variations between samples; well, the answer is yes as described in the paper "Standard Dilution Analysis," the full reference for which is Jones, W. B.; Donati, G. L.; Calloway, C. P.; Jones, B. T. *Anal. Chem.* **2015**, *87*, 2321-2327.

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5.4: Linear Regression and Calibration Curves

In a single-point external standardization we determine the value of k_A by measuring the signal for a single standard that contains a known concentration of analyte. Using this value of k_A and our sample's signal, we then calculate the concentration of analyte in our sample (see Example 5.3.1). With only a single determination of k_A, a quantitative analysis using a single-point external standardization is straightforward.

A multiple-point standardization presents a more difficult problem. Consider the data in Table 5.4.1 for a multiple-point external standardization. What is our best estimate of the relationship between S_{std} and C_{std}? It is tempting to treat this data as five separate single-point standardizations, determining k_A for each standard, and reporting the mean value for the five trials. Despite it simplicity, this is not an appropriate way to treat a multiple-point standardization.

Table 5.4.1 : Data for a Hypothetical Multiple-Point External Standardization

C_{std} (arbitrary units)	S_{std} (arbitrary units)	$k_A = S_{std}/C_{std}$
0.000	0.00	—
0.100	12.36	123.6
0.200	24.83	124.2
0.300	35.91	119.7
0.400	48.79	122.0
0.500	60.42	122.8
		mean k_A = 122.5

So why is it inappropriate to calculate an average value for k_A using the data in Table 5.4.1 ? In a single-point standardization we assume that the reagent blank (the first row in Table 5.4.1) corrects for all constant sources of determinate error. If this is not the case, then the value of k_A from a single-point standardization has a constant determinate error. Table 5.4.2 demonstrates how an uncorrected constant error affects our determination of k_A. The first three columns show the concentration of analyte in a set of standards, C_{std}, the signal without any source of constant error, S_{std}, and the actual value of k_A for five standards. As we expect, the value of k_A is the same for each standard. In the fourth column we add a constant determinate error of +0.50 to the signals, $(S_{std})_e$. The last column contains the corresponding apparent values of k_A. Note that we obtain a different value of k_A for each standard and that each apparent k_A is greater than the true value.

Table 5.4.2 : Effect of a Constant Determinate Error on the Value of k_A From a Single-Point Standardization

C_{std}	S_{std} (without constant error)	$k_A = S_{std}/C_{std}$ (actual)	$(S_{std})_e$ (with constant error)	$k_A = (S_{std})_e/C_{std}$ (apparent)
1.00	1.00	1.00	1.50	1.50
2.00	2.00	1.00	2.50	1.25
3.00	3.00	1.00	3.50	1.17
4.00	4.00	1.00	4.50	1.13
5.00	5.00	1.00	5.50	1.10
		mean k_A (true) = 1.00		mean k_A (apparent) = 1.23

How do we find the best estimate for the relationship between the signal and the concentration of analyte in a multiple-point standardization? Figure 5.4.1 shows the data in Table 5.4.1 plotted as a normal calibration curve. Although the data certainly appear

to fall along a straight line, the actual calibration curve is not intuitively obvious. The process of determining the best equation for the calibration curve is called linear regression.

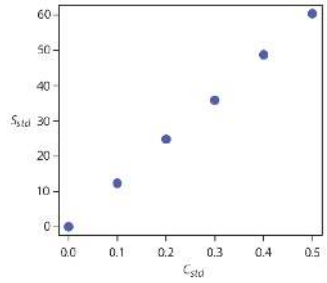


Figure 5.4.1 : Normal calibration curve data for the hypothetical multiple-point external standardization in Table 5.4.1 .

Linear Regression of Straight Line Calibration Curves

When a calibration curve is a straight-line, we represent it using the following mathematical equation

$$y = \beta_0 + \beta_1 x \tag{5.4.1}$$

where y is the analyte's signal, S_{std}, and x is the analyte's concentration, C_{std}. The constants β_0 and β_1 are, respectively, the calibration curve's expected y-intercept and its expected slope. Because of uncertainty in our measurements, the best we can do is to estimate values for β_0 and β_1, which we represent as b_0 and b_1. The goal of a ***linear regression*** analysis is to determine the best estimates for b_0 and b_1. How we do this depends on the uncertainty in our measurements.

Unweighted Linear Regression with Errors in *y*

The most common method for completing the linear regression for Equation 5.4.1 makes three assumptions:

1. that the difference between our experimental data and the calculated regression line is the result of indeterminate errors that affect y
2. that indeterminate errors that affect y are normally distributed
3. that the indeterminate errors in y are independent of the value of x

Because we assume that the indeterminate errors are the same for all standards, each standard contributes equally in our estimate of the slope and the y-intercept. For this reason the result is considered an ***unweighted linear regression***.

The second assumption generally is true because of the central limit theorem, which we considered in Chapter 4. The validity of the two remaining assumptions is less obvious and you should evaluate them before you accept the results of a linear regression. In particular the first assumption always is suspect because there certainly is some indeterminate error in the measurement of x. When we prepare a calibration curve, however, it is not unusual to find that the uncertainty in the signal, S_{std}, is significantly larger than the uncertainty in the analyte's concentration, C_{std}. In such circumstances the first assumption is usually reasonable.

How a Linear Regression Works

To understand the logic of a linear regression consider the example shown in Figure 5.4.2 , which shows three data points and two possible straight-lines that might reasonably explain the data. How do we decide how well these straight-lines fit the data, and how do we determine the best straight-line?

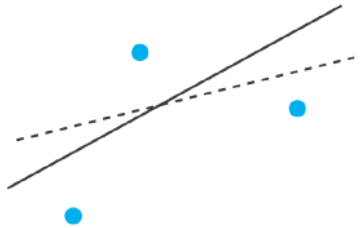


Figure 5.4.2 : Illustration showing three data points and two possible straight-lines that might explain the data. The goal of a linear regression is to find the mathematical model, in this case a straight-line, that best explains the data.

Let's focus on the solid line in Figure 5.4.2 . The equation for this line is

$$\hat{y} = b_0 + b_1 x \tag{5.4.2}$$

where b_0 and b_1 are estimates for the y-intercept and the slope, and $\hat{y}$ is the predicted value of y for any value of x. Because we assume that all uncertainty is the result of indeterminate errors in y, the difference between y and $\hat{y}$ for each value of x is the residual error, r, in our mathematical model.

$$r_i = (y_i - \hat{y}_i)$$

Figure 5.4.3 shows the residual errors for the three data points. The smaller the total residual error, R, which we define as

$$R = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2 \tag{5.4.3}$$

the better the fit between the straight-line and the data. In a linear regression analysis, we seek values of b_0 and b_1 that give the smallest total residual error.

> The reason for squaring the individual residual errors is to prevent a positive residual error from canceling out a negative residual error. You have seen this before in the equations for the sample and population standard deviations. You also can see from this equation why a linear regression is sometimes called the method of least squares.

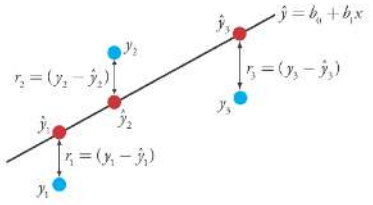


Figure 5.4.3 : Illustration showing the evaluation of a linear regression in which we assume that all uncertainty is the result of indeterminate errors in y. The points in blue, y , are the original data and the points in red, y_i , are the predicted values from the regression equation, $\hat{y} = b_0 + b_1 x$.The smaller the total residual error (Equation 5.4.3), the better the fit of the straight-line to the data.

Finding the Slope and y-Intercept

Although we will not formally develop the mathematical equations for a linear regression analysis, you can find the derivations in many standard statistical texts [See, for example, Draper, N. R.; Smith, H. Applied Regression Analysis, 3rd ed.; Wiley: New York, 1998]. The resulting equation for the slope, b_1, is

$$b_1 = \frac{n\sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n\sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i\right)^2} \tag{5.4.4}$$

and the equation for the y-intercept, b_0, is

$$b_0 = \frac{\sum_{i=1}^{n} y_i - b_1 \sum_{i=1}^{n} x_i}{n} \tag{5.4.5}$$

Although Equation 5.4.4 and Equation 5.4.5 appear formidable, it is necessary only to evaluate the following four summations

$$\sum_{i=1}^{n} x_i \quad \sum_{i=1}^{n} y_i \quad \sum_{i=1}^{n} x_i y_i \quad \sum_{i=1}^{n} x_i^2$$

Many calculators, spreadsheets, and other statistical software packages are capable of performing a linear regression analysis based on this model. To save time and to avoid tedious calculations, learn how to use one of these tools (and see Section 5.6 for details on completing a linear regression analysis using Excel and R.). For illustrative purposes the necessary calculations are shown in detail in the following example.

Equation 5.4.4 and Equation 5.4.5 are written in terms of the general variables x and y. As you work through this example, remember that x corresponds to C_{std}, and that y corresponds to S_{std}.

Example 5.4.1

Using the data from Table 5.4.1 , determine the relationship between S_{std} and C_{std} using an unweighted linear regression.

Solution

We begin by setting up a table to help us organize the calculation.

x_i	y_i	$x_i y_i$	x_i^2
0.000	0.00	0.000	0.000
0.100	12.36	1.236	0.010
0.200	24.83	4.966	0.040
0.300	35.91	10.773	0.090
0.400	48.79	19.516	0.160
0.500	60.42	30.210	0.250

Adding the values in each column gives

$$\sum_{i=1}^{n} x_i = 1.500 \quad \sum_{i=1}^{n} y_i = 182.31 \quad \sum_{i=1}^{n} x_i y_i = 66.701 \quad \sum_{i=1}^{n} x_i^2 = 0.550$$

Substituting these values into Equation 5.4.4 and Equation 5.4.5, we find that the slope and the y-intercept are

$$b_1 = \frac{(6 \times 66.701) - (1.500 \times 182.31)}{(6 \times 0.550) - (1.500)^2} = 120.706 \approx 120.71$$

$$b_0 = \frac{182.31 - (120.706 \times 1.500)}{6} = 0.209 \approx 0.21$$

The relationship between the signal and the analyte, therefore, is

$$S_{std} = 120.71 \times C_{std} + 0.21$$

For now we keep two decimal places to match the number of decimal places in the signal. The resulting calibration curve is shown in Figure 5.4.4 .

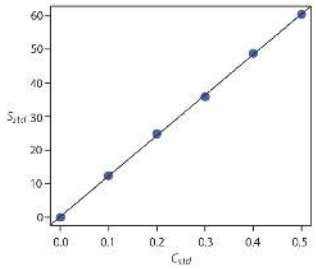


Figure 5.4.4 : Calibration curve for the data in Table 5.4.1 and Example 5.4.1 .

Uncertainty in the Regression Analysis

As shown in Figure 5.4.4 , because indeterminate errors in the signal, the regression line may not pass through the exact center of each data point. The cumulative deviation of our data from the regression line—that is, the total residual error—is proportional to the uncertainty in the regression. We call this uncertainty the ***standard deviation about the regression***, s_r, which is equal to

$$s_r = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n-2}} \tag{5.4.6}$$

where y_i is the i^{th} experimental value, and $\hat{y}_i$ is the corresponding value predicted by the regression line in Equation 5.4.2. Note that the denominator of Equation 5.4.6 indicates that our regression analysis has n – 2 degrees of freedom—we lose two degree of freedom because we use two parameters, the slope and the y-intercept, to calculate $\hat{y}_i$.

> Did you notice the similarity between the standard deviation about the regression (Equation 5.4.6) and the standard deviation for a sample (Equation 4.1.1)?

A more useful representation of the uncertainty in our regression analysis is to consider the effect of indeterminate errors on the slope, b_1, and the y-intercept, b_0, which we express as standard deviations.

$$s_{b_1} = \sqrt{\frac{n s_r^2}{n\sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i\right)^2}} = \sqrt{\frac{s_r^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}} \tag{5.4.7}$$

$$s_{b_0} = \sqrt{\frac{s_r^2 \sum_{i=1}^{n} x_i^2}{n\sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i\right)^2}} = \sqrt{\frac{s_r^2 \sum_{i=1}^{n} x_i^2}{n\sum_{i=1}^{n}(x_i - \bar{x})^2}} \tag{5.4.8}$$

We use these standard deviations to establish confidence intervals for the expected slope, β_1, and the expected y-intercept, β_0

$$\beta_1 = b_1 \pm t s_{b_1} \tag{5.4.9}$$

$$\beta_0 = b_0 \pm t s_{b_0} \tag{5.4.10}$$

where we select t for a significance level of α and for n – 2 degrees of freedom. Note that Equation 5.4.9 and Equation 5.4.10 do not contain a factor of $(\sqrt{n})^{-1}$ because the confidence interval is based on a single regression line.

Example 5.4.2

Calculate the 95% confidence intervals for the slope and y-intercept from Example 5.4.1 .

Solution

We begin by calculating the standard deviation about the regression. To do this we must calculate the predicted signals, $\hat{y}_i$, using the slope and y-intercept from Example 5.4.1 , and the squares of the residual error, $(y_i - \hat{y}_i)^2$. Using the last standard as an example, we find that the predicted signal is

$$\hat{y}_6 = b_0 + b_1 x_6 = 0.209 + (120.706 \times 0.500) = 60.562$$

and that the square of the residual error is

$$(y_i - \hat{y}_i)^2 = (60.42 - 60.562)^2 = 0.2016 \approx 0.202$$

The following table displays the results for all six solutions.

x_i	y_i	$\hat{y}_i$	$(y_i - \hat{y}_i)^2$
0.000	0.00	0.209	0.0437
0.100	12.36	12.280	0.0064
0.200	24.83	24.350	0.2304
0.300	35.91	36.421	0.2611
0.400	48.79	48.491	0.0894
0.500	60.42	60.562	0.0202

Adding together the data in the last column gives the numerator of Equation 5.4.6 as 0.6512; thus, the standard deviation about the regression is

$$s_r = \sqrt{\frac{0.6512}{6-2}} = 0.4035$$

Next we calculate the standard deviations for the slope and the *y*-intercept using Equation 5.4.7 and Equation 5.4.8. The values for the summation terms are from Example 5.4.1 .

$$s_{b_1} = \sqrt{\frac{6 \times (0.4035)^2}{(6 \times 0.550) - (1.500)^2}} = 0.965$$

$$s_{b_0} = \sqrt{\frac{(0.4035)^2 \times 0.550}{(6 \times 0.550) - (1.500)^2}} = 0.292$$

Finally, the 95% confidence intervals ($\alpha = 0.05$, 4 degrees of freedom) for the slope and *y*-intercept are

$$\beta_1 = b_1 \pm t s_{b_1} = 120.706 \pm (2.78 \times 0.965) = 120.7 \pm 2.7$$

$$\beta_0 = b_0 \pm t s_{b_0} = 0.209 \pm (2.78 \times 0.292) = 0.2 \pm 0.80$$

where $t(0.05, 4)$ from Appendix 4 is 2.78. The standard deviation about the regression, s_r, suggests that the signal, S_{std}, is precise to one decimal place. For this reason we report the slope and the *y*-intercept to a single decimal place.

Minimizing Uncertainty in Calibration Model

To minimize the uncertainty in a calibration curve's slope and *y*-intercept, we evenly space our standards over a wide range of analyte concentrations. A close examination of Equation 5.4.7 and Equation 5.4.8 help us appreciate why this is true. The denominators of both equations include the term $\sum_{i=1}^{n}(x_i - \overline{x}_i)^2$. The larger the value of this term—which we accomplish by increasing the range of *x* around its mean value—the smaller the standard deviations in the slope and the *y*-intercept. Furthermore, to minimize the uncertainty in the *y*-intercept, it helps to decrease the value of the term $\sum_{i=1}^{n} x_i$ in Equation 5.4.8, which we accomplish by including standards for lower concentrations of the analyte.

Obtaining the Analyte's Concentration From a Regression Equation

Once we have our regression equation, it is easy to determine the concentration of analyte in a sample. When we use a normal calibration curve, for example, we measure the signal for our sample, S_{samp}, and calculate the analyte's concentration, C_A, using the regression equation.

$$C_A = \frac{S_{samp} - b_0}{b_1} \tag{5.4.11}$$

What is less obvious is how to report a confidence interval for C_A that expresses the uncertainty in our analysis. To calculate a confidence interval we need to know the standard deviation in the analyte's concentration, s_{C_A}, which is given by the following equation

$$s_{C_A} = \frac{s_r}{b_1}\sqrt{\frac{1}{m} + \frac{1}{n} + \frac{\left(\overline{S}_{samp} - \overline{S}_{std}\right)^2}{(b_1)^2 \sum_{i=1}^{n}\left(C_{std_i} - \overline{C}_{std}\right)^2}} \tag{5.4.12}$$

where m is the number of replicate we use to establish the sample's average signal, S_{samp}, n is the number of calibration standards, S_{std} is the average signal for the calibration standards, and C_{std_1} and $\overline{C}_{std}$ are the individual and the mean concentrations for the calibration standards. Knowing the value of s_{C_A}, the confidence interval for the analyte's concentration is

$$\mu_{C_A} = C_A \pm t s_{C_A}$$

where μ_{C_A} is the expected value of C_A in the absence of determinate errors, and with the value of t is based on the desired level of confidence and $n - 2$ degrees of freedom.

Equation 5.4.12 is written in terms of a calibration experiment. A more general form of the equation, written in terms of x and y, is given here.

$$s_x = \frac{s_r}{b_1}\sqrt{\frac{1}{m} + \frac{1}{n} + \frac{\left(\overline{Y} - \overline{y}\right)^2}{(b_1)^2 \sum_{i=1}^{n}\left(x_i - \overline{x}\right)^2}}$$

A close examination of Equation 5.4.12 should convince you that the uncertainty in C_A is smallest when the sample's average signal, $\overline{S}_{samp}$, is equal to the average signal for the standards, $\overline{S}_{std}$. When practical, you should plan your calibration curve so that S_{samp} falls in the middle of the calibration curve. For more information about these regression equations see (a) Miller, J. N. *Analyst* **1991**, *116*, 3–14; (b) Sharaf, M. A.; Illman, D. L.; Kowalski, B. R. *Chemometrics*, Wiley-Interscience: New York, 1986, pp. 126-127; (c) Analytical Methods Committee "Uncertainties in concentrations estimated from calibration experiments," AMC Technical Brief, March 2006.

Example 5.4.3

Three replicate analyses for a sample that contains an unknown concentration of analyte, yield values for S_{samp} of 29.32, 29.16 and 29.51 (arbitrary units). Using the results from Example 5.4.1 and Example 5.4.2 , determine the analyte's concentration, C_A, and its 95% confidence interval.

Solution

The average signal, $\overline{S}_{samp}$, is 29.33, which, using Equation 5.4.11 and the slope and the y-intercept from Example 5.4.1 , gives the analyte's concentration as

$$C_A = \frac{\overline{S}_{samp} - b_0}{b_1} = \frac{29.33 - 0.209}{120.706} = 0.241$$

To calculate the standard deviation for the analyte's concentration we must determine the values for $\overline{S}_{std}$ and for $\sum_{i=1}^{2}(C_{std_i} - \overline{C}_{std})^2$. The former is just the average signal for the calibration standards, which, using the data in Table 5.4.1 , is 30.385. Calculating $\sum_{i=1}^{2}(C_{std_i} - \overline{C}_{std})^2$ looks formidable, but we can simplify its calculation by recognizing that this sum-of-squares is the numerator in a standard deviation equation; thus,

$$\sum_{i=1}^{n}(C_{std_i} - \overline{C}_{std})^2 = (s_{C_{std}})^2 \times (n-1)$$

where $s_{C_{std}}$ is the standard deviation for the concentration of analyte in the calibration standards. Using the data in Table 5.4.1 we find that $s_{C_{std}}$ is 0.1871 and

$$\sum_{i=1}^{n}(C_{std_i}-\overline{C}_{std})^2 = (0.1872)^2 \times (6-1) = 0.175$$

Substituting known values into Equation 5.4.12 gives

$$s_{C_A} = \frac{0.4035}{120.706}\sqrt{\frac{1}{3}+\frac{1}{6}+\frac{(29.33-30.385)^2}{(120.706)^2 \times 0.175}} = 0.0024$$

Finally, the 95% confidence interval for 4 degrees of freedom is

$$\mu_{C_A} = C_A \pm ts_{C_A} = 0.241 \pm (2.78 \times 0.0024) = 0.241 \pm 0.007$$

Figure 5.4.5 shows the calibration curve with curves showing the 95% confidence interval for C_A.

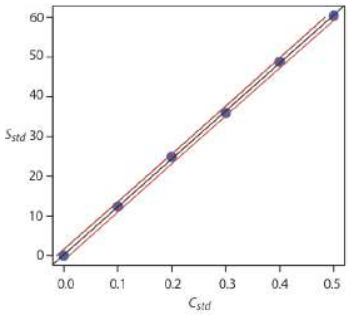


Figure 5.4.5 : Example of a normal calibration curve with a superimposed confidence interval for the analyte's concentration. The points in blue are the original data from Table 5.4.1 . The black line is the normal calibration curve as determined in Example 5.4.1 . The red lines show the 95% confidence interval for C_A assuming a single determination of S_{samp}.

In a standard addition we determine the analyte's concentration by extrapolating the calibration curve to the *x*-intercept. In this case the value of C_A is

$$C_A = x\text{-intercept} = \frac{-b_0}{b_1}$$

and the standard deviation in C_A is

$$s_{C_A} = \frac{s_r}{b_1}\sqrt{\frac{1}{n}+\frac{(\overline{S}_{std})^2}{(b_1)^2\sum_{i=1}^{n}(C_{std_i}-\overline{C}_{std})^2}}$$

where n is the number of standard additions (including the sample with no added standard), and $\overline{S}_{std}$ is the average signal for the n standards. Because we determine the analyte's concentration by extrapolation, rather than by interpolation, s_{C_A} for the method of standard additions generally is larger than for a normal calibration curve.

? Exercise 5.4.1

Figure 5.4.2 shows a normal calibration curve for the quantitative analysis of Cu^{2+}. The data for the calibration curve are shown here.

$[Cu^{2+}]$ (M)	Absorbance
0	0
1.55×10^{-3}	0.050
3.16×10^{-3}	0.093
4.74×10^{-3}	0.143
6.34×10^{-3}	0.188
7.92×10^{-3}	0.236

Complete a linear regression analysis for this calibration data, reporting the calibration equation and the 95% confidence interval for the slope and the *y*-intercept. If three replicate samples give an S_{samp} of 0.114, what is the concentration of analyte in the sample and its 95% confidence interval?

Answer

We begin by setting up a table to help us organize the calculation

x_i	y_i	$x_i y_i$	x_i^2
0.000	0.000	0.000	0.000
1.55×10^{-3}	0.050	7.750×10^{-5}	2.403×10^{-6}
3.16×10^{-3}	0.093	2.939×10^{-4}	9.986×10^{-6}
4.74×10^{-3}	0.143	6.778×10^{-4}	2.247×10^{-5}
6.34×10^{-3}	0.188	1.192×10^{-3}	4.020×10^{-5}
7.92×10^{-3}	0.236	1.869×10^{-3}	6.273×10^{-5}

Adding the values in each column gives

$$\sum_{i=1}^{n} x_i = 2.371 \times 10^{-2} \quad \sum_{i=1}^{n} y_i = 0.710 \quad \sum_{i=1}^{n} x_i y_i = 4.110 \times 10^{-3} \quad \sum_{i=1}^{n} x_i^2 = 1.378 \times 10^{-4}$$

When we substitute these values into Equation 5.4.4 and Equation 5.4.5, we find that the slope and the *y*-intercept are

$$b_1 = \frac{6 \times (4.110 \times 10^{-3}) - (2.371 \times 10^{-2}) \times 0.710}{6 \times (1.378 \times 10^{-4}) - (2.371 \times 10^{-2})^2}) = 29.57$$

$$b_0 = \frac{0.710 - 29.57 \times (2.371 \times 10^{-2}}{6} = 0.0015$$

and that the regression equation is

$$S_{std} = 29.57 \times C_{std} + 0.0015$$

To calculate the 95% confidence intervals, we first need to determine the standard deviation about the regression. The following table helps us organize the calculation.

x_i	y_i	$\hat{y}_i$	$(y_i - \hat{y}_i)^2$
0.000	0.000	0.0015	2.250×10^{-6}
1.55×10^{-3}	0.050	0.0473	7.110×10^{-6}
3.16×10^{-3}	0.093	0.0949	3.768×10^{-6}
4.74×10^{-3}	0.143	0.1417	1.791×10^{-6}
6.34×10^{-3}	0.188	0.1890	9.483×10^{-6}
7.92×10^{-3}	0.236	0.2357	9.339×10^{-6}

Adding together the data in the last column gives the numerator of Equation 5.4.6 as 1.596×10^{-5}. The standard deviation about the regression, therefore, is

$$s_r = \sqrt{\frac{1.596 \times 10^{-5}}{6 - 2}} = 1.997 \times 10^{-3}$$

Next, we need to calculate the standard deviations for the slope and the *y*-intercept using Equation 5.4.7 and Equation 5.4.8.

$$s_{b_1} = \sqrt{\frac{6 \times (1.997 \times 10^{-3})^2}{6 \times (1.378 \times 10^{-4}) - (2.371 \times 10^{-2})^2}} = 0.3007$$

$$s_{b_0} = \sqrt{\frac{(1.997 \times 10^{-3})^2 \times (1.378 \times 10^{-4})}{6 \times (1.378 \times 10^{-4}) - (2.371 \times 10^{-2})^2}} = 1.441 \times 10^{-3}$$

and use them to calculate the 95% confidence intervals for the slope and the *y*-intercept

$$\beta_1 = b_1 \pm t s_{b_1} = 29.57 \pm (2.78 \times 0.3007) = 29.57 \text{ M}^{-1} \pm 0.84 \text{ M}^{-1}$$

$$\beta_0 = b_0 \pm t s_{b_0} = 0.0015 \pm (2.78 \times 1.441 \times 10^{-3}) = 0.0015 \pm 0.0040$$

With an average S_{samp} of 0.114, the concentration of analyte, C_A, is

$$C_A = \frac{S_{samp} - b_0}{b_1} = \frac{0.114 - 0.0015}{29.57 \text{ M}^{-1}} = 3.80 \times 10^{-3} \text{ M}$$

The standard deviation in C_A is

$$s_{C_A} = \frac{1.997 \times 10^{-3}}{29.57} \sqrt{\frac{1}{3} + \frac{1}{6} + \frac{(0.114 - 0.1183)^2}{(29.57)^2 \times (4.408 \times 10^{-5})}} = 4.778 \times 10^{-5}$$

and the 95% confidence interval is

$$\mu = C_A \pm t s_{C_A} = 3.80 \times 10^{-3} \pm \{2.78 \times (4.778 \times 10^{-5})\}$$

$$\mu = 3.80 \times 10^{-3} \text{ M} \pm 0.13 \times 10^{-3} \text{ M}$$

Evaluating a Linear Regression Model

You should never accept the result of a linear regression analysis without evaluating the validity of the model. Perhaps the simplest way to evaluate a regression analysis is to examine the residual errors. As we saw earlier, the residual error for a single calibration standard, r_i, is

$$r_i = (y_i - \hat{y}_i)$$

If the regression model is valid, then the residual errors should be distributed randomly about an average residual error of zero, with no apparent trend toward either smaller or larger residual errors (Figure 5.4.6 a). Trends such as those in Figure 5.4.6 b and Figure 5.4.6 c provide evidence that at least one of the model's assumptions is incorrect. For example, a trend toward larger

residual errors at higher concentrations, Figure 5.4.6 b, suggests that the indeterminate errors affecting the signal are not independent of the analyte's concentration. In Figure 5.4.6 c, the residual errors are not random, which suggests we cannot model the data using a straight-line relationship. Regression methods for the latter two cases are discussed in the following sections.

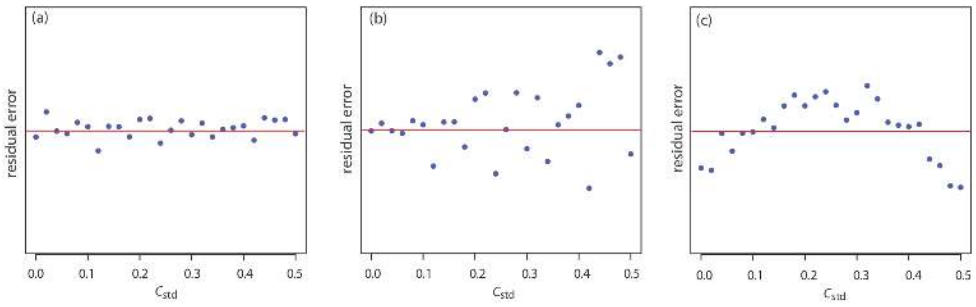


Figure 5.4.6 : Plots of the residual error in the signal, S_{std}, as a function of the concentration of analyte, C_{std}, for an unweighted straight-line regression model. The red line shows a residual error of zero. The distribution of the residual errors in (a) indicates that the unweighted linear regression model is appropriate. The increase in the residual errors in (b) for higher concentrations of analyte, suggests that a weighted straight-line regression is more appropriate. For (c), the curved pattern to the residuals suggests that a straight-line model is inappropriate; linear regression using a quadratic model might produce a better fit.

Exercise 5.4.2

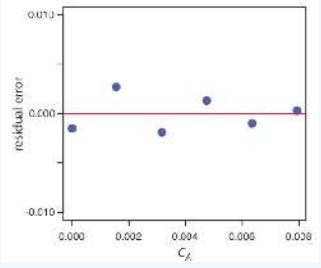
Using your results from Exercise 5.4.1 , construct a residual plot and explain its significance.

Answer

To create a residual plot, we need to calculate the residual error for each standard. The following table contains the relevant information.

x_i	y_i	$\hat{y}_i$	$y_i - \hat{y}_i$
0.000	0.000	0.0015	–0.0015
1.55×10^{-3}	0.050	0.0473	0.0027
3.16×10^{-3}	0.093	0.0949	–0.0019
4.74×10^{-3}	0.143	0.1417	0.0013
6.34×10^{-3}	0.188	0.1890	–0.0010
7.92×10^{-3}	0.236	0.2357	0.0003

The figure below shows a plot of the resulting residual errors. The residual errors appear random, although they do alternate in sign, and that do not show any significant dependence on the analyte's concentration. Taken together, these observations suggest that our regression model is appropriate.



Weighted Linear Regression with Errors in *y*

Our treatment of linear regression to this point assumes that indeterminate errors affecting *y* are independent of the value of *x*. If this assumption is false, as is the case for the data in Figure 5.4.6 b, then we must include the variance for each value of *y* into our determination of the *y*-intercept, b_0, and the slope, b_1; thus

$$b_0 = \frac{\sum_{i=1}^{n} w_i y_i - b_1 \sum_{i=1}^{n} w_i x_i}{n} \tag{5.4.13}$$

$$b_1 = \frac{n\sum_{i=1}^{n} w_i x_i y_i - \sum_{i=1}^{n} w_i x_i \sum_{i=1}^{n} w_i y_i}{n\sum_{i=1}^{n} w_i x_i^2 - \left(\sum_{i=1}^{n} w_i x_i\right)^2} \tag{5.4.14}$$

where w_i is a weighting factor that accounts for the variance in y_i

$$w_i = \frac{n(s_{y_i})^{-2}}{\sum_{i=1}^{n}(s_{y_i})^{-2}} \tag{5.4.15}$$

and s_{y_i} is the standard deviation for y_i. In a ***weighted linear regression***, each *xy*-pair's contribution to the regression line is inversely proportional to the precision of y_i; that is, the more precise the value of *y*, the greater its contribution to the regression.

Example 5.4.4

Shown here are data for an external standardization in which *sstd* is the standard deviation for three replicate determination of the signal. This is the same data used in Example 5.4.1 with additional information about the standard deviations in the signal.

C_{std} (arbitrary units)	S_{std} (arbitrary units)	s_i
0.000	0.00	0.02
0.100	12.36	0.02
0.200	24.83	0.07
0.300	35.91	0.13
0.400	48.79	0.22
0.500	60.42	0.33

Determine the calibration curve's equation using a weighted linear regression. As you work through this example, remember that *x* corresponds to C_{std}, and that *y* corresponds to S_{std}.

Solution

We begin by setting up a table to aid in calculating the weighting factors.

C_{std} (arbitrary units)	S_{std} (arbitrary units)	s_i	$(s_i)^{-2}$	w_i
0.000	0.00	0.02	2500.00	2.8339
0.100	12.36	0.02	2500.00	2.8339
0.200	24.83	0.07	204.08	0.2313
0.300	35.91	0.13	59.17	0.0671
0.400	48.79	0.22	20.66	0.0234
0.500	60.42	0.33	9.18	0.0104

Adding together the values in the fourth column gives

$$\sum_{i=1}^{n}(s_{y_i})^{-2}$$

which we use to calculate the individual weights in the last column. As a check on your calculations, the sum of the individual weights must equal the number of calibration standards, *n*. The sum of the entries in the last column is 6.0000, so all is well. After we calculate the individual weights, we use a second table to aid in calculating the four summation terms in Equation 5.4.13 and Equation 5.4.14.

x_i	y_i	w_i	w_ix_i	w_iy_i	$w_ix_i^2$	$w_ix_iy_i$
0.000	0.00	2.8339	0.0000	0.0000	0.0000	0.0000
0.100	12.36	2.8339	0.2834	35.0270	0.0283	3.5027
0.200	24.83	0.2313	0.0463	5.7432	0.0093	1.1486
0.300	35.91	0.0671	0.0201	2.4096	0.0060	0.7229
0.400	48.79	0.0234	0.0094	1.1417	0.0037	0.4567
0.500	60.42	0.0104	0.0052	0.6284	0.0026	0.3142

Adding the values in the last four columns gives

$$\sum_{i=1}^{n}w_ix_i = 0.3644 \quad \sum_{i=1}^{n}w_iy_i = 44.9499 \quad \sum_{i=1}^{n}w_ix_i^2 = 0.0499 \quad \sum_{i=1}^{n}w_ix_iy_i = 6.1451$$

Substituting these values into the Equation 5.4.13 and Equation 5.4.14 gives the estimated slope and estimated *y*-intercept as

$$b_1 = \frac{(6 \times 6.1451) - (0.3644 \times 44.9499)}{(6 \times 0.0499) - (0.3644)^2} = 122.985$$

$$b_0 = \frac{44.9499 - (122.985 \times 0.3644)}{6} = 0.0224$$

The calibration equation is

$$S_{std} = 122.98 \times C_{std} + 0.2$$

Figure 5.4.7 shows the calibration curve for the weighted regression and the calibration curve for the unweighted regression in Example 5.4.1 . Although the two calibration curves are very similar, there are slight differences in the slope and in the *y*-intercept. Most notably, the *y*-intercept for the weighted linear regression is closer to the expected value of zero. Because the standard deviation for the signal, S_{std}, is smaller for smaller concentrations of analyte, C_{std}, a weighted linear regression gives more emphasis to these standards, allowing for a better estimate of the *y*-intercept.

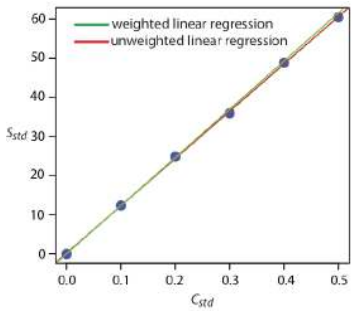


Figure 5.4.7 : A comparison of the unweighted and the weighted normal calibration curves. See Example 5.4.1 for details of the unweighted linear regression and Example 5.4.4 for details of the weighted linear regression.

Equations for calculating confidence intervals for the slope, the *y*-intercept, and the concentration of analyte when using a weighted linear regression are not as easy to define as for an unweighted linear regression [Bonate, P. J. *Anal. Chem.* **1993**, *65*, 1367–1372].

The confidence interval for the analyte's concentration, however, is at its optimum value when the analyte's signal is near the weighted centroid, y_c , of the calibration curve.

$$y_c = \frac{1}{n}\sum_{i=1}^{n} w_i x_i$$

Weighted Linear Regression with Errors in Both *x* and *y*

If we remove our assumption that indeterminate errors affecting a calibration curve are present only in the signal (*y*), then we also must factor into the regression model the indeterminate errors that affect the analyte's concentration in the calibration standards (*x*). The solution for the resulting regression line is computationally more involved than that for either the unweighted or weighted regression lines. Although we will not consider the details in this textbook, you should be aware that neglecting the presence of indeterminate errors in *x* can bias the results of a linear regression.

> See, for example, Analytical Methods Committee, "Fitting a linear functional relationship to data with error on both variable," AMC Technical Brief, March, 2002), as well as this chapter's Additional Resources.

Curvilinear and Multivariate Regression

A straight-line regression model, despite its apparent complexity, is the simplest functional relationship between two variables. What do we do if our calibration curve is curvilinear—that is, if it is a curved-line instead of a straight-line? One approach is to try transforming the data into a straight-line. Logarithms, exponentials, reciprocals, square roots, and trigonometric functions have been used in this way. A plot of log(*y*) versus *x* is a typical example. Such transformations are not without complications, of which the most obvious is that data with a uniform variance in *y* will not maintain that uniform variance after it is transformed.

> It is worth noting that the term "linear" does not mean a straight-line. A linear function may contain more than one additive term, but each such term has one and only one adjustable multiplicative parameter. The function
>
> $$y = ax + bx^2$$
>
> is an example of a linear function because the terms *x* and x^2 each include a single multiplicative parameter, *a* and *b*, respectively. The function
>
> $$y = x^b$$
>
> is nonlinear because *b* is not a multiplicative parameter; it is, instead, a power. This is why you can use linear regression to fit a polynomial equation to your data.
>
> Sometimes it is possible to transform a nonlinear function into a linear function. For example, taking the log of both sides of the nonlinear function above gives a linear function.
>
> $$\log(y) = b\log(x)$$

Another approach to developing a linear regression model is to fit a polynomial equation to the data, such as $y = a + bx + cx^2$. You can use linear regression to calculate the parameters *a*, *b*, and *c*, although the equations are different than those for the linear regression of a straight-line. If you cannot fit your data using a single polynomial equation, it may be possible to fit separate polynomial equations to short segments of the calibration curve. The result is a single continuous calibration curve known as a spline function.

> For details about curvilinear regression, see (a) Sharaf, M. A.; Illman, D. L.; Kowalski, B. R. *Chemometrics*, Wiley-Interscience: New York, 1986; (b) Deming, S. N.; Morgan, S. L. *Experimental Design: A Chemometric Approach*, Elsevier: Amsterdam, 1987.

The regression models in this chapter apply only to functions that contain a single independent variable, such as a signal that depends upon the analyte's concentration. In the presence of an interferent, however, the signal may depend on the concentrations of both the analyte and the interferent

$$S = k_A C_A + k_I C I + S_{reag}$$

where k_I is the interferent's sensitivity and C_I is the interferent's concentration. Multivariate calibration curves are prepared using standards that contain known amounts of both the analyte and the interferent, and modeled using multivariate regression.

> See Beebe, K. R.; Kowalski, B. R. *Anal. Chem.* **1987**, *59*, 1007A–1017A. for additional details, and check out this chapter's Additional Resources for more information about linear regression with errors in both variables, curvilinear regression, and multivariate regression.

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5.5: Compensating for the Reagent Blank

Thus far in our discussion of strategies for standardizing analytical methods, we have assumed that a suitable reagent blank is available to correct for signals arising from sources other than the analyte. We did not, however ask an important question: "What constitutes an appropriate reagent blank?" Surprisingly, the answer is not immediately obvious.

In one study, approximately 200 analytical chemists were asked to evaluate a data set consisting of a normal calibration curve, a separate analyte-free blank, and three samples with different sizes, but drawn from the same source [Cardone, M. J. *Anal. Chem.* **1986**, *58*, 433–438]. The first two columns in Table 5.5.1 shows a series of external standards and their corresponding signals. The normal calibration curve for the data is

$$S_{std} = 0.0750 \times W_{std} + 0.1250$$

where the *y*-intercept of 0.1250 is the calibration blank. A separate reagent blank gives the signal for an analyte-free sample.

Table 5.5.1 : Data Used to Study the Blank in an Analytical Method

W_{std}	S_{std}	Sample Number	W_{samp}	S_{samp}
1.6667	0.2500	1	62.4746	0.8000
5.0000	0.5000	2	82.7915	1.0000
8.3333	0.7500	3	103.1085	1.2000
11.6667	0.8413			
18.1600	1.4870		reagent blank	0.1000
19.9333	1.6200			
$S_{std} = 0.0750 \times W_{std} + 0.1250$				
W_{std}: weight of analyte used to prepare the external standard; diluted to a volume, V				
W_{samp}: weight of sample used to prepare sample as analyzed; diluted to a volume, V				

In working up this data, the analytical chemists used at least four different approaches to correct the signals: (a) ignoring both the calibration blank, *CB*, and the reagent blank, *RB*, which clearly is incorrect; (b) using the calibration blank only; (c) using the reagent blank only; and (d) using both the calibration blank and the reagent blank. The first four rows of Table 5.5.2 shows the equations for calculating the analyte's concentration using each approach, along with the reported concentrations for the analyte in each sample.

Table 5.5.2 : Equations and Resulting Concentrations of Analyte for Different Approaches to Correcting for the Blank

	Concentration of Analyte in...

Approach for Correcting the Signal	Equation	Concentration of Analyte in...		
		Sample 1	Sample 2	Sample 3
ignore calibration and reagent blanks	$C_A = \frac{W_A}{W_{samp}} = \frac{S_{samp}}{k_A W_{samp}}$	0.1707	0.1610	0.1552
use calibration blank only	$C_A = \frac{W_A}{W_{samp}} = \frac{S_{samp} - CB}{k_A W_{samp}}$	0.1441	0.1409	0.1390
use reagent blank only	$C_A = \frac{W_A}{W_{samp}} = \frac{S_{samp} - RB}{k_A W_{samp}}$	0.1494	0.1449	0.1422
use both calibration and reagent blanks	$C_A = \frac{W_A}{W_{samp}} = \frac{S_{samp} - CB - RB}{k_A W_{samp}}$	0.1227	0.1248	0.1266
use total Youden blank	$C_A = \frac{W_A}{W_{samp}} = \frac{S_{samp} - TYB}{k_A W_{samp}}$	0.1313	0.1313	0.1313

$C_A =$ concentration of analyte; $W_A =$ weight of analyte; W_{samp} weight of sample; $k_A =$ slope of calibration curve (0.0750; slope of calibration equation); $CB =$ calibration blank (0.125; intercept of calibration equation); $RB =$ reagent blank (0.100); $TYB =$ total Youden blank (0.185; see text)

That all four methods give a different result for the analyte's concentration underscores the importance of choosing a proper blank, but does not tell us which blank is correct. Because all four methods fail to predict the same concentration of analyte for each sample, none of these blank corrections properly accounts for an underlying constant source of determinate error.

To correct for a constant method error, a blank must account for signals from any reagents and solvents used in the analysis and any bias that results from interactions between the analyte and the sample's matrix. Both the calibration blank and the reagent blank compensate for signals from reagents and solvents. Any difference in their values is due to indeterminate errors in preparing and analyzing the standards.

> Because we are considering a matrix effect of sorts, you might think that the method of standard additions is one way to overcome this problem. Although the method of standard additions can compensate for proportional determinate errors, it cannot correct for a constant determinate error; see Ellison, S. L. R.; Thompson, M. T. "Standard additions: myth and reality," *Analyst*, **2008**, *133*, 992–997.

Unfortunately, neither a calibration blank nor a reagent blank can correct for a bias that results from an interaction between the analyte and the sample's matrix. To be effective, the blank must include both the sample's matrix and the analyte and, consequently, it must be determined using the sample itself. One approach is to measure the signal for samples of different size, and to determine the regression line for a plot of S_{samp} versus the amount of sample. The resulting *y*-intercept gives the signal in the absence of sample, and is known as the ***total Youden blank*** [Cardone, M. J. *Anal. Chem.* **1986**, *58*, 438–445]. This is the true blank correction. The regression line for the three samples in Table 5.5.1 is

$$S_{samp} = 0.009844 \times W_{samp} + 0.185$$

giving a true blank correction of 0.185. As shown in Table 5.5.2 , using this value to correct S_{samp} gives identical values for the concentration of analyte in all three samples.

The use of the total Youden blank is not common in analytical work, with most chemists relying on a calibration blank when using a calibration curve and a reagent blank when using a single-point standardization. As long we can ignore any constant bias due to interactions between the analyte and the sample's matrix, which is often the case, the accuracy of an analytical method will not suffer. It is a good idea, however, to check for constant sources of error before relying on either a calibration blank or a reagent blank.

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5.6: Using Excel for a Linear Regression

Although the calculations in this chapter are relatively straightforward—consisting, as they do, mostly of summations—it is tedious to work through problems using nothing more than a calculator. Excel includes functions for completing a linear regression analysis and for visually evaluating the resulting model.

Excel

Let's use Excel to fit the following straight-line model to the data in Example 5.4.1.

$$y = \beta_0 + \beta_1 x$$

Enter the data into a spreadsheet, as shown in Figure 5.6.1. Depending upon your needs, there are many ways that you can use Excel to complete a linear regression analysis. We will consider three approaches here.

	A	B
1	Cstd	Sstd
2	0.000	0.00
3	0.100	12.36
4	0.200	24.83
5	0.300	35.91
6	0.400	48.79
7	0.500	60.42

Figure 5.6.1: Portion of a spreadsheet containing data from Example 5.4.1 (Cstd = C_{std}; Sstd = S_{std}).

Using Excel's Built-In Functions

If all you need are values for the slope, β_1, and the *y*-intercept, β_0, you can use the following functions:

= intercept(*known_y's*, *known_x's*)

= slope(*known_y's*, *known_x's*)

where *known_y's* is the range of cells that contain the signals (*y*), and *known_x's* is the range of cells that contain the concentrations (*x*). For example, if you click on an empty cell and enter

= slope(B2:B7, A2:A7)

Excel returns exact calculation for the slope (120.705 714 3).

Using Excel's LINEST Function

To obtain the slope and the *y*-intercept, along with additional statistical details, you can use the LINEST function. The LINEST function is standard in Excel on both Macs and PC's and a version of it is available in Google docs.

Once you have your data entered in Excel, highlight a blank area of 2 columns by 5 row and type =LINEST. The syntax for the LINEST function is LINEST(known_y's, [known_x's], [const], [stats]) where known_y's and known x's can be drag entered, the [const] can have the values of TRUE where the intercept is treated normally or FALSE where the intercept is set to have the value of 0, and the [stats] can have the values of True where a set of regression statistics is returned with the slope and intercept or FALSE where the regression statistics are not returned.

Because the LINEST function is an array function, the contents of the active cell bar on the same line above the spreadsheet to the right of the word LINEST and the symbol f_x which will show something like =LINEST(C2:C7,B2:B7,TRUE,TRUE) need to be highlighted and enetered with the Cntrl, Shift and Enter keys all pressed at the same time so the results appear and the contents of of the active cell box appear in curved brackets like {=LINEST(C2:C7,B2:B7,TRUE,TRUE)}. The entry of an array function on a Mac uses the cloverleaf key instead of Cntrl.

The output of the LINEST function should appear for the sample data as the table shown below:

120.7057	0.208571
0.964065	0.291885
0.999745	0.403297
15676.3	4
2549.727	0.650594

Column 1, from top to bottom, shows the slope, the error in the slope, the coefficient of determination, the F statistic or F-observed value , and the the regression sum of squares ss_{reg}). Column 2, from top to bottom, shows the intercept, the error in the intercept, the standard error in the regression or y-estimate, the degrees of freedom, and the residual sum of squares (ss_{resid})

Using Excel's Data Analysis Tools

To obtain the slope and the *y*-intercept, along with additional statistical details, you can use the data analysis tools in the Data Analysis ToolPak. The ToolPak is not a standard part of Excel's instillation and is only available for PCs. To see if you have access to the Analysis ToolPak on your computer, select **Tools** from the menu bar and look for the **Data Analysis...** option. If you do not see **Data Analysis...**, select **Add-ins...** from the **Tools** menu. Check the box for the **Analysis ToolPak** and click on **OK** to install them.

Select **Data Analysis...** from the **Tools** menu, which opens the *Data Analysis* window. Scroll through the window, select **Regression** from the available options, and press **OK**. Place the cursor in the box for *Input Y range* and then click and drag over cells B1:B7. Place the cursor in the box for *Input X range* and click and drag over cells A1:A7. Because cells A1 and B1 contain labels, check the box for *Labels*.

> Including labels is a good idea. Excel's summary output uses the *x*-axis label to identify the slope.

Select the radio button for *Output range* and click on any empty cell; this is where Excel will place the results. Clicking **OK** generates the information shown in Figure **5.6.2**.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.99987244
R Square	0.9997449
Adjusted R Square	0.99968113
Standard Error	0.40329713
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2549.727156	2549.72716	15676.296	2.4405E-08
Residual	4	0.650594286	0.16264857		
Total	5	2550.37775			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.20857143	0.29188503	0.71456706	0.51436267	-0.60183133	1.01897419	-0.60183133	1.01897419
Cstd	120.705714	0.964064525	125.205016	2.4405E-08	118.029042	123.382387	118.029042	123.382387

Figure 5.6.2: Output from Excel's Regression command in the Analysis ToolPak. See the text for a discussion of how to interpret the information in these tables.

There are three parts to Excel's summary of a regression analysis. At the top of Figure **5.6.2** is a table of *Regression Statistics*. The *standard error* is the standard deviation about the regression, s_r. Also of interest is the value for *Multiple R*, which is the model's correlation coefficient, *r*, a term with which you may already be familiar. The correlation coefficient is a measure of the extent to which the regression model explains the variation in *y*. Values of *r* range from –1 to +1. The closer the correlation coefficient is to ±1, the better the model is at explaining the data. A correlation coefficient of 0 means there is no relationship between *x* and *y*. In developing the calculations for linear regression, we did not consider the correlation coefficient. There is a reason for this. For most straight-line calibration curves the correlation coefficient is very close to +1, typically 0.99 or better. There is a tendency, however, to put too much faith in the correlation coefficient's significance, and to assume that an *r* greater than 0.99 means the linear regression model is appropriate. Figure **5.6.3** provides a useful counterexample. Although the regression line has a correlation

coefficient of 0.993, the data clearly is curvilinear. The take-home lesson here is simple: do not fall in love with the correlation coefficient!

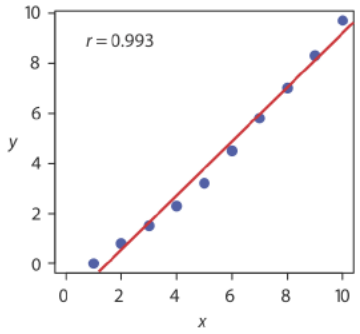


Figure 5.6.3: Example of fitting a straight-line (in red) to curvilinear data (in blue).

The second table in Figure **5.6.2** is entitled *ANOVA*, which stands for analysis of variance. We will take a closer look at ANOVA in Chapter 14. For now, it is sufficient to understand that this part of Excel's summary provides information on whether the linear regression model explains a significant portion of the variation in the values of *y*. The value for *F* is the result of an *F*-test of the following null and alternative hypotheses.

H_0: the regression model does not explain the variation in *y*

H_A: the regression model does explain the variation in *y*

The value in the column for *Significance F* is the probability for retaining the null hypothesis. In this example, the probability is $2.5 \times 10^{-6}\%$, which is strong evidence for accepting the regression model. As is the case with the correlation coefficient, a small value for the probability is a likely outcome for any calibration curve, even when the model is inappropriate. The probability for retaining the null hypothesis for the data in Figure **5.6.3**, for example, is $9.0 \times 10^{-7}\%$.

> See Chapter 4.6 for a review of the *F*-test.

The third table in Figure **5.6.2** provides a summary of the model itself. The values for the model's coefficients—the slope, β_1, and the *y*-intercept, β_0—are identified as *intercept* and with your label for the *x*-axis data, which in this example is C_{std}. The standard deviations for the coefficients, s_{b_0} and s_{b_1}, are in the column labeled *Standard error*. The column *t Stat* and the column *P-value* are for the following *t*-tests.

$$\text{slope: } H_0\text{: } \beta_1 = 0 \quad H_A\text{: } \beta_1 \neq 0$$

$$y\text{-intercept: } H_0\text{: } \beta_0 = 0 \quad H_A\text{: } \beta_0 \neq 0$$

The results of these *t*-tests provide convincing evidence that the slope is not zero, but there is no evidence that the *y*-intercept differs significantly from zero. Also shown are the 95% confidence intervals for the slope and the *y*-intercept (*lower 95%* and *upper 95%*).

> See Chapter 4.6 for a review of the *t*-test.

Programming the Formulas Yourself

A third approach to completing a regression analysis is to program a spreadsheet using Excel's built-in formula for a summation

=sum(*first cell*:*last cell*)

and its ability to parse mathematical equations. The resulting spreadsheet is shown in Figure **5.6.4**.

	A	B	C	D	E	F
1	x	y	xy	x^2	n =	6
2	0.000	0.00	=A2*B2	=A2^2	slope =	=(F1*C8 - A8*B8)/(F1*D8-A8^2)
3	0.100	12.36	=A3*B3	=A3^2	y-int =	=(B8-F2*A8)/F1
4	0.200	24.83	=A4*B4	=A4^2		
5	0.300	35.91	=A5*B5	=A5^2		
6	0.400	48.79	=A6*B6	=A6^2		
7	0.500	60.42	=A7*B7	=A7^2		
8						
9	=sum(A2:A7)	=sum(B2:B7)	=sum(C2:C7)	=sum(D2:D7)	<--sums	

Figure 5.6.4: Spreadsheet showing the formulas for calculating the slope and the *y*-intercept for the data in Example 5.4.1. The shaded cells contain formulas that you must enter. Enter the formulas in cells C3 to C7, and cells D3 to D7. Next, enter the formulas for cells A9 to D9. Finally, enter the formulas in cells F2 and F3. When you enter a formula, Excel replaces it with the resulting calculation. The values in these cells should agree with the results in Example 5.4.1. You can simplify the entering of formulas by copying and pasting. For example, enter the formula in cell C2. Select **Edit: Copy**, click and drag your cursor over cells C3 to C7, and select **Edit: Paste**. Excel automatically updates the cell referencing.

Using Excel to Visualize the Regression Model

You can use Excel to examine your data and the regression line. Begin by plotting the data. Organize your data in two columns, placing the *x* values in the left-most column. Click and drag over the data and select **Charts** from the ribbon. Select **Scatter**, choosing the option without lines that connect the points. To add a regression line to the chart, click on the chart's data and select **Chart: Add Trendline...** from the main men. Pick the straight-line model and click **OK** to add the line to your chart. By default, Excel displays the regression line from your first point to your last point. Figure 5.6.5 shows the result for the data in Figure 5.6.1.

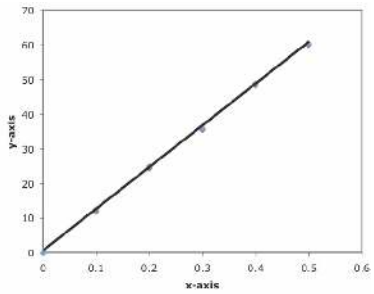


Figure 5.6.5: Example of an Excel scatterplot showing the data and a regression line.

Excel also will create a plot of the regression model's residual errors. To create the plot, build the regression model using the Analysis ToolPak, as described earlier. Clicking on the option for *Residual plots* creates the plot shown in Figure 5.6.6.

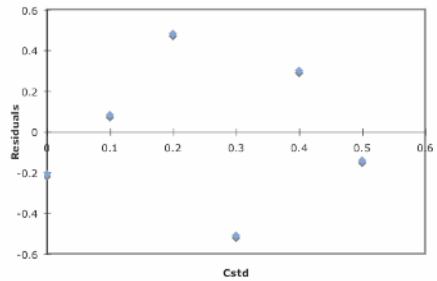


Figure 5.6.6: Example of Excel's plot of a regression model's residual errors.

Limitations to Using Excel for a Regression Analysis

Excel's biggest limitation for a regression analysis is that it does not provide a function to calculate the uncertainty when predicting values of *x*. In terms of this chapter, Excel can not calculate the uncertainty for the analyte's concentration, C_A, given the signal for a sample, S_{samp}. Another limitation is that Excel does not have a built-in function for a weighted linear regression. You can, however, program a spreadsheet to handle these calculations.

Exercise 5.6.1

Use Excel to complete the regression analysis in Exercise 5.4.1.

Answer

Begin by entering the data into an Excel spreadsheet, following the format shown in Figure 5.6.1. Because Excel's Data Analysis tools provide most of the information we need, we will use it here. The resulting output, which is shown below, provides the slope and the *y*-intercept, along with their respective 95% confidence intervals.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.99979366
R Square	0.99958737
Adjusted R Sq	0.99948421
Standard Erroı	0.00199602
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.0386054	0.0386054	9689.9103	6.3858E-08
Residual	4	1.5936E-05	3.9841E-06		
Total	5	0.03862133			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.00139272	0.00144059	0.96677158	0.38840479	-0.00260699	0.00539242	-0.00260699	0.00539242
Cstd	29.5927329	0.30062507	98.437342	6.3858E-08	28.7580639	30.4274019	28.7580639	30.4274019

Excel does not provide a function for calculating the uncertainty in the analyte's concentration, C_A, given the signal for a sample, S_{samp}. You must complete these calculations by hand. With an S_{samp} of 0.114, we find that C_A is

$$C_A = \frac{S_{samp} - b_0}{b_1} = \frac{0.114 - 0.0014}{29.59\ \text{M}^{-1}} = 3.80 \times 10^{-3}\ \text{M}$$

The standard deviation in C_A is

$$s_{C_A} = \frac{1.996 \times 10^{-3}}{29.59}\sqrt{\frac{1}{3} + \frac{1}{6} + \frac{(0.114 - 0.1183)^2}{(29.59)^2 \times 4.408 \times 10^{-5})}} = 4.772 \times 10^{-5}$$

and the 95% confidence interval is

$$\mu = C_A \pm ts_{C_A} = 3.80 \times 10^{-3} \pm \{2.78 \times (4.772 \times 10^{-5})\}$$

$$\mu = 3.80 \times 10^{-3}\ \text{M} \pm 0.13 \times 10^{-3}\ \text{M}$$

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5.7: Problems

1. Suppose you use a serial dilution to prepare 100 mL each of a series of standards with concentrations of 1.00×10^{-5}, 1.00×10^{-4}, 1.00×10^{-3}, and 1.00×10^{-2} M from a 0.100 M stock solution. Calculate the uncertainty for each solution using a propagation of uncertainty, and compare to the uncertainty if you prepare each solution as a single dilution of the stock solution. You will find tolerances for different types of volumetric glassware and digital pipets in Table 4.2.1 and Table 4.2.2. Assume that the uncertainty in the stock solution's molarity is ±0.0002.

2. Three replicate determinations of S_{total} for a standard solution that is 10.0 ppm in analyte give values of 0.163, 0.157, and 0.161 (arbitrary units). The signal for the reagent blank is 0.002. Calculate the concentration of analyte in a sample with a signal of 0.118.

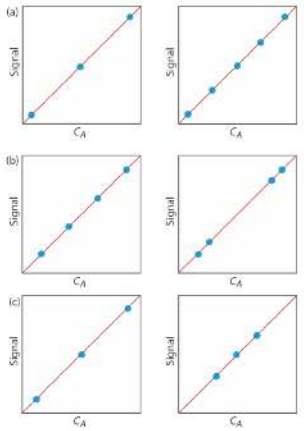
3. A 10.00-g sample that contains an analyte is transferred to a 250-mL volumetric flask and diluted to volume. When a 10.00 mL aliquot of the resulting solution is diluted to 25.00 mL it gives a signal of 0.235 (arbitrary units). A second 10.00-mL portion of the solution is spiked with 10.00 mL of a 1.00-ppm standard solution of the analyte and diluted to 25.00 mL. The signal for the spiked sample is 0.502. Calculate the weight percent of analyte in the original sample.

4. A 50.00 mL sample that contains an analyte gives a signal of 11.5 (arbitrary units). A second 50 mL aliquot of the sample, which is spiked with 1.00 mL of a 10.0-ppm standard solution of the analyte, gives a signal of 23.1. What is the analyte's concentration in the original sample?

5. A standard additions calibration curve based on Equation 5.3.10 places $S_{spike} \times (V_o + V_{std})$ on the *y*-axis and $C_{std} \times V_{std}$ on the *x*-axis. Derive equations for the slope and the *y*-intercept and explain how you can determine the amount of analyte in a sample from the calibration curve. In addition, clearly explain why you cannot plot S_{spike} on the *y*-axis and $C_{std} \times \{V_{std}/(V_o + V_{std})\}$ on the *x*-axis.

6. A standard sample contains 10.0 mg/L of analyte and 15.0 mg/L of internal standard. Analysis of the sample gives signals for the analyte and the internal standard of 0.155 and 0.233 (arbitrary units), respectively. Sufficient internal standard is added to a sample to make its concentration 15.0 mg/L. Analysis of the sample yields signals for the analyte and the internal standard of 0.274 and 0.198, respectively. Report the analyte's concentration in the sample.

7. For each of the pair of calibration curves shown ibelow, select the calibration curve that uses the more appropriate set of standards. Briefly explain the reasons for your selections. The scales for the *x*-axis and the *y*-axis are the same for each pair.



8. The following data are for a series of external standards of Cd^{2+} buffered to a pH of 4.6.

$[Cd^{2+}]$ (nM)	15.4	30.4	44.9	59.0	72.7	86.0
S_{meas} (nA)	4.8	11.4	18.2	26.6	32.3	37.7

(a) Use a linear regression analysis to determine the equation for the calibration curve and report confidence intervals for the slope and the *y*-intercept.

(b) Construct a plot of the residuals and comment on their significance.

At a pH of 3.7 the following data were recorded for the same set of external standards.

$[Cd^{2+}]$ (nM)	15.4	30.4	44.9	59.0	72.7	86.0
S_{meas} (nA)	15.0	42.7	58.5	77.0	101	118

(c) How much more or less sensitive is this method at the lower pH?

(d) A single sample is buffered to a pH of 3.7 and analyzed for cadmium, yielding a signal of 66.3 nA. Report the concentration of Cd^{2+} in the sample and its 95% confidence interval.

The data in this problem are from Wojciechowski, M.; Balcerzak, J. *Anal. Chim. Acta* **1991**, *249*, 433–445.

9. To determine the concentration of analyte in a sample, a standard addition is performed. A 5.00-mL portion of sample is analyzed and then successive 0.10-mL spikes of a 600.0 ppb standard of the analyte are added, analyzing after each spike. All samples were diluted to 10.00 mL before measuring the signal. The following table shows the results of this analysis.

V_{spike} (mL)	0.00	0.10	0.20	0.30
S_{total} (arbitrary units)	0.119	0.231	0.339	0.442

Construct an appropriate standard additions calibration curve and use a linear regression analysis to determine the concentration of analyte in the original sample and its 95% confidence interval.

10. Troost and Olavsesn investigated the application of an internal standardization to the quantitative analysis of polynuclear aromatic hydrocarbons. The following results were obtained for the analysis of phenanthrene using isotopically labeled phenanthrene as an internal standard. Each solution was analyzed twice.

C_A/C_{IS}	0.50	1.25	2.00	3.00	4.00
S_A/S_{IS}	0.514 0.522	0.993 1.024	1.486 1.471	2.044 2.080	2.342 2.550

(a) Determine the equation for the calibration curve using a linear regression, and report confidence intervals for the slope and the *y*-intercept. Average the replicate signals for each standard before you complete the linear regression analysis.

(b) Based on your results explain why the authors concluded that the internal standardization was inappropriate.

The data in this problem are from Troost, J. R.; Olavesen, E. Y. *Anal. Chem.* **1996**, *68*, 708–711.

11. In Chapter 4.6. we used a paired *t*-test to compare two analytical methods that were used to analyze independently a series of samples of variable composition. An alternative approach is to plot the results for one method versus the results for the other method. If the two methods yield identical results, then the plot should have an expected slope, β_1, of 1.00 and an expected *y*-intercept, β_0, of 0.0. We can use a *t*-test to compare the slope and the *y*-intercept from a linear regression to the expected values. The appropriate test statistic for the *y*-intercept is found by rearranging Equation 5.4.10.

$$t_{exp} = \frac{|\beta_0 - b_0|}{s_{b_0}} = \frac{|b_0|}{s_{b_0}}$$

Rearranging Equation 5.4.9 gives the test statistic for the slope.

$$t_{exp} = \frac{|\beta_1 - b_1}{s_{b_1}} = \frac{|b_1|}{s_{b_1}}$$

Reevaluate the data in Problem 25 from Chapter 4 using the same significance level as in the original problem.

> Although this is a common approach for comparing two analytical methods, it does violate one of the requirements for an unweighted linear regression—that indeterminate errors affect *y* only. Because indeterminate errors affect both analytical methods, the result of an unweighted linear regression is biased. More specifically, the regression underestimates the slope, b_1,

and overestimates the y-intercept, b_0. We can minimize the effect of this bias by placing the more precise analytical method on the x-axis, by using more samples to increase the degrees of freedom, and by using samples that uniformly cover the range of concentrations.

For more information, see Miller, J. C.; Miller, J. N. *Statistics for Analytical Chemistry*, 3rd ed. Ellis Horwood PTR Prentice-Hall: New York, 1993. Alternative approaches are found in Hartman, C.; Smeyers-Verbeke, J.; Penninckx, W.; Massart, D. L. *Anal. Chim. Acta* **1997**, *338*, 19–40, and Zwanziger, H. W.; Sârbu, C. *Anal. Chem.* **1998**, *70*, 1277–1280.

12. Consider the following three data sets, each of which gives values of y for the same values of x.

x	y_1	y_2	y_3
10.00	8.04	9.14	7.46
8.00	6.95	8.14	6.77
13.00	7.58	8.74	12.74
9.00	8.81	8.77	7.11
11.00	8.33	9.26	7.81
14.00	9.96	8.10	8.84
6.00	7.24	6.13	6.08
4.00	4.26	3.10	5.39
12.00	10.84	9.13	8.15
7.00	4.82	7.26	6.42
5.00	5.68	4.74	5.73

(a) An unweighted linear regression analysis for the three data sets gives nearly identical results. To three significant figures, each data set has a slope of 0.500 and a y-intercept of 3.00. The standard deviations in the slope and the y-intercept are 0.118 and 1.125 for each data set. All three standard deviations about the regression are 1.24. Based on these results for a linear regression analysis, comment on the similarity of the data sets.

(b) Complete a linear regression analysis for each data set and verify that the results from part (a) are correct. Construct a residual plot for each data set. Do these plots change your conclusion from part (a)? Explain.

(c) Plot each data set along with the regression line and comment on your results.

(d) Data set 3 appears to contain an outlier. Remove the apparent outlier and reanalyze the data using a linear regression. Comment on your result.

(e) Briefly comment on the importance of visually examining your data.

These three data sets are taken from Anscombe, F. J. “Graphs in Statistical Analysis,” *Amer. Statis.* **1973**, *27*, 17-21.

13. Fanke and co-workers evaluated a standard additions method for a voltammetric determination of Tl. A summary of their results is tabulated in the following table.

ppm Tl added	Instrument Response (μA)						
0.000	2.53	2.50	2.70	2.63	2.70	2.80	2.52
0.387	8.42	7.96	8.54	8.18	7.70	8.34	7.98
1.851	29.65	28.70	29.05	28.30	29.20	29.95	28.95
5.734	84.8	85.6	86.0	85.2	84.2	86.4	87.8

Use a weighted linear regression to determine the standardization relationship for this data.

The data in this problem are from Franke, J. P.; de Zeeuw, R. A.; Hakkert, R. *Anal. Chem.* **1978**, *50*, 1374–1380.

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5.8: Additional Resources

Although there are many experiments in the literature that incorporate external standards, the method of standard additions, or internal standards, the issue of choosing a method standardization is not the experiment's focus. One experiment designed to consider the issue of selecting a method of standardization is given here.

- Harvey, D. T. "External Standards or Standard Additions? Selecting and Validating a Method of Standardization," *J. Chem. Educ.* **2002**, *79*, 613–615.

In addition to the texts listed as suggested readings in Chapter 4, the following text provide additional details on linear regression.

- Draper, N. R.; Smith, H. *Applied Regression Analysis*, 2nd. ed.; Wiley: New York, 1981.

The following articles providing more details about linear regression.

- Analytical Methods Committee "Is my calibration linear?" AMC Technical Brief, December 2005.
- Analytical Methods Committee "Robust regression: an introduction, "AMCTB 50, 2012.
- Badertscher, M.; Pretsch, E. "Bad results from good data," *Trends Anal. Chem.* **2006**, *25*, 1131–1138.
- Boqué, R.; Rius, F. X.; Massart, D. L. "Straight Line Calibration: Something More Than Slopes, Intercepts, and Correlation Coefficients," *J. Chem. Educ.* **1993**, *70*, 230–232.
- Danzer, K.; Currie, L. A. "Guidelines for Calibration in Analytical Chemistry. Part 1. Fundamentals and Single Component Calibration," *Pure Appl. Chem.* **1998**, *70*, 993–1014.
- Henderson, G. "Lecture Graphic Aids for Least-Squares Analysis," *J. Chem. Educ.* **1988**, *65*, 1001–1003.
- Logan, S. R. "How to Determine the Best Straight Line," *J. Chem. Educ.* **1995**, *72*, 896–898.
- Mashkina, E.; Oldman, K. B. "Linear Regressions to Which the Standard Formulas do not Apply," *ChemTexts*, **2015**, *1*, 1–11.
- Miller, J. N. "Basic Statistical Methods for Analytical Chemistry. Part 2. Calibration and Regression Methods," *Analyst* **1991**, *116*, 3–14.
- Raposo, F. "Evaluation of analytical calibration based on least-squares linear regression for instrumental techniques: A tutorial review," *Trends Anal. Chem.* **2016**, *77*, 167–185.
- Renman, L., Jagner, D. "Asymmetric Distribution of Results in Calibration Curve and Standard Addition Evaluations," *Anal. Chim. Acta* **1997**, *357*, 157–166.
- Rodriguez, L. C.; Gamiz-Gracia; Almansa-Lopez, E. M.; Bosque-Sendra, J. M. "Calibration in chemical measurement processes. II. A methodological approach," *Trends Anal. Chem*. **2001**, *20*, 620–636.

Useful papers providing additional details on the method of standard additions are gathered here.

- Bader, M. "A Systematic Approach to Standard Addition Methods in Instrumental Analysis," *J. Chem. Educ.* **1980**, *57*, 703–706.
- Brown, R. J. C.; Roberts, M. R.; Milton, M. J. T. "Systematic error arising form 'Sequential' Standard Addition Calibrations. 2. Determination of Analyte Mass Fraction in Blank Solutions," *Anal. Chim. Acta* **2009**, *648*, 153–156.
- Brown, R. J. C.; Roberts, M. R.; Milton, M. J. T. "Systematic error arising form 'Sequential' Standard Addition Calibrations: Quantification and correction," *Anal. Chim. Acta* **2007**, *587*, 158–163.
- Bruce, G. R.; Gill, P. S. "Estimates of Precision in a Standard Additions Analysis," *J. Chem. Educ.* **1999**, *76*, 805–807.
- Kelly, W. R.; MacDonald, B. S.; Guthrie "Gravimetric Approach to the Standard Addition Method in Instrumental Analysis. 1." *Anal. Chem.* **2008**, *80*, 6154–6158.
- Meija, J.; Pagliano, E.; Mester, Z. "Coordinate Swapping in Standard Addition Graphs for Analytical Chemistry: A Simplified Path for Uncertainty Calculation in Linear and Nonlinear Plots," Anal. Chem. 2014, 86, 8563–8567.
- Nimura, Y.; Carr, M. R. "Reduction of the Relative Error in the Standard Additions Method," *Analyst* **1990**, *115*, 1589–1595.

Approaches that combine a standard addition with an internal standard are described in the following paper.

- Jones, W. B.; Donati, G. L.; Calloway, C. P.; Jones, B. T. "Standard Dilution Analysis," Anal. Chem. 2015, 87, 2321–2327.

The following papers discusses the importance of weighting experimental data when use linear regression.

- Analytical Methods Committee "Why are we weighting?" AMC Technical Brief, June 2007.
- Karolczak, M. "To Weight or Not to Weight? An Analyst's Dilemma," *Current Separations* **1995**, *13*, 98–104.

Algorithms for performing a linear regression with errors in both *X* and *Y* are discussed in the following papers. Also included here are papers that address the difficulty of using linear regression to compare two analytical methods.

- Irvin, J. A.; Quickenden, T. L. "Linear Least Squares Treatment When There are Errors in Both x and y," *J. Chem. Educ.* **1983**, *60*, 711–712.
- Kalantar, A. H. "Kerrich's Method for y = ax Data When Both y and x Are Uncertain," *J. Chem. Educ.* **1991**, *68*, 368–370.
- Macdonald, J. R.; Thompson, W. J. "Least-Squares Fitting When Both Variables Contain Errors: Pitfalls and Possibilities," *Am. J. Phys.* **1992**, *60*, 66–73.
- Martin, R. F. "General Deming Regression for Estimating Systematic Bias and Its Confidence Interval in Method-Comparison Studies," *Clin. Chem.* **2000**, *46*, 100–104.
- Ogren, P. J.; Norton, J. R. "Applying a Simple Linear Least-Squares Algorithm to Data with Uncertain- ties in Both Variables," *J. Chem. Educ.* **1992**, *69*, A130–A131.
- Ripley, B. D.; Thompson, M. "Regression Techniques for the Detection of Analytical Bias," *Analyst* **1987**, *112*, 377–383.
- Tellinghuisen, J. "Least Squares in Calibration: Dealing with Uncertainty in *x*," *Analyst*, **2010**, *135*, 1961–1969.

Outliers present a problem for a linear regression analysis. The following papers discuss the use of robust linear regression techniques.

- Glaister, P. "Robust Linear Regression Using Thiel's Method," *J. Chem. Educ.* **2005**, *82*, 1472–1473.
- Glasser, L. "Dealing with Outliers: Robust, Resistant Regression," *J. Chem. Educ.* **2007**, *84*, 533–534.
- Ortiz, M. C.; Sarabia, L. A.; Herrero, A. "Robust regression techniques. A useful alternative for the detection of outlier data in chemical analysis," *Talanta* **2006**, *70*, 499–512.

The following papers discusses some of the problems with using linear regression to analyze data that has been mathematically transformed into a linear form, as well as alternative methods of evaluating curvilinear data.

- Chong, D. P. "On the Use of Least Squares to Fit Data in Linear Form," *J. Chem. Educ.* **1994**, *71*, 489–490.
- Hinshaw, J. V. "Nonlinear Calibration," *LCGC* **2002**, *20*, 350–355.
- Lieb, S. G. "Simplex Method of Nonlinear Least-Squares - A Logical Complementary Method to Linear Least-Squares Analysis of Data," *J. Chem. Educ.* **1997**, *74*, 1008–1011.
- Zielinski, T. J.; Allendoerfer, R. D. "Least Squares Fitting of Nonlinear Data in the Undergraduate Laboratory," *J. Chem. Educ.* **1997**, *74*, 1001–1007.

More information on multivariate and multiple regression can be found in the following papers.

- Danzer, K.; Otto, M.; Currie, L. A. "Guidelines for Calibration in Analytical Chemistry. Part 2. Multispecies Calibration," *Pure Appl. Chem.* **2004**, *76*, 1215–1225.
- Escandar, G. M.; Faber, N. M.; Goicoechea, H. C.; de la Pena, A. M.; Olivieri, A.; Poppi, R. J. "Second- and third-order multivariate calibration: data, algorithms and applications," *Trends Anal. Chem.* **2007**, *26*, 752–765.
- Kowalski, B. R.; Seasholtz, M. B. "Recent Developments in Multivariate Calibration," *J. Chemometrics* **1991**, *5*, 129–145.
- Lang, P. M.; Kalivas, J. H. "A Global Perspective on Multivariate Calibration Methods," *J. Chemomet- rics* **1993**, *7*, 153–164.
- Madden, S. P.; Wilson, W.; Dong, A.; Geiger, L.; Mecklin, C. J. "Multiple Linear Regression Using a Graphing Calculator," J. Chem. Educ. 2004, 81, 903–907.
- Olivieri, A. C.; Faber, N. M.; Ferré, J.; Boqué, R.; Kalivas, J. H.; Mark, H. "Uncertainty Estimation and Figures of Merit for Multivariate Calibration," *Pure Appl. Chem.* **2006**, *78*, 633–661.

An additional discussion on method blanks, including the use of the total Youden blank, is found in the fol- lowing papers.

- Cardone, M. J. "Detection and Determination of Error in Analytical Methodology. Part II. Correc- tion for Corrigible Systematic Error in the Course of Real Sample Analysis," *J. Assoc. Off. Anal. Chem.* **1983**, *66*, 1283–1294.
- Cardone, M. J. "Detection and Determination of Error in Analytical Methodology. Part IIB. Direct Calculational Technique for Making Corrigible Systematic Error Corrections," *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 199–202.
- Ferrus, R.; Torrades, F. "Bias-Free Adjustment of Analytical Methods to Laboratory Samples in Routine Analytical Procedures," *Anal. Chem.* **1988**, *60*, 1281–1285.

- Vitha, M. F.; Carr, P. W.; Mabbott, G. A. “Appropriate Use of Blanks, Standards, and Controls in Chemical Measurements,” *J. Chem. Educ.* **2005**, *82*, 901–902.

There are a variety of computational packages for completing linear regression analyses. These papers provide details on there use in a variety of contexts.

- Espinosa-Mansilla, A.; de la Peña, A. M.; González-Gómez, D. “Using Univariate Linear Regression Calibration Software in the MATLAB Environment. Application to Chemistry Laboratory Practices,” *Chem. Educator* **2005**, *10*, 1–9.
- Harris, D. C. “Nonlinear Least-Squares Curve Fitting with Microsoft Excel Solver,” *J. Chem. Educ.* **1998**, *75*, 119–121.
- Kim, M. S.; Bukart, M.; Kim, M. H. “A Method Visual Interactive Regression,” *J. Chem. Educ.* **2006**, *83*, 1884.
- Machuca-Herrera, J. G. “Nonlinear Curve Fitting with Spreadsheets,” *J. Chem. Educ.* **1997**, *74*, 448–449.
- Smith, E. T.; Belogay, E. A.; Hõim “Linear Regression and Error Analysis for Calibration Curves and Standard Additions: An Excel Spreadsheet Exercise for Undergraduates,” Chem. Educator 2010, 15, 100–102.
- Smith, E. T.; Belogay, E. A.; Hõim “Using Multiple Linear Regression to Analyze Mixtures: An Excel Spreadsheet Exercise for Undergraduates,” *Chem. Educator* **2010**, *15*, 103–107.
- Young, S. H.; Wierzbicki, A. “Mathcad in the Chemistry Curriculum. Linear Least-Squares Regres- sion,” *J. Chem. Educ.* **2000**, *77*, 669.
- Young, S. H.; Wierzbicki, A. “Mathcad in the Chemistry Curriculum. Non-Linear Least-Squares Re- gression,” *J. Chem. Educ.* **2000**, *77*, 669.

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5.9: Chapter Summary and Key Terms

Summary

In a quantitative analysis we measure a signal, S_{total}, and calculate the amount of analyte, n_A or C_A, using one of the following equations.

$$S_{total} = k_A n_A + S_{reag}$$

$$S_{total} = k_A C_A + S_{reag}$$

To obtain an accurate result we must eliminate determinate errors that affect the signal, S_{total}, the method's sensitivity, k_A, and the signal due to the reagents, S_{reag}.

To ensure that we accurately measure S_{total}, we calibrate our equipment and instruments. To calibrate a balance, for example, we use a standard weight of known mass. The manufacturer of an instrument usually suggests appropriate calibration standards and calibration methods.

To standardize an analytical method we determine its sensitivity. There are several standardization strategies available to us, including external standards, the method of standard addition, and internal standards. The most common strategy is a multiple-point external standardization and a normal calibration curve. We use the method of standard additions, in which we add known amounts of analyte to the sample, when the sample's matrix complicates the analysis. When it is difficult to reproducibly handle samples and standards, we may choose to add an internal standard.

Single-point standardizations are common, but are subject to greater uncertainty. Whenever possible, a multiple-point standardization is preferred, with results displayed as a calibration curve. A linear regression analysis provides an equation for the standardization.

A reagent blank corrects for any contribution to the signal from the reagents used in the analysis. The most common reagent blank is one in which an analyte-free sample is taken through the analysis. When a simple reagent blank does not compensate for all constant sources of determinate error, other types of blanks, such as the total Youden blank, are used.

Key Terms

calibration curve	external standard	internal standard
linear regression	matrix matching	method of standard additions
multiple-point standardization	normal calibration curve	primary standard
reagent grade	residual error	secondary standard
serial dilution	single-point standardization	standard deviation about the regression
total Youden blank	unweighted linear regression	weighted linear regression

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CHAPTER OVERVIEW

6: General Properties of Electromagnetic Radiation

6.1: Overview of Spectroscopy
6.2: The Nature of Light
6.2.1: The Propagation of Light
6.2.2: The Law of Reflection
6.2.3: Refraction
6.2.4: Dispersion
6.2.5: Superposition and Interference
6.2.6: Diffraction
6.2.7: Polarization
6.3: Light as a Particle
6.4: The Nature of Light (Exercises)
6.4.1: The Nature of Light (Answers)

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6.1: Overview of Spectroscopy

The focus of this chapter is on the interaction of ultraviolet, visible, and infrared radiation with matter. Because these techniques use optical materials to disperse and focus the radiation, they often are identified as optical spectroscopies. For convenience we will use the simpler term spectroscopy in place of optical ***spectroscopy***; however, you should understand we will consider only a limited piece of what is a much broader area of analytical techniques.

Despite the difference in instrumentation, all spectroscopic techniques share several common features. Before we consider individual examples in greater detail, let's take a moment to consider some of these similarities. As you work through the chapter, this overview will help you focus on the similarities between different spectroscopic methods of analysis. You will find it easier to understand a new analytical method when you can see its relationship to other similar methods.

What is Electromagnetic Radiation?

Electromagnetic radiation—light—is a form of energy whose behavior is described by the properties of both waves and particles. Some properties of electromagnetic radiation, such as its refraction when it passes from one medium to another (Figure 6.1.1), are explained best when we describe light as a wave. Other properties, such as absorption and emission, are better described by treating light as a particle. The exact nature of electromagnetic radiation remains unclear, as it has since the development of quantum mechanics in the first quarter of the 20th century [Home, D.; Gribbin, J. *New Scientist* 1991, 2 Nov. 30–33]. Nevertheless, this dual model of wave and particle behavior provide a useful description for electromagnetic radiation.



Figure 6.1.1. The Golden Gate bridge as seen through rain drops. Refraction of light by the rain drops produces the distorted images. Source: Brocken Inaglory (commons. wikipedia.org).

Wave Properties of Electromagnetic Radiation

Electromagnetic radiation consists of oscillating electric and magnetic fields that propagate through space along a linear path and with a constant velocity. In a vacuum, electromagnetic radiation travels at the speed of light, c, which is 2.99792×10^8 m/s. When electromagnetic radiation moves through a medium other than a vacuum, its velocity, v, is less than the speed of light in a vacuum. The difference between v and c is sufficiently small (<0.1%) that the speed of light to three significant figures, 3.00×10^8 m/s, is accurate enough for most purposes.

The oscillations in the electric field and the magnetic field are perpendicular to each other and to the direction of the wave's propagation. Figure 6.1.2 shows an example of plane-polarized electromagnetic radiation, which consists of a single oscillating electric field and a single oscillating magnetic field.

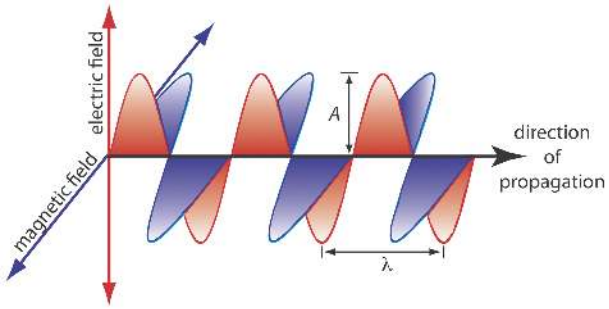


Figure 6.1.2. Plane-polarized electromagnetic radiation showing the oscillating electric field in blue and the oscillating magnetic field in red. The radiation's amplitude, A, and its wavelength, λ, are shown. Normally, electromagnetic radiation is unpolarized, with oscillating electric and magnetic fields present in all possible planes perpendicular to the direction of propagation.

An electromagnetic wave is characterized by several fundamental properties, including its velocity, amplitude, frequency, phase angle, polarization, and direction of propagation [Ball, D. W. *Spectroscopy* **1994**, *9(5)*, 24–25]. For example, the amplitude of the oscillating electric field at any point along the propagating wave is

$$A_t = A_e \sin(2\pi\nu t + \Phi)$$

where A_t is the magnitude of the electric field at time t, A_e is the electric field's maximum ***amplitude***, ν is the wave's ***frequency***—the number of oscillations in the electric field per unit time—and Φ is a ***phase angle*** that accounts for the fact that A_t need not have a value of zero at $t = 0$. The identical equation for the magnetic field is

$$A_t = A_m \sin(2\pi\nu t + \Phi)$$

where A_m is the magnetic field's maximum amplitude.

Other properties also are useful for characterizing the wave behavior of electromagnetic radiation. The ***wavelength***, λ, is defined as the distance between successive maxima (see Figure 6.1.2). For ultraviolet and visible electromagnetic radiation the wavelength usually is expressed in nanometers (1 nm = 10^{-9} m), and for infrared radiation it is expressed in microns (1 mm = 10^{-6} m). The relationship between wavelength and frequency is

$$\lambda = \frac{c}{\nu}$$

Another unit useful unit is the ***wavenumber***, $\bar{\nu}$, which is the reciprocal of wavelength

$$\bar{\nu} = \frac{1}{\lambda}$$

Wavenumbers frequently are used to characterize infrared radiation, with the units given in cm^{-1}.

When electromagnetic radiation moves between different media—for example, when it moves from air into water—its frequency, ν, remains constant. Because its velocity depends upon the medium in which it is traveling, the electromagnetic radiation's wavelength, λ, changes. If we replace the speed of light in a vacuum, c, with its speed in the medium, v, then the wavelength is

$$\lambda = \frac{v}{\nu}$$

This change in wavelength as light passes between two media explains the refraction of electromagnetic radiation shown in Figure 6.1.1.

Example 6.1.1

In 1817, Josef Fraunhofer studied the spectrum of solar radiation, observing a continuous spectrum with numerous dark lines. Fraunhofer labeled the most prominent of the dark lines with letters. In 1859, Gustav Kirchhoff showed that the D line in the sun's spectrum was due to the absorption of solar radiation by sodium atoms. The wavelength of the sodium D line is 589 nm. What are the frequency and the wavenumber for this line?

Solution

The frequency and wavenumber of the sodium D line are

$$\nu = \frac{c}{\lambda} = \frac{3.00 \times 10^8 \text{ m/s}}{589 \times 10^{-9} \text{ m}} = 5.09 \times 10^{14} \text{ s}^{-1}$$

$$\overline{\nu} = \frac{1}{\lambda} = \frac{1}{589 \times 10^{-9} \text{ m}} \times \frac{1 \text{ m}}{100 \text{ cm}} = 1.70 \times 10^4 \text{ cm}^{-1}$$

Exercise 6.1.1

Another historically important series of spectral lines is the Balmer series of emission lines from hydrogen. One of its lines has a wavelength of 656.3 nm. What are the frequency and the wavenumber for this line?

Answer

The frequency and wavenumber for the line are

$$\nu = \frac{c}{\lambda} = \frac{3.00 \times 10^8 \text{ m/s}}{656.3 \times 10^{-9} \text{ m}} = 4.57 \times 10^{14} \text{ s}^{-1}$$

$$\overline{\nu} = \frac{1}{\lambda} = \frac{1}{656.3 \times 10^{-9} \text{ m}} \times \frac{1 \text{ m}}{100 \text{ cm}} = 1.524 \times 10^4 \text{ cm}^{-1}$$

The Electromagnetic Spectrum

The frequency and the wavelength of electromagnetic radiation vary over many orders of magnitude. For convenience, we divide electromagnetic radiation into different regions—the ***electromagnetic spectrum***—based on the type of atomic or molecular transitions that gives rise to the absorption or emission of photons (Figure **6.1.3**). The boundaries between the regions of the electromagnetic spectrum are not rigid and overlap between spectral regions is possible.

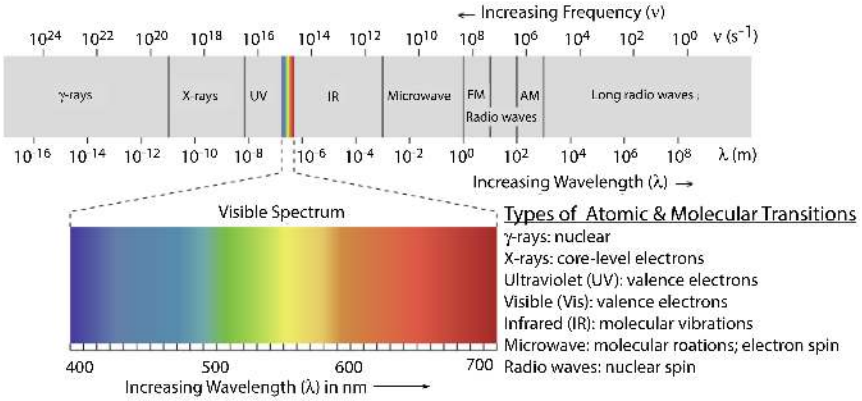


Figure **6.1.3**. The electromagnetic spectrum showing the boundaries between different regions and the type of atomic or molecular transitions responsible for the change in energy. The colored inset shows the visible spectrum. Source: modified from Zedh (www.commons.wikipedia.org).

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SECTION OVERVIEW

6.2: The Nature of Light

In this chapter, we study the basic properties of light. In the next few chapters, we investigate the behavior of light when it interacts with optical devices such as mirrors, lenses, and apertures.

6.2.1: The Propagation of Light

6.2.2: The Law of Reflection

6.2.3: Refraction

6.2.4: Dispersion

6.2.5: Superposition and Interference

6.2.6: Diffraction

6.2.7: Polarization

Thumbnail: An EM wave, such as light, is a transverse wave. The electric $\vec{E}$ *and magnetic* $\vec{B}$ *fields are perpendicular to the direction of propagation. The direction of polarization of the wave is the direction of the electric field.*

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6.2.1: The Propagation of Light

Learning Objectives

By the end of this section, you will be able to:

- Determine the index of refraction, given the speed of light in a medium
- List the ways in which light travels from a source to another location

The Speed of Light: Early Measurements

The first measurement of the speed of light was made by the Danish astronomer Ole Roemer (1644–1710) in 1675. He studied the orbit of Io, one of the four large moons of Jupiter, and found that it had a period of revolution of 42.5 h around Jupiter. He also discovered that this value fluctuated by a few seconds, depending on the position of Earth in its orbit around the Sun. Roemer realized that this fluctuation was due to the finite speed of light and could be used to determine **c**.

Roemer found the period of revolution of Io by measuring the time interval between successive eclipses by Jupiter. Figure **6.2.1.1*a*** shows the planetary configurations when such a measurement is made from Earth in the part of its orbit where it is receding from Jupiter. When Earth is at point **A**, Earth, Jupiter, and Io are aligned. The next time this alignment occurs, Earth is at point **B**, and the light carrying that information to Earth must travel to that point. Since **B** is farther from Jupiter than **A**, light takes more time to reach Earth when Earth is at **B**. Now imagine it is about 6 months later, and the planets are arranged as in Figure **6.2.1.1*b***. The measurement of Io's period begins with Earth at point A' and Io eclipsed by Jupiter. The next eclipse then occurs when Earth is at point B', to which the light carrying the information of this eclipse must travel. Since B' is closer to Jupiter than A', light takes less time to reach Earth when it is at B'. This time interval between the successive eclipses of Io seen at A' and B' is therefore less than the time interval between the eclipses seen at **A** and **B**. By measuring the difference in these time intervals and with appropriate knowledge of the distance between Jupiter and Earth, Roemer calculated that the speed of light was $2.0 \times 10^8\ m/s$, which is only 33% below the value accepted today.

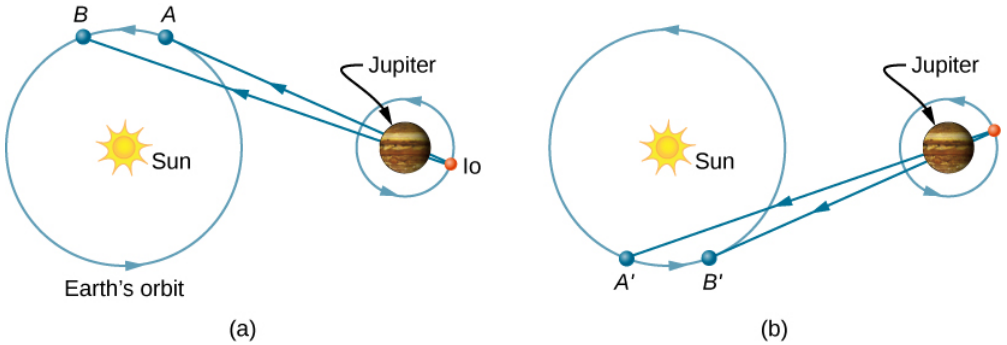


Figure **6.2.1.1**: Roemer's astronomical method for determining the speed of light. Measurements of Io's period done with the configurations of parts (a) and (b) differ, because the light path length and associated travel time increase from A to B (a) but decrease from A'A′ to B'B′ (b).

The first successful terrestrial measurement of the speed of light was made by Armand Fizeau (1819–1896) in 1849. He placed a toothed wheel that could be rotated very rapidly on one hilltop and a mirror on a second hilltop 8 km away (Figure **6.2.1.2**). An intense light source was placed behind the wheel, so that when the wheel rotated, it chopped the light beam into a succession of pulses. The speed of the wheel was then adjusted until no light returned to the observer located behind the wheel. This could only happen if the wheel rotated through an angle corresponding to a displacement of (n+½) teeth, while the pulses traveled down to the mirror and back. Knowing the rotational speed of the wheel, the number of teeth on the wheel, and the distance to the mirror, Fizeau determined the speed of light to be $3.15 \times 10^8\ m/s$, which is only 5% too high.

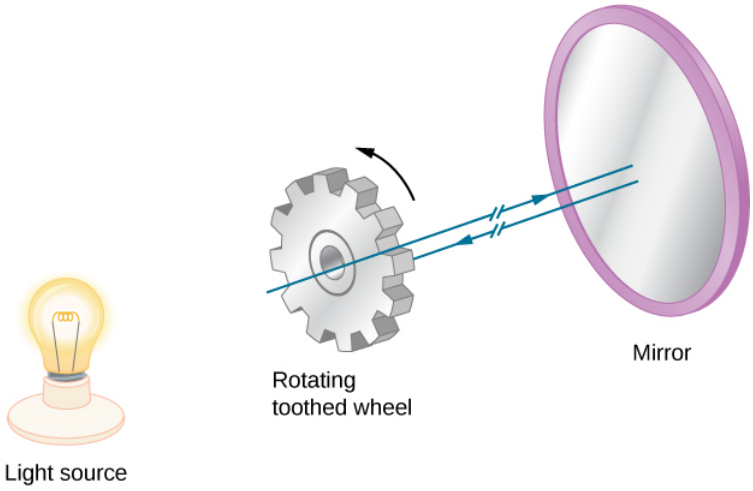


Figure **6.2.1.2**: Fizeau's method for measuring the speed of light. The teeth of the wheel block the reflected light upon return when the wheel is rotated at a rate that matches the light travel time to and from the mirror.

The French physicist Jean Bernard Léon Foucault (1819–1868) modified Fizeau's apparatus by replacing the toothed wheel with a rotating mirror. In 1862, he measured the speed of light to be 2.98×10^8m/s, which is within 0.6% of the presently accepted value. Albert Michelson (1852–1931) also used Foucault's method on several occasions to measure the speed of light. His first experiments were performed in 1878; by 1926, he had refined the technique so well that he found **c** to be $(2.99796\pm4)\times10^8$m/s.

Today, the speed of light is known to great precision. In fact, the speed of light in a vacuum **c** is so important that it is accepted as one of the basic physical quantities and has the value

$$c = 2.99792458 \times 10^8 m/s \equiv 3.00 \times 10^8 m/s \tag{6.2.1.1}$$

where the approximate value of 3.00×10^8m/s is used whenever three-digit accuracy is sufficient.

Speed of Light in Matter

The speed of light through matter is less than it is in a vacuum, because light interacts with atoms in a material. The speed of light depends strongly on the type of material, since its interaction varies with different atoms, crystal lattices, and other substructures. We can define a constant of a material that describes the speed of light in it, called the index of refraction η :

$$\eta = \frac{c}{v} \tag{6.2.1.2}$$

where v is the observed speed of light in the material.

Since the speed of light is always less than **c** in matter and equals **c** only in a vacuum, the index of refraction is always greater than or equal to one; that is, $\eta \geq 1$. Table **6.2.1.1** gives the indices of refraction for some representative substances. The values are listed for a particular wavelength of light, because they vary slightly with wavelength. (This can have important effects, such as colors separated by a prism, as we will see in Dispersion.) Note that for gases, η is close to 1.0. This seems reasonable, since atoms in gases are widely separated, and light travels at **c** in the vacuum between atoms. It is common to take $n = 1$ for gases unless great precision is needed. Although the speed of light **v** in a medium varies considerably from its value **c** in a vacuum, it is still a large speed.

Figure **6.2.1.1**: Index of Refraction in Various Media For light with a wavelength of 589 nm in a vacuum

Medium	η
Gases at 0°C, 1 atm	
Air	1.000293
Carbon dioxide	1.00045
Hydrogen	1.000139
Oxygen	1.000271
Liquids at 20°C	
Benzene	1.501
Carbon disulfide	1.628
Carbon tetrachloride	1.461
Ethanol	1.361
Glycerine	1.473
Water, fresh	1.333
Solids at 20°C	
Diamond	2.419
Fluorite	1.434
Glass, crown	1.52
Glass, flint	1.66
Ice (at 0°C)0°C)	1.309
Polystyrene	1.49
Plexiglas	1.51
Quartz, crystalline	1.544
Quartz, fused	1.458
Sodium chloride	1.544
Zircon	1.923

Example 6.2.1.1: Speed of Light in Jewelry

Calculate the speed of light in zircon, a material used in jewelry to imitate diamond.

Strategy

We can calculate the speed of light in a material v from the index of refraction η of the material, using Equation \red{index}

Solution

Rearranging Equation 6.2.1.2 for v gives us

$$v = \frac{c}{n}. \tag{6.2.1.3}$$

The index of refraction for zircon is given as 1.923 in Table 6.2.1.1, and c is given in Equation 6.2.1.1. Entering these values in the equation gives

$$v = \frac{3.00 \times 10^8 m/s}{1.923}$$
$$= 1.56 \times 10^8 m/s.$$

Significance

This speed is slightly larger than half the speed of light in a vacuum and is still high compared with speeds we normally experience. The only substance listed in Table **6.2.1.1** that has a greater index of refraction than zircon is diamond. We shall see later that the large index of refraction for zircon makes it sparkle more than glass, but less than diamond.

Exercise 6.2.1.1

Table **6.2.1.1** shows that ethanol and fresh water have very similar indices of refraction. By what percentage do the speeds of light in these liquids differ?

Answer

2.1% (to two significant figures)

The Ray Model of Light

You have already studied some of the wave characteristics of light in the previous chapter on Electromagnetic Waves. In this chapter, we start mainly with the ray characteristics. There are three ways in which light can travel from a source to another location (Figure **6.2.1.1**). It can come directly from the source through empty space, such as from the Sun to Earth. Or light can travel through various media, such as air and glass, to the observer. Light can also arrive after being reflected, such as by a mirror. In all of these cases, we can model the path of light as a straight line called a ray.

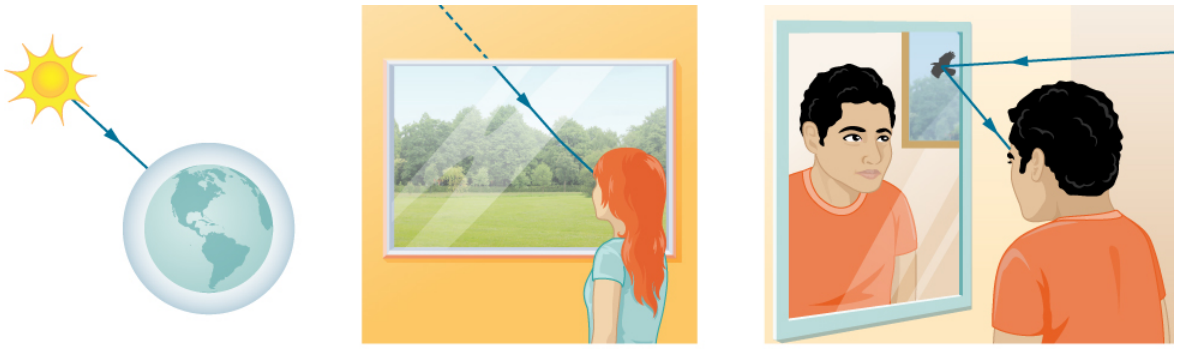


Figure **6.2.1.3**: Three methods for light to travel from a source to another location. (a) Light reaches the upper atmosphere of Earth, traveling through empty space directly from the source. (b) Light can reach a person by traveling through media like air and glass. (c) Light can also reflect from an object like a mirror. In the situations shown here, light interacts with objects large enough that it travels in straight lines, like a ray.

Experiments show that when light interacts with an object several times larger than its wavelength, it travels in straight lines and acts like a ray. Its wave characteristics are not pronounced in such situations. Since the wavelength of visible light is less than a micron (a thousandth of a millimeter), it acts like a ray in the many common situations in which it encounters objects larger than a micron. For example, when visible light encounters anything large enough that we can observe it with unaided eyes, such as a coin, it acts like a ray, with generally negligible wave characteristics.

In all of these cases, we can model the path of light as straight lines. Light may change direction when it encounters objects (such as a mirror) or in passing from one material to another (such as in passing from air to glass), but it then continues in a straight line or as a ray. The word “ray” comes from mathematics and here means a straight line that originates at some point. It is acceptable to visualize light rays as laser rays. The **ray model of light** describes the path of light as straight lines.

Since light moves in straight lines, changing directions when it interacts with materials, its path is described by geometry and simple trigonometry. This part of optics, where the ray aspect of light dominates, is therefore called geometric optics. Two laws

govern how light changes direction when it interacts with matter. These are the **law of reflection**, for situations in which light bounces off matter, and the **law of refraction**, for situations in which light passes through matter. We will examine more about each of these laws in upcoming sections of this chapter.

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6.2.2: The Law of Reflection

Learning Objectives

By the end of this section, you will be able to:

- Explain the reflection of light from polished and rough surfaces
- Describe the reflection of the interface between two transparent media

Whenever we look into a mirror, or squint at sunlight glinting from a lake, we are seeing a reflection. When you look at a piece of white paper, you are seeing light scattered from it. Large telescopes use reflection to form an image of stars and other astronomical objects.

The **law of reflection** states that the angle of reflection equals the angle of incidence:

$$\theta_r = \theta_i \tag{6.2.2.1}$$

The law of reflection is illustrated in Figure **6.2.2.1**, which also shows how the angle of incidence and angle of reflection are measured relative to the perpendicular to the surface at the point where the light ray strikes.

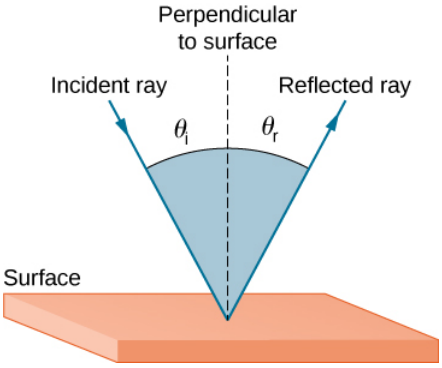


Figure **6.2.2.1**: The law of reflection states that the angle of reflection equals the angle of incidence $\theta_r = \theta_i$. The angles are measured relative to the perpendicular to the surface at the point where the ray strikes the surface.

We expect to see reflections from smooth surfaces, but Figure **6.2.2.2** illustrates how a rough surface reflects light. Since the light strikes different parts of the surface at different angles, it is reflected in many different directions, or diffused. Diffused light is what allows us to see a sheet of paper from any angle, as shown in Figure **6.2.2.1*a***.

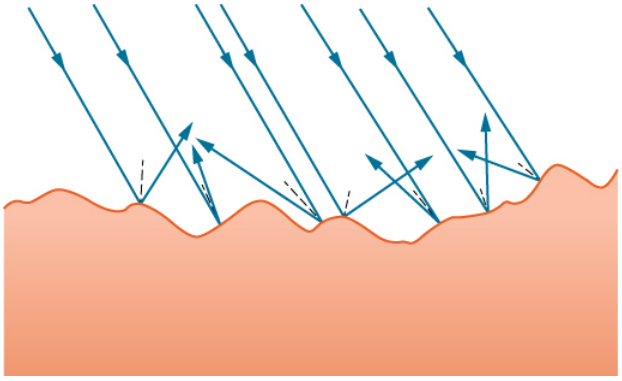


Figure **6.2.2.2**: Light is diffused when it reflects from a rough surface. Here, many parallel rays are incident, but they are reflected at many different angles, because the surface is rough.

People, clothing, leaves, and walls all have rough surfaces and can be seen from all sides. A mirror, on the other hand, has a smooth surface (compared with the wavelength of light) and reflects light at specific angles, as illustrated in Figure **6.2.2.3*b***. When the Moon reflects from a lake, as shown in Figure **6.2.2.1*c***, a combination of these effects takes place.

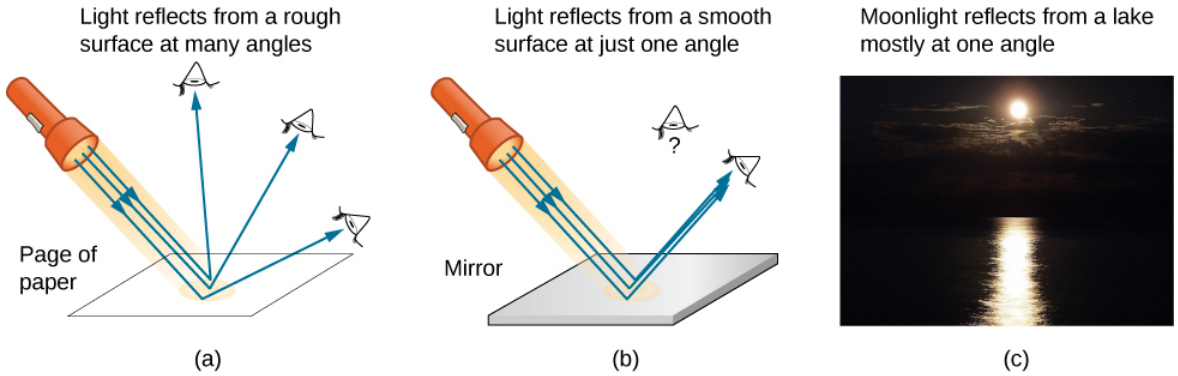


Figure **6.2.2.3**: (a) When a sheet of paper is illuminated with many parallel incident rays, it can be seen at many different angles, because its surface is rough and diffuses the light. (b) A mirror illuminated by many parallel rays reflects them in only one direction, because its surface is very smooth. Only the observer at a particular angle sees the reflected light. (c) Moonlight is spread out when it is reflected by the lake, because the surface is shiny but uneven. (credit c: modification of work by Diego Torres Silvestre)

Reflection from the interface between two transmissive materials

Figure **6.2.2.4**:illustrates what happens when electromagnetic radiation crosses a smooth interface into a dielectric medium that has a higher refractive index ($\eta_t > \eta_i$). We see two phenomena: reflection and refraction (a specific type of transmission). The angle of reflection, θ_r, equals the angle of incidence, θ^i, where each is defined with respect to the surface normal. The angle of refraction, θ_t, (t for transmitted) is described by Snell's law (of Refraction) which is described in the next section.

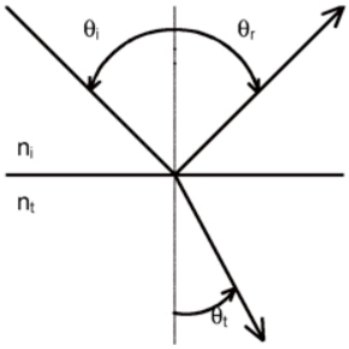


Figure **6.2.2.4**: Reflection and refraction at the interface between two transmissive materials of differing refractive indices.

The radiant power of the reflected and refracted light depend on several factors. The Fresnel equations describe the dependence of the reflected light on the angle of incidence, the refractive indices of the two media and, in some cases, the polarization of the incident beam. In the case of normal incidence, the fraction of light reflected reflectance ($\rho=I_r/I_0$) is

$$\rho = \left[\frac{n_t - n_i}{n_t + n_i}\right]^2$$

where I_r is the intensity of light reflected and I_0 is the incident intensity. The reflectance is thus related to the difference in refractive indices between the two media. For glass and air, which have refractive indices of 1.50 and 1.00, respectively, the fraction of reflected light is 0.04. For water and diamond, which has a refractive index of 2.4, the fraction of light reflected is 0.08. It is therefore easier to see diamonds in water than it is to see glass in air.

It should be noted that reflection occurs upon each pass through an interface. Consequently for light passing through a cuvette reflection will occur at 4 interfaces and the fraction of light lost due to reflection will $(\rho)^4$.

An of interesting tangent that is important for the ATR accessory with our FTIR

Snell's law, $\eta_i \sin\theta_i = \eta_t \sin\theta_t$, predicts the phenomenon of total internal reflection, depicted in Figure **6.2.2.5**. Whenever light is traveling from a more optically dense medium to a less optically dense medium $\eta_i > \eta_t$, θ_t can appear to exceed 90. In reality, most of the incident beam is totally internally reflected. At incident angles above a critical angle specified by Eqn. 4.1, the transmitted

beam becomes vanishingly small and propagates parallel to the interface. This small electric field extends beyond the boundary of the interface into the lower refractive index medium for a distance approximately equal to the wavelength of light. This electric field beyond the interface is called the evanescent field, and it can be used to excite molecules on the other side (η_t) of the interface, provided they are within a distance on the order of the wavelength of light. The evanescent field strength is dependent upon the polarization (the orientation of the electric field), the angle of the incident beam and the refractive indices of the two media. The technique of "attenuated total internal reflectance", or ATR, is commonly used in infrared spectroscopy to make measurements of films or solids by launching an IR evanescent wave into a thin layer of the material of interest adsorbed or clamped to a crystal in which an IR beam undergoes multiple internal reflections.

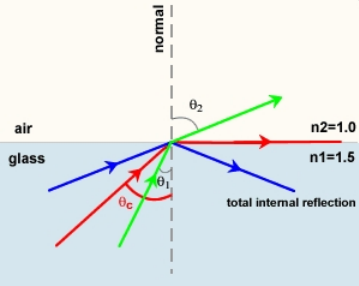


Figure 6.2.2.5: Total internal reflection for light traveling from glass to air.

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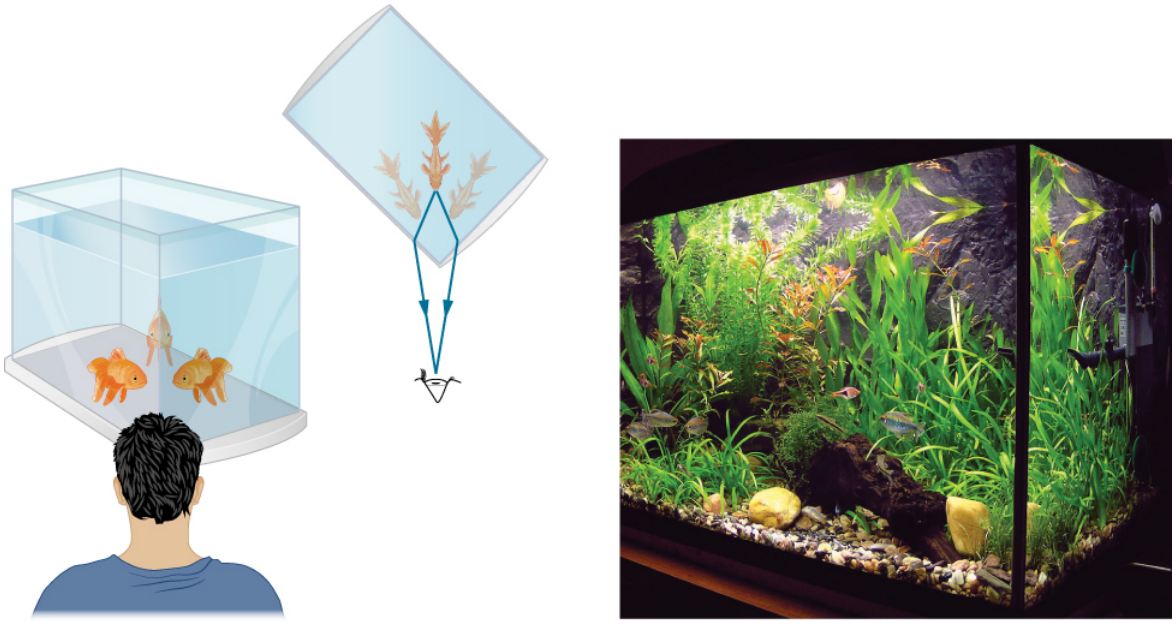
6.2.3: Refraction

Learning Objectives

By the end of this section, you will be able to:

- Describe how rays change direction upon entering a medium
- Apply the law of refraction in problem solving

You may often notice some odd things when looking into a fish tank. For example, you may see the same fish appearing to be in two different places (Figure 6.2.3.1). This happens because light coming from the fish to you changes direction when it leaves the tank, and in this case, it can travel two different paths to get to your eyes. The changing of a light ray's direction (loosely called bending) when it passes through substances of different refractive indices is called **refraction** and is related to changes in the speed of light, $v = c/\eta$. Refraction is responsible for a tremendous range of optical phenomena, from the action of lenses to data transmission through optical fibers.



(a) (b)

Figure 6.2.3.1: (a) Looking at the fish tank as shown, we can see the same fish in two different locations, because light changes directions when it passes from water to air. In this case, the light can reach the observer by two different paths, so the fish seems to be in two different places. This bending of light is called refraction and is responsible for many optical phenomena. (b) This image shows refraction of light from a fish near the top of a fish tank.

Figure 6.2.3.2 shows how a ray of light changes direction when it passes from one medium to another. As before, the angles are measured relative to a perpendicular to the surface at the point where the light ray crosses it. (Some of the incident light is reflected from the surface, but for now we concentrate on the light that is transmitted.) The change in direction of the light ray depends on the relative values of the indices of refraction of the two media involved. In the situations shown, medium 2 has a greater index of refraction than medium 1. Note that as shown in Figure 6.2.3.1*a*, the direction of the ray moves closer to the perpendicular when it progresses from a medium with a lower index of refraction to one with a higher index of refraction. Conversely, as shown in Figure 6.2.3.1*b*, the direction of the ray moves away from the perpendicular when it progresses from a medium with a higher index of refraction to one with a lower index of refraction. The path is exactly reversible.

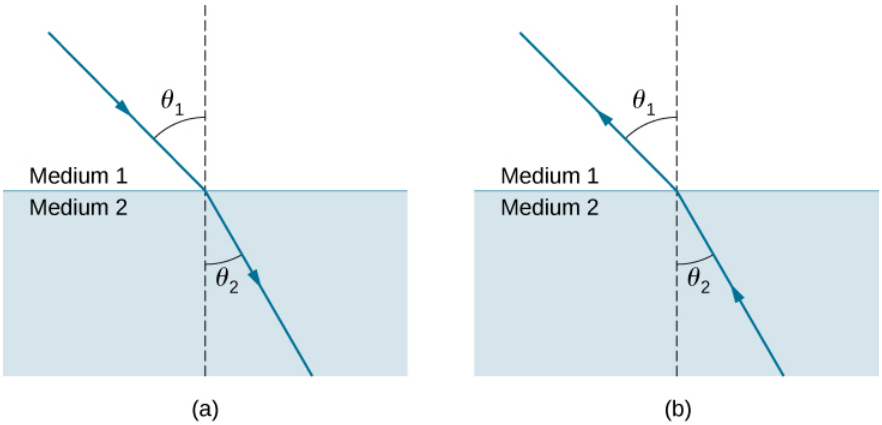


Figure **6.2.3.2**: The change in direction of a light ray depends on how the index of refraction changes when it crosses from one medium to another. In the situations shown here, the index of refraction is greater in medium 2 than in medium 1. (a) A ray of light moves closer to the perpendicular when entering a medium with a higher index of refraction. (b) A ray of light moves away from the perpendicular when entering a medium with a lower index of refraction.

The amount that a light ray changes its direction depends both on the incident angle and the amount that the speed changes. For a ray at a given incident angle, a large change in speed causes a large change in direction and thus a large change in angle. The exact mathematical relationship is the law of refraction, or Snell's law, after the Dutch mathematician Willebrord Snell (1591–1626), who discovered it in 1621. The law of refraction is stated in equation form as

$$\eta_1 \sin\theta_1 = \eta_2 \sin\theta_2. \tag{6.2.3.1}$$

Here η_1 and η_2 are the indices of refraction for media 1 and 2, and θ_1 and θ_2 are the angles between the rays and the perpendicular in media 1 and 2. The incoming ray is called the incident ray, the outgoing ray is called the refracted ray, and the associated angles are the incident angle and the refracted angle, respectively.

Snell's experiments showed that the law of refraction is obeyed and that a characteristic index of refraction η could be assigned to a given medium and its value measured. Snell was not aware that the speed of light varied in different media, a key fact used when we derive the law of refraction theoretically using Huygens's Principle.

Example 6.2.3.1: Determining the Index of Refraction

Find the index of refraction for medium 2 in Figure **6.2.3.1***a*, assuming medium 1 is air and given that the incident angle is 30.0° and the angle of refraction is 22.0°.

Strategy

The index of refraction for air is taken to be 1 in most cases (and up to four significant figures, it is 1.000). Thus, $\eta_1 = 1.00$ here. From the given information, $\theta_1 = 30.0°$ and $\theta_2 = 22.0°$. With this information, the only unknown in Snell's law is η_2, so we can use Snell's law (Equation 6.2.3.1) to find it.

Solution

From Snell's law (Equation 6.2.3.1), we have

$$\eta_1 \sin\theta_1 = \eta_2 \sin\theta_2$$
$$\eta_2 = \eta_1 \frac{\sin\theta_1}{\sin\theta_2}.$$

Entering known values,

$$\eta_2 = 1.00 \frac{\sin 30.0°}{\sin 22.0°}$$
$$= \frac{0.500}{0.375}$$
$$= 1.33.$$

Significance

This is the index of refraction for water, and Snell could have determined it by measuring the angles and performing this calculation. He would then have found 1.33 to be the appropriate index of refraction for water in all other situations, such as when a ray passes from water to glass. Today, we can verify that the index of refraction is related to the speed of light in a medium by measuring that speed directly.

Explore bending of light between two media with different indices of refraction. Use the "Intro" simulation and see how changing from air to water to glass changes the bending angle. Use the protractor tool to measure the angles and see if you can recreate the configuration in Example **6.2.3.1**. Also by measurement, confirm that the angle of reflection equals the angle of incidence.

Example 6.2.3.2: A Larger Change in Direction

Suppose that in a situation like that in Example **6.2.3.1**, light goes from air to diamond and that the incident angle is 30.0°. Calculate the angle of refraction θ_2 in the diamond.

Strategy

Again, the index of refraction for air is taken to be η_1=1.00, and we are given θ_1=30.0°. We can look up the index of refraction for diamond, finding η_2=2.419. The only unknown in Snell's law is θ_2, which we wish to determine.

Solution

Solving Snell's law (Equation 6.2.3.1) for $\sin\theta_2$ yields

$$\sin\theta_2 = \frac{\eta_1}{\eta_2}\sin\theta_1.$$

Entering known values,

$$\sin\theta_2 = \frac{1.00}{2.419}\sin 30.0° = (0.413)(0.500) = 0.207.$$

The angle is thus

$$\theta_2 = \sin^{-1}(0.207) = 11.9°.$$

Significance

For the same 30.0° angle of incidence, the angle of refraction in diamond is significantly smaller than in water (11.9° rather than 22.0°—see Example **6.2.3.2**). This means there is a larger change in direction in diamond. The cause of a large change in direction is a large change in the index of refraction (or speed). In general, the larger the change in speed, the greater the effect on the direction of the ray.

Exercise 6.2.3.1: Zircon

The solid with the next highest index of refraction after diamond is zircon. If the diamond in Example **6.2.3.2** were replaced with a piece of zircon, what would be the new angle of refraction?

Answer

15.1°

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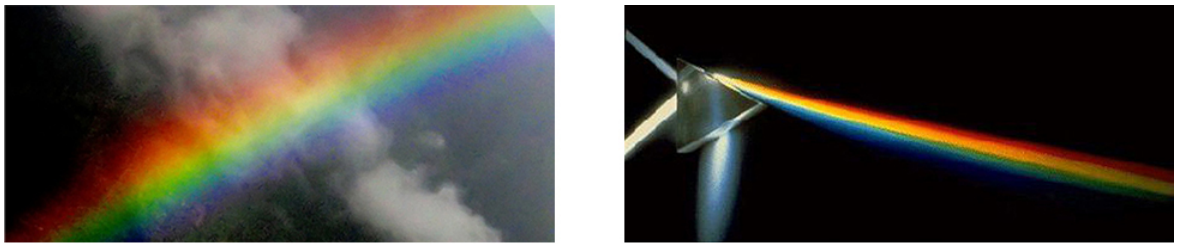
6.2.4: Dispersion

Learning Objectives

By the end of this section, you will be able to:

- Explain the cause of dispersion in a prism
- Describe the effects of dispersion in producing rainbows
- Summarize the advantages and disadvantages of dispersion

Everyone enjoys the spectacle of a rainbow glimmering against a dark stormy sky. How does sunlight falling on clear drops of rain get broken into the rainbow of colors we see? The same process causes white light to be broken into colors by a clear glass prism or a diamond (Figure **6.2.4.1**).



(a) (b)

Figure **6.2.4.1**: The colors of the rainbow (a) and those produced by a prism (b) are identical. (credit a: modification of work by "Alfredo55"/Wikimedia Commons; credit b: modification of work by NASA)

We see about six colors in a rainbow—red, orange, yellow, green, blue, and violet; sometimes indigo is listed, too. These colors are associated with different wavelengths of light, as shown in Figure **6.2.4.2**. When our eye receives pure-wavelength light, we tend to see only one of the six colors, depending on wavelength. The thousands of other hues we can sense in other situations are our eye's response to various mixtures of wavelengths. White light, in particular, is a fairly uniform mixture of all visible wavelengths. Sunlight, considered to be white, actually appears to be a bit yellow, because of its mixture of wavelengths, but it does contain all visible wavelengths. The sequence of colors in rainbows is the same sequence as the colors shown in the figure. This implies that white light is spread out in a rainbow according to wavelength. Dispersion is defined as the spreading of white light into its full spectrum of wavelengths. More technically, dispersion occurs whenever the propagation of light depends on wavelength.

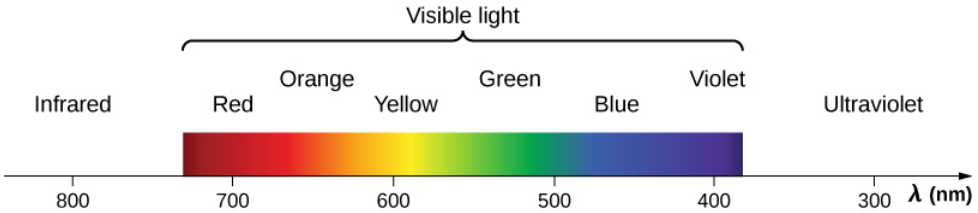


Figure **6.2.4.2**: Even though rainbows are associated with six colors, the rainbow is a continuous distribution of colors according to wavelengths.

Any type of wave can exhibit dispersion. For example, sound waves, all types of electromagnetic waves, and water waves can be dispersed according to wavelength. Dispersion may require special circumstances and can result in spectacular displays such as in the production of a rainbow. This is also true for sound, since all frequencies ordinarily travel at the same speed. If you listen to sound through a long tube, such as a vacuum cleaner hose, you can easily hear it dispersed by interaction with the tube. Dispersion, in fact, can reveal a great deal about what the wave has encountered that disperses its wavelengths. The dispersion of electromagnetic radiation from outer space, for example, has revealed much about what exists between the stars—the so-called interstellar medium.

Nick Moore's video discu s Moore replays the high-speed footage showing ww.youtube.com/watch?v=KbmOcT5sX7I

Refraction is responsible fo n depends on the index of refraction, as we know from . But for a given medium, η also depends on wavelengt

Medium	Red (660 nm)	Orange (610 nm)	Yellow (580 nm)	Green (550 nm)	Blue (470 nm)	Violet (410 nm)
Water	1.331	1.332	1.333	1.335	1.338	1.342
Diamond	2.410	2.415	2.417	2.426	2.444	2.458
Glass, crown	1.512	1.514	1.518	1.519	1.524	1.530
Glass, flint	1.662	1.665	1.667	1.674	1.684	1.698
Polystyrene	1.488	1.490	1.492	1.493	1.499	1.506
Quartz, fused	1.455	1.456	1.458	1.459	1.462	1.468

Note that for a given medium, η increases as wavelength decreases and is greatest for violet light. Thus, violet light is bent more than red light, as shown for a prism in Figure 6.2.4.3*b*. White light is dispersed into the same sequence of wavelengths as seen in Figures 6.2.4.1 and 6.2.4.2.

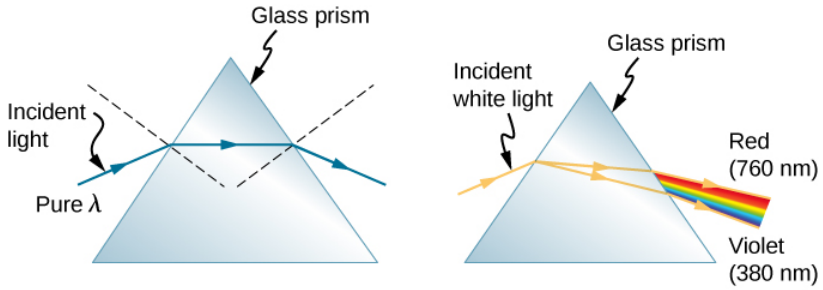
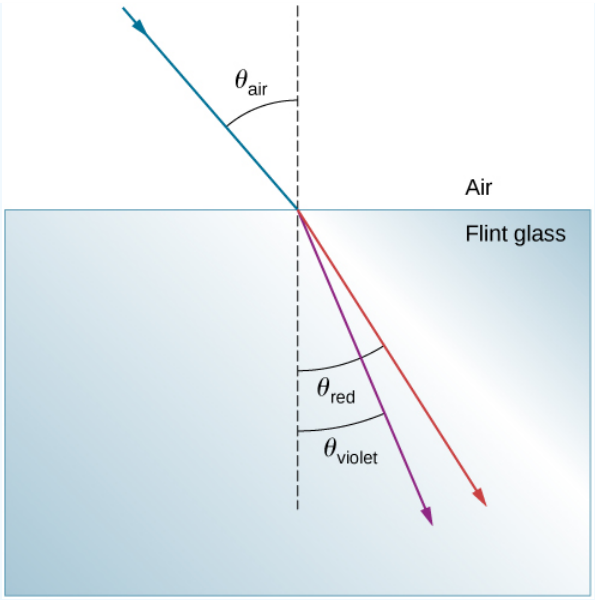


Figure 6.2.4.3: (a) A pure wavelength of light falls onto a prism and is refracted at both surfaces. (b) White light is dispersed by the prism (shown exaggerated). Since the index of refraction varies with wavelength, the angles of refraction vary with wavelength. A sequence of red to violet is produced, because the index of refraction increases steadily with decreasing wavelength.

Example 6.2.4.1: Dispersion of White Light by Flint Glass

A beam of white light goes from air into flint glass at an incidence angle of 43.2°. What is the angle between the red (660 nm) and violet (410 nm) parts of the refracted light?



Strategy

Values for the indices of refraction for flint glass at various wavelengths are listed in Table **6.2.4.1**. Use these values for calculate the angle of refraction for each color and then take the difference to find the dispersion angle.

Solution

Applying the law of refraction for the red part of the beam

$$\eta_{air} \sin \theta_{air} = \eta_{red} \sin \theta_{red}, \tag{6.2.4.1}$$

we can solve for the angle of refraction as

$$\theta_{red} = \sin^{-1}\left(\frac{\eta_{air} \sin \theta_{air}}{\eta_{red}}\right) = \sin^{-1}\left[\frac{(1.000) \sin 43.2°}{(1.512)}\right] = 27.0°. \tag{6.2.4.2}$$

Similarly, the angle of incidence for the violet part of the beam is

$$\theta_{violet} = \sin^{-1}\left(\frac{\eta_{air} sin\theta_{air}}{\eta_{violet}}\right) = \sin^{-1}\left[\frac{(1.000) \sin 43.2°}{(1.530)}\right] = 26.4°. \tag{6.2.4.3}$$

The difference between these two angles is

$$\theta_{red} - \theta_{violet} = 27.0° - 26.4° = 0.6°. \tag{6.2.4.4}$$

Significance

Although 0.6° may seem like a negligibly small angle, if this beam is allowed to propagate a long enough distance, the dispersion of colors becomes quite noticeable.

Exercise 6.2.4.1

In the preceding example, how much distance inside the block of flint glass would the red and the violet rays have to progress before they are separated by 1.0 mm?

Answer

9.3 cm

Rainbows are produced by a combination of refraction and reflection. You may have noticed that you see a rainbow only when you look away from the Sun. Light enters a drop of water and is reflected from the back of the drop (Figure **6.2.4.4**).

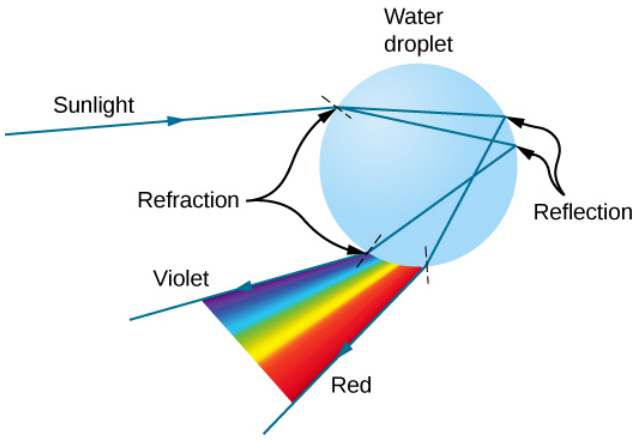


Figure **6.2.4.4**: A ray of light falling on this water drop enters and is reflected from the back of the drop. This light is refracted and dispersed both as it enters and as it leaves the drop.

The light is refracted both as it enters and as it leaves the drop. Since the index of refraction of water varies with wavelength, the light is dispersed, and a rainbow is observed (Figure **6.2.4.4***a*). (No dispersion occurs at the back surface, because the law of reflection does not depend on wavelength.) The actual rainbow of colors seen by an observer depends on the myriad rays being refracted and reflected toward the observer's eyes from numerous drops of water. The effect is most spectacular when the background is dark, as in stormy weather, but can also be observed in waterfalls and lawn sprinklers. The arc of a rainbow comes from the need to be looking at a specific angle relative to the direction of the Sun, as illustrated in Figure **6.2.4.4***b*. If two reflections of light occur within the water drop, another "secondary" rainbow is produced. This rare event produces an arc that lies above the primary rainbow arc, as in Figure **6.2.4.4***c*, and produces colors in the reverse order of the primary rainbow, with red at the lowest angle and violet at the largest angle.

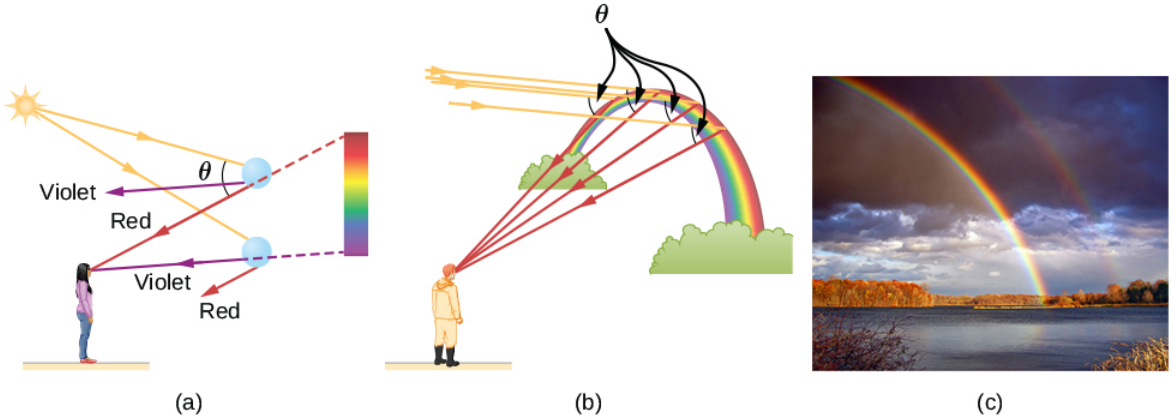


Figure **6.2.4.5**: (a) Different colors emerge in different directions, and so you must look at different locations to see the various colors of a rainbow. (b) The arc of a rainbow results from the fact that a line between the observer and any point on the arc must make the correct angle with the parallel rays of sunlight for the observer to receive the refracted rays. (c) Double rainbow. (credit c: modification of work by "Nicholas"/Wikimedia Commons)

Dispersion may produce beautiful rainbows, but it can cause problems in optical systems. White light used to transmit messages in a fiber is dispersed, spreading out in time and eventually overlapping with other messages. Since a laser produces a nearly pure wavelength, its light experiences little dispersion, an advantage over white light for transmission of information. In contrast, dispersion of electromagnetic waves coming to us from outer space can be used to determine the amount of matter they pass through.

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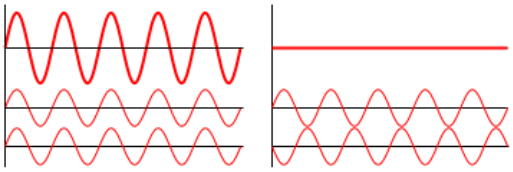
6.2.5: Superposition and Interference

learning objectives

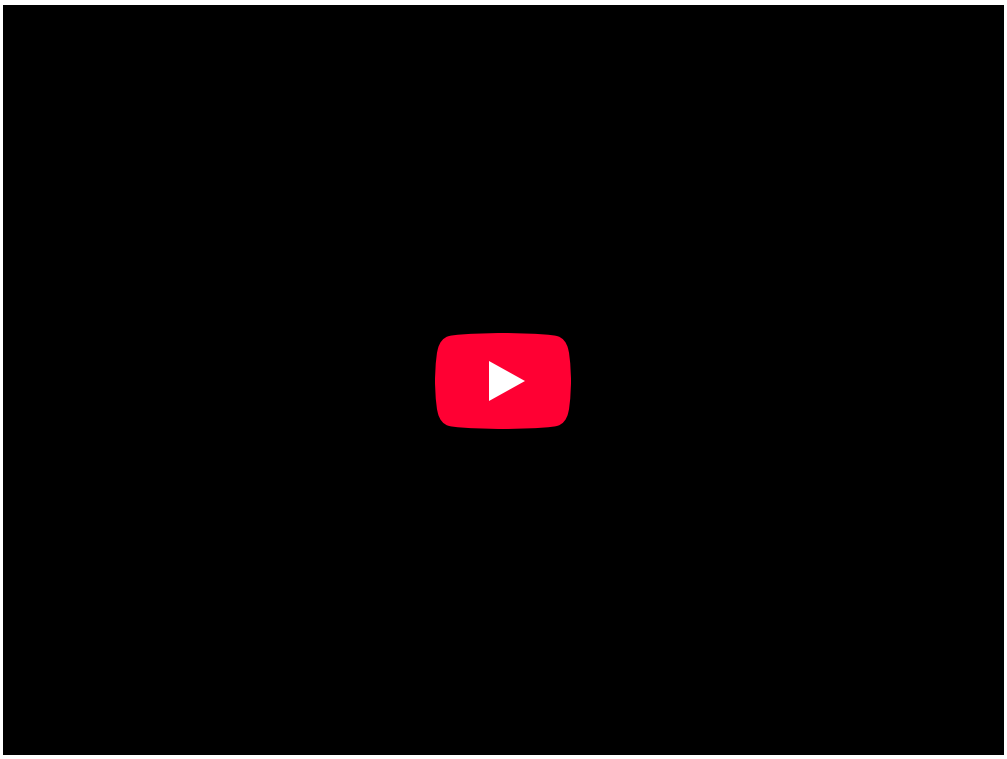
- Contrast the effects of constructive and destructive interference

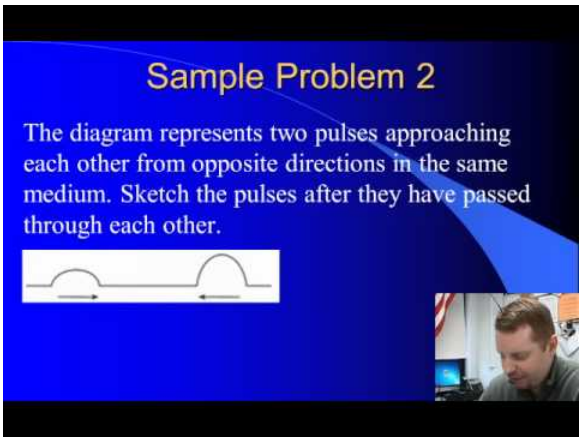
Conditions for Wave Interference

Interference is a phenomenon in which two waves superimpose to form a resultant wave of greater or lesser amplitude. Its effects can be observed in all types of waves (for example, light, acoustic waves and water waves). Interference usually refers to the interaction of waves that are correlated (coherent) with each other because they originate from the same source, or they have the same or nearly the same frequency. When two or more waves are incident on the same point, the total displacement at that point is equal to the vector sum of the displacements of the individual waves. If a crest of one wave meets a crest of another wave of the same frequency at the same point, then the magnitude of the displacement is the sum of the individual magnitudes. This is *constructive interference* and occurs when the phase difference between the waves is a multiple of 2π. *Destructive interference* occurs when the crest of one wave meets a trough of another wave. In this case, the magnitude of the displacements is equal to the difference in the individual magnitudes, and occurs when this difference is an odd multiple of π. Examples of constructive and destructive interference are shown in. If the difference between the phases is intermediate between these two extremes, then the magnitude of the displacement of the summed waves lies between the minimum and maximum values.



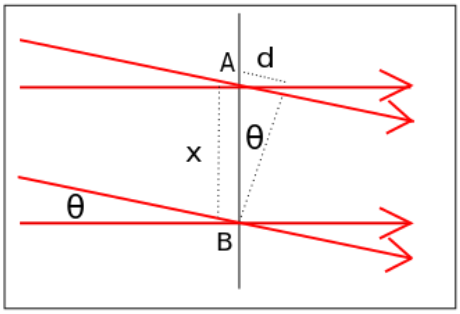
Wave Interference: Examples of constructive (left) and destructive (right) wave interference.





Wave Interference: A brief introduction to constructive and destructive wave interference and the principle of superposition.

A simple form of wave interference is observed when two waves of the same frequency (also called a plane wave) intersect at an angle, as shown in. Assuming the two waves are in phase at point B, then the relative phase changes along the *x*-axis. The phase difference at point A is given by:



Interference of Plane Waves: Geometrical arrangement for two plane wave interference.

$$\Delta\varphi = \frac{2\pi d}{\lambda} = \frac{2\pi x \sin\theta}{\lambda} \tag{6.2.5.1}$$

Constructive interference occurs when the waves are in phase, or

$$\frac{x\sin\theta}{\lambda} = 0, \pm 1, \pm 2, \ldots \tag{6.2.5.2}$$

Destructive interference occurs when the waves are half a cycle out of phase, or

$$\frac{x\sin\theta}{\lambda} = \pm\frac{1}{2}, \pm\frac{3}{2}, \ldots \tag{6.2.5.3}$$

Reflection Due to Phase Change

Light exhibits wave characteristics in various media as well as in a vacuum. When light goes from a vacuum to some medium (like water) its speed and wavelength change, but its frequency *f* remains the same. The speed of light in a medium is $v = c/n$, where n is the index of refraction. For example, water has an index of refraction of $n = 1.333$. When light is reflected off a medium with a higher index of refraction, crests get reflected as troughs and troughs get reflected as crests. In other words, the wave undergoes a 180 degree change of phase upon reflection, and the reflected ray “jumps” ahead by half a wavelength.

Air Wedge

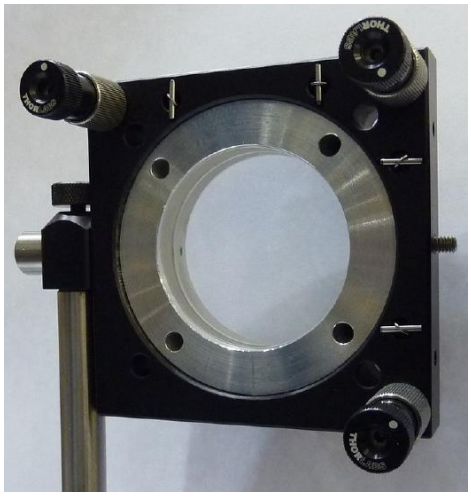
An air wedge is a simple interferometer used to visualize the disturbance of the wave front after propagation through a test object.

learning objectives

- Describe how an air wedge is used to visualize the disturbance of a wave front after proagation

Air Wedge

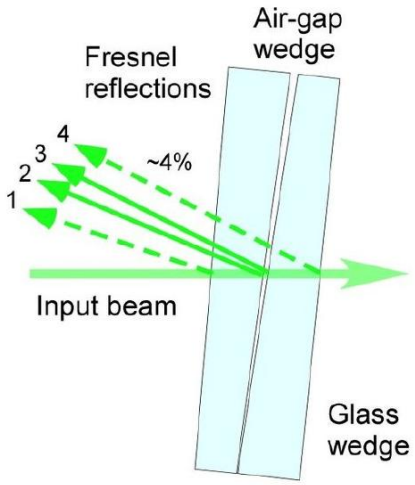
An air wedge is one of the simplest designs of shearing interferometers used to visualize the disturbance of the wave front after propagation through a test object. An air wedge can be used with nearly any light source, including non-coherent white light. The interferometer consists of two optical glass wedges (~2-5 degrees), pushed together and then slightly separated from one side to create a thin air-gap wedge. An example of an air wedge interferometer is shown in.



Air Wedge: Example of air wedge interferometer

The air gap between the two glass plates has two unique properties: it is very thin (micrometer scale) and has perfect flatness. Because of this extremely thin air-gap, the air wedge interferometer has been successfully used in experiments with femto-second high-power lasers.

An incident beam of light encounters four boundaries at which the index of refraction of the media changes, causing four reflected beams (or Fresnel reflections) as shown in. The first reflection occurs when the beam enters the first glass plate. The second reflection occurs when the beam exits the first plate and enters the air wedge, and the third reflection occurs when the beam exits the air wedge and enters the second glass plate. The fourth beam is reflected when it encounters the boundary of the second glass plate. The air wedge angle, between the second and third Fresnel reflections, can be adjusted, causing the reflected light beams to constructively and destructively interfere and create a fringe pattern. To minimize image aberrations of the resulting fringes, the angle plane of the glass wedges has to be placed orthogonal to the angle plane of the air-wedge.



Light Reflections Inside an Air Wedge Interferometer: Beam path inside of air wedge interferometer

Newton's Rings

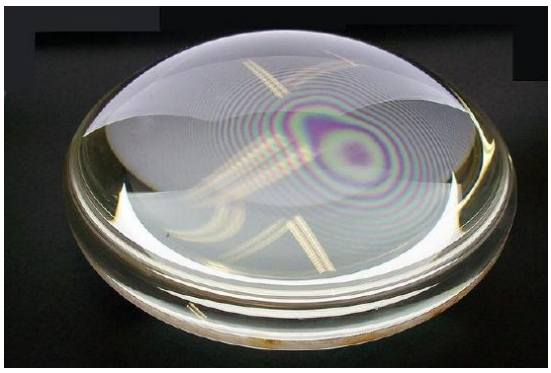
Newton's rings are a series of concentric circles centered at the point of contact between a spherical and a flat surface.

learning objectives

- Apply Newton's rings to determine light characteristics of a lens

Newton's Rings

In 1717, Isaac Newton first analyzed an interference pattern caused by the reflection of light between a spherical surface and an adjacent flat surface. Although first observed by Robert Hooke in 1664, this pattern is called Newton's rings, as Newton was the first to analyze and explain the phenomena. Newton's rings appear as a series of concentric circles centered at the point of contact between the spherical and flat surfaces. When viewed with monochromatic light, Newton's rings appear as alternating bright and dark rings; when viewed with white light, a concentric ring pattern of rainbow colors is observed. An example of Newton's rings when viewed with white light is shown in the figure below.



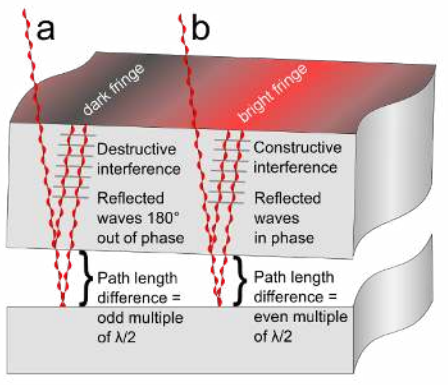
Newton's Rings in a drop of water: Newton's rings seen in two plano-convex lenses with their flat surfaces in contact. One surface is slightly convex, creating the rings. In white light, the rings are rainbow-colored, because the different wavelengths of each color interfere at different locations.

The light rings are caused by constructive interference between the light rays reflected from both surfaces, while the dark rings are caused by destructive interference. The outer rings are spaced more closely than the inner ones because the slope of the curved lens surface increases outwards. The radius of the Nth bright ring is given by:

$$r_N = \left[\left(N - \frac{1}{2}\lambda R\right)\right]^{1/2} \tag{6.2.5.4}$$

where N is the bright-ring number, R is the radius of curvature of the lens the light is passing through, and λ is the wavelength of the light passing through the glass.

A spherical lens is placed on top of a flat glass surface. An incident ray of light passes through the curved lens until it comes to the glass-air boundary, at which point it passes from a region of higher refractive index *n* (the glass) to a region of lower *n* (air). At this boundary, some light is transmitted into the air, while some light is reflected. The light that is transmitted into the air does not experience a change in phase and travels a a distance, *d*, before it is reflected at the flat glass surface below. This second air-glass boundary imparts a half-cycle phase shift to the reflected light ray because air has a lower *n* than the glass. The two reflected light rays now travel in the same direction to be detected. As one gets farther from the point at which the two surfaces touch, the distance *d*increases because the lens is curving away from the flat surface.



Formation of Interference Fringes: This figure shows how interference fringes form.

If the path length difference between the two reflected light beams is an odd multiple of the wavelength divided by two, $\lambda/2$, the reflected waves will be 180 degrees out of phase and destructively interfere, causing a dark fringe. If the path-length difference is an even multiple of $\lambda/2$, the reflected waves will be in phase with one another. The constructive interference of the two reflected waves creates a bright fringe.

Key Points

- When two or more waves are incident on the same point, the total displacement at that point is equal to the vector sum of the displacements of the individual waves.
- Light exhibits wave characteristics in various media as well as in a vacuum. When light goes from a vacuum to some medium, like water, its speed and wavelength change, but its frequency *f* remains the same.
- When light is reflected off a medium with a higher index of refraction, crests get reflected as troughs and troughs get reflected as crests. In other words, the wave undergoes a 180 degree change of phase upon reflection, and the reflected ray "jumps" ahead by half a wavelength.
- An air wedge interferometer consists of two optical glass wedges (~2-5 degrees), pushed together and then slightly separated from one side to create a thin air-gap wedge.
- The air gap between the two glass plates has two unique properties: it is very thin (micrometer scale) and has perfect flatness.
- To minimize image aberrations of the resulting fringes, the angle plane of the glass wedges has to be placed orthogonal to the angle plane of the air wedge.
- When viewed with monochromatic light, Newton's rings appear as alternating bright and dark rings; when viewed with white light, a concentric ring pattern of rainbow colors is observed.
- If the path length difference between the two reflected light beams is an odd multiple of the wavelength divided by two, $\lambda/2$, the reflected waves will be 180 degrees out of phase and destructively interfere, causing a dark fringe.
- If the path length difference is an even multiple of $\lambda/2$, the reflected waves will be in-phase with one another. The constructive interference of the two reflected waves creates a bright fringe.

Key Terms

- **coherent**: Of waves having the same direction, wavelength and phase, as light in a laser.
- **plane wave**: A constant-frequency wave whose wavefronts (surfaces of constant phase) are infinite parallel planes of constant peak-to-peak amplitude normal to the phase velocity vector.
- **orthogonal**: Of two objects, at right angles; perpendicular to each other.
- **interferometer**: Any of several instruments that use the interference of waves to determine wavelengths and wave velocities, determine refractive indices, and measure small distances, temperature changes, stresses, and many other useful measurements.
- **wavelength**: The length of a single cycle of a wave, as measured by the distance between one peak or trough of a wave and the next; it is often designated in physics as λ, and corresponds to the velocity of the wave divided by its frequency.
- **lens**: an object, usually made of glass, that focuses or defocuses the light that passes through it
- **monochromatic**: Describes a beam of light with a single wavelength (i.e., of one specific color or frequency).

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6.2.6: Diffraction

learning objectives

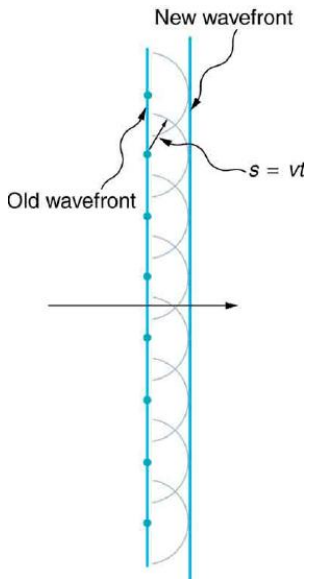
- Understanding diffraction

Overview

The Huygens-Fresnel principle states that every point on a wavefront is a source of wavelets. These wavelets spread out in the forward direction, at the same speed as the source wave. The new wavefront is a line tangent to all of the wavelets.

Background

Christiaan Huygens was a Dutch scientist who developed a useful technique for determining how and where waves propagate. In 1678, he proposed that every point that a luminous disturbance touches becomes itself a source of a spherical wave. The sum of the secondary waves (waves that are a result of the disturbance) determines the form of the new wave. shows secondary waves traveling forward from their point of origin. He was able to come up with an explanation of the linear and spherical wave propagation, and derive the laws of reflection and refraction (covered in previous atoms) using this principle. He could not, however, explain what is commonly known as diffraction effects. Diffraction effects are the deviations from rectilinear propagation that occurs when light encounters edges, screens and apertures. These effects were explained in 1816 by French physicist Augustin-Jean Fresnel.



Straight Wavefront: Huygens's principle applied to a straight wavefront. Each point on the wavefront emits a semicircular wavelet that moves a distance s=vt. The new wavefront is a line tangent to the wavelets.

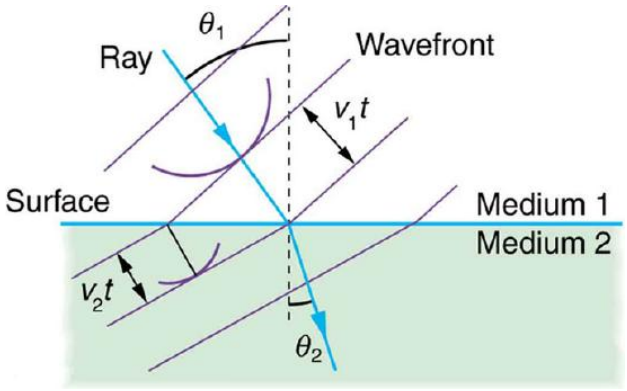
Huygens's Principle

Figure 1 shows a simple example of the Huygens's Principle of diffraction. The principle can be shown with the equation below:

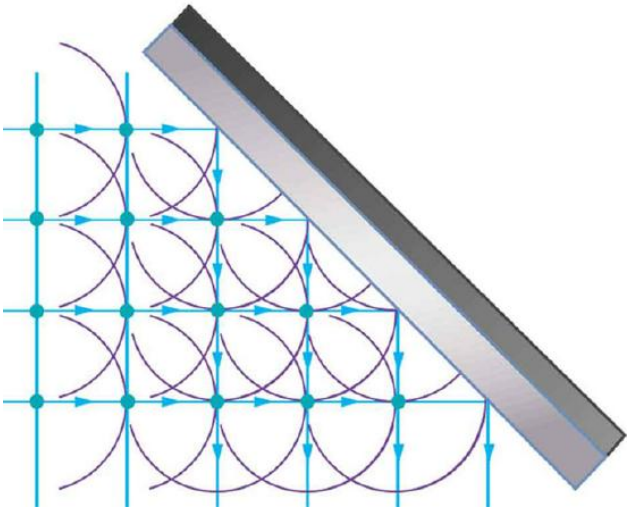
$$s = vt \tag{6.2.6.1}$$

where s is the distance, v is the propagation speed, and t is time.

Each point on the wavefront emits a wave at speed, v. The emitted waves are semicircular, and occur at t, time later. The new wavefront is tangent to the wavelets. This principle works for all wave types, not just light waves. The principle is helpful in describing reflection, refraction and interference. shows visually how Huygens's Principle can be used to explain reflection, and shows how it can be applied to refraction.



Huygens's Refraction: Huygens's principle applied to a straight wavefront traveling from one medium to another where its speed is less. The ray bends toward the perpendicular, since the wavelets have a lower speed in the second medium.



Reflection: Huygens's principle applied to a straight wavefront striking a mirror. The wavelets shown were emitted as each point on the wavefront struck the mirror. The tangent to these wavelets shows that the new wavefront has been reflected at an angle equal to the incident angle. The direction of propagation is perpendicular to the wavefront, as shown by the downward-pointing arrows.

Example 6.2.6.1:

This principle is actually something you have seen or experienced often, but just don't realize. Although this principle applies to all types of waves, it is easier to explain using sound waves, since sound waves have longer wavelengths. If someone is playing music in their room, with the door closed, you might not be able to hear it while walking past the room. However, if that person where to open their door while playing music, you could hear it not only when directly in front of the door opening, but also on a considerable distance down the hall to either side. is a direct effect of diffraction. When light passes through much smaller openings, called slits, Huygens's principle shows that light bends similar to the way sound does, just on a much smaller scale. We will examine in later atoms single slit diffraction and double slit diffraction, but for now it is just important that we understand the basic concept of diffraction.

Diffraction

As we explained in the previous paragraph, diffraction is defined as the bending of a wave around the edges of an opening or an obstacle.

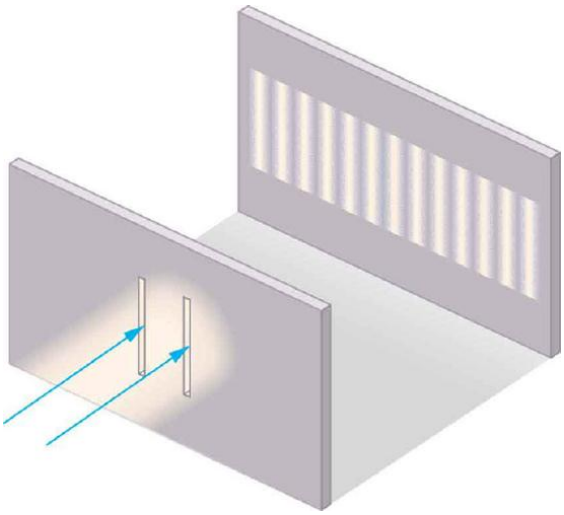
Young's Double Slit Experiment

The double-slit experiment, also called Young's experiment, shows that matter and energy can display both wave and particle characteristics.

learning objectives

- Explain why Young's experiment more credible than Huygens'

The double-slit experiment, also called Young's experiment, shows that matter and energy can display both wave and particle characteristics. As we discussed in the atom about the Huygens principle, Christiaan Huygens proved in 1628 that light was a wave. But some people disagreed with him, most notably Isaac Newton. Newton felt that color, interference, and diffraction effects needed a better explanation. People did not accept the theory that light was a wave until 1801, when English physicist Thomas Young performed his double-slit experiment. In his experiment, he sent light through two closely spaced vertical slits and observed the resulting pattern on the wall behind them. The pattern that resulted can be seen in.



Young's Double Slit Experiment: Light is sent through two vertical slits and is diffracted into a pattern of vertical lines spread out horizontally. Without diffraction and interference, the light would simply make two lines on the screen.

Wave-Particle Duality

The wave characteristics of light cause the light to pass through the slits and interfere with itself, producing the light and dark areas on the wall behind the slits. The light that appears on the wall behind the slits is scattered and absorbed by the wall, which is a characteristic of a particle.

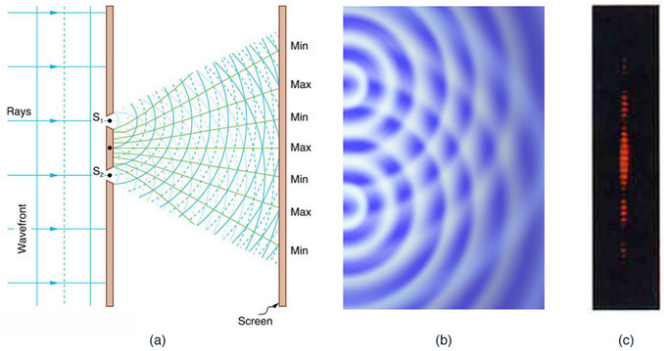
Young's Experiment

Why was Young's experiment so much more credible than Huygens'? Because, while Huygens' was correct, he could not demonstrate that light acted as a wave, while the double-slit experiment shows this very clearly. Since light has relatively short wavelengths, to show wave effects it must interact with something small — Young's small, closely spaced slits worked.

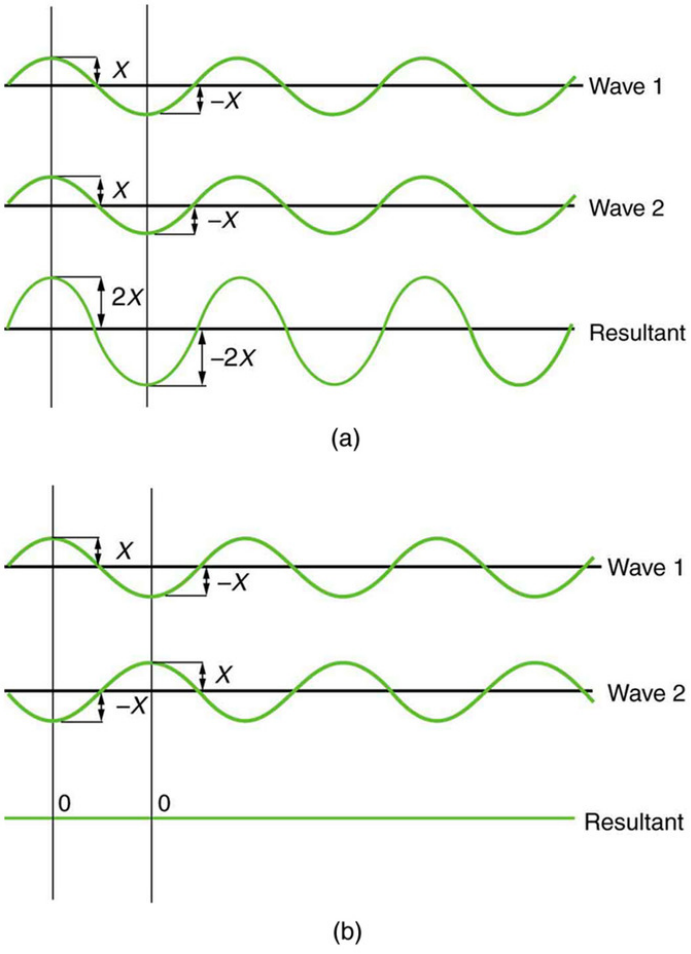
The example in uses two coherent light sources of a single monochromatic wavelength for simplicity. (This means that the light sources were in the same phase.) The two slits cause the two coherent light sources to interfere with each other either constructively or destructively.

Constructive and Destructive Wave Interference

Constructive wave interference occurs when waves interfere with each other crest-to-crest (peak-to-peak) or trough-to-trough (valley-to-valley) and the waves are exactly in phase with each other. This *amplifies* the resultant wave. Destructive wave interference occurs when waves interfere with each other crest-to-trough (peak-to-valley) and are exactly out of phase with each other. This cancels out any wave and results in no light. These concepts are shown in. It should be noted that this example uses a single, monochromatic wavelength, which is not common in real life; a more practical example is shown in.



Practical Constructive and Destructive Wave Interference: Double slits produce two coherent sources of waves that interfere. (a) Light spreads out (diffracts) from each slit because the slits are narrow. These waves overlap and interfere constructively (bright lines) and destructively (dark regions). We can only see this if the light falls onto a screen and is scattered into our eyes. (b) Double-slit interference pattern for water waves are nearly identical to that for light. Wave action is greatest in regions of constructive interference and least in regions of destructive interference. (c) When light that has passed through double slits falls on a screen, we see a pattern such as this.



Theoretical Constructive and Destructive Wave Interference: The amplitudes of waves add together. (a) Pure constructive interference is obtained when identical waves are in phase. (b) Pure destructive interference occurs when identical waves are exactly out of phase (shifted by half a wavelength).

The pattern that results from double-slit diffraction is not random, although it may seem that way. Each slit is a different distance from a given point on the wall behind it. For each different distance, a different number of wavelengths fit into that path. The waves

all start out in phase (matching crest-to-crest), but depending on the distance of the point on the wall from the slit, they could be in phase at that point and interfere constructively, or they could end up out of phase and interfere with each other destructively.

Diffraction Gratings: X-Ray, Grating, Reflection

Diffraction grating has periodic structure that splits and diffracts light into several beams travelling in different directions.

learning objectives

- Describe function of the diffraction grating

Diffraction Grating

A diffraction grating is an optical component with a periodic structure that splits and diffracts light into several beams travelling in different directions. The directions of these beams depend on the spacing of the grating and the wavelength of the light so that the grating acts as the dispersive element. Because of this, gratings are often used in monochromators, spectrometers, wavelength division multiplexing devices, optical pulse compressing devices, and many other optical instruments.

A photographic slide with a fine pattern of purple lines forms a complex grating. For practical applications, gratings generally have ridges or rulings on their surface rather than dark lines. Such gratings can be either transmissive or reflective. Gratings which modulate the phase rather than the amplitude of the incident light are also produced, frequently using holography.

Ordinary pressed CD and DVD media are every-day examples of diffraction gratings and can be used to demonstrate the effect by reflecting sunlight off them onto a white wall. (see). This is a side effect of their manufacture, as one surface of a CD has many small pits in the plastic, arranged in a spiral; that surface has a thin layer of metal applied to make the pits more visible. The structure of a DVD is optically similar, although it may have more than one pitted surface, and all pitted surfaces are inside the disc. In a standard pressed vinyl record when viewed from a low angle perpendicular to the grooves, one can see a similar, but less defined effect to that in a CD/DVD. This is due to viewing angle (less than the critical angle of reflection of the black vinyl) and the path of the light being reflected due to being changed by the grooves, leaving a rainbow relief pattern behind.



Readable Surface of a CD: The readable surface of a Compact Disc includes a spiral track wound tightly enough to cause light to diffract into a full visible spectrum.

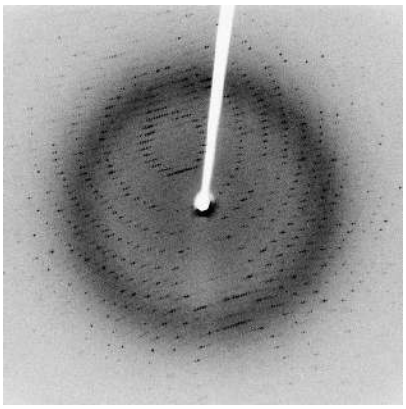
Some bird feathers use natural diffraction grating which produce constructive interference, giving the feathers an iridescent effect. Iridescence is the effect where surfaces seem to change color when the angle of illumination is changed. An opal is another example of diffraction grating that reflects the light into different colors.

X-Ray Diffraction

X-ray crystallography is a method of determining the atomic and molecular structure of a crystal, in which the crystalline atoms cause a beam of X-rays to diffract into many specific directions. By measuring the angles and intensities of these diffracted beams, a crystallographer can produce a three-dimensional picture of the density of electrons within the crystal. From this electron density, the mean positions of the atoms in the crystal can be determined, as well as their chemical bonds, their disorder and various other information.

In an X-ray diffraction measurement, a crystal is mounted on a goniometer and gradually rotated while being bombarded with X-rays, producing a diffraction pattern of regularly spaced spots known as reflections (see). The two-dimensional images taken at

different rotations are converted into a three-dimensional model of the density of electrons within the crystal using the mathematical method of Fourier transforms, combined with chemical data known for the sample.



Reflections in Diffraction Patterns: Each dot, called a reflection, in this diffraction pattern forms from the constructive interference of scattered X-rays passing through a crystal. The data can be used to determine the crystalline structure.

Single Slit Diffraction

Single slit diffraction is the phenomenon that occurs when waves pass through a narrow gap and bend, forming an interference pattern.

learning objectives

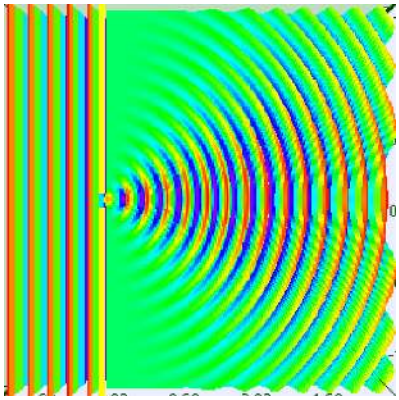
- Formulate the Huygens's Principle

Diffraction

As we explained in a previous atom, diffraction is defined as the bending of a wave around the edges of an opening or obstacle. Diffraction is a phenomenon all wave types can experience. It is explained by the *Huygens-Fresnel Principle*, and the principal of superposition of waves. The former states that every point on a wavefront is a source of wavelets. These wavelets spread out in the forward direction, at the same speed as the source wave. The new wavefront is a line tangent to all of the wavelets. The superposition principle states that at any point, the net result of multiple stimuli is the sum of all stimuli.

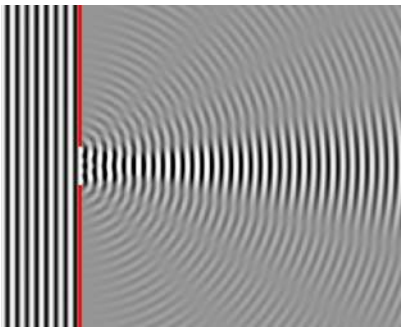
Single Slit Diffraction

In single slit diffraction, the diffraction pattern is determined by the wavelength and by the length of the slit. Figure 1 shows a visualization of this pattern. This is the most simplistic way of using the Huygens-Fresnel Principle, which was covered in a previous atom, and applying it to slit diffraction. But what happens when the slit is NOT the exact (or close to exact) length of a single wave?



Single Slit Diffraction – One Wavelength: Visualization of single slit diffraction when the slit is equal to one wavelength.

A slit that is wider than a single wave will produce interference -like effects downstream from the slit. It is easier to understand by thinking of the slit not as a long slit, but as a number of point sources spaced evenly across the width of the slit. This can be seen in Figure 2.



Single Slit Diffraction – Four Wavelengths: This figure shows single slit diffraction, but the slit is the length of 4 wavelengths.

To examine this effect better, lets consider a single monochromatic wavelength. This will produce a wavefront that is all in the same phase. Downstream from the slit, the light at any given point is made up of contributions from each of these point sources. The resulting phase differences are caused by the different in path lengths that the contributing portions of the rays traveled from the slit.

The variation in wave intensity can be mathematically modeled. From the center of the slit, the diffracting waves propagate radially. The angle of the minimum intensity (θ_{min}) can be related to wavelength (λ) and the slit's width (d) such that:

$$d \sin \theta_{min} = \lambda \tag{6.2.6.2}$$

The intensity (I) of waves at any angle can also be calculated as a relation to slit width, wavelength and intensity of the original waves before passing through the slit:

$$I(\theta) = I_0 \left(\frac{\sin(\pi x)}{\pi x} \right)^2 \tag{6.2.6.3}$$

where x is equal to:

$$\frac{d}{\lambda} \sin \theta \tag{6.2.6.4}$$

The Rayleigh Criterion

The Rayleigh criterion determines the separation angle between two light sources which are distinguishable from each other. Consider to reflecting points from an object under a microscope

learning objectives

- Explain meaning of the Rayleigh criterion

Resolution Limits

Along with the diffraction effects that we have discussed in previous subsections this section, diffraction also limits the detail that we can obtain in images. shows three different circumstances of resolution limits due to diffraction:



(a) (b) (c)

Resolution Limits: (a) Monochromatic light passed through a small circular aperture produces this diffraction pattern. (b) Two point light sources that are close to one another produce overlapping images because of diffraction. (c) If they are closer together, they cannot be resolved or distinguished.

- (a) shows a light passing through a small circular aperture. You do not see a sharp circular outline, but a spot with fuzzy edges. This is due to diffraction similar to that through a single slit.
- (b) shows two point sources close together, producing overlapping images. Due to the diffraction, you can just barely distinguish between the two point sources.
- (c) shows two point sources which are so close together that you can no longer distinguish between them.

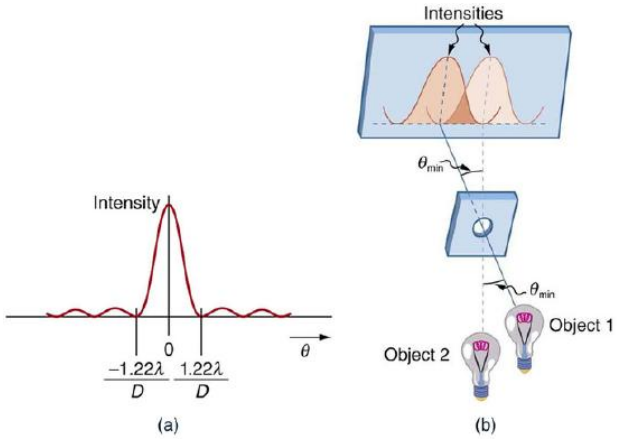
This effect can be seen with light passing through small apertures or larger apertures. This same effect happens when light passes through our pupils, and this is why the human eye has limited acuity.

Rayleigh Criterion

In the 19th century, Lord Rayleigh invented a criteria for determining when two light sources were distinguishable from each other, or resolved. According to the criteria, two point sources are considered just resolved (just distinguishable enough from each other to recognize two sources) if the center of the diffraction pattern of one is directly overlapped by the first minimum of the diffraction pattern of the other. If the distance is greater between these points, the sources are well resolved (i.e., they are easy to distingush from each other). If the distance is smaller, they are not resolved (i.e., they cannot be distinguished from each other). The equation to determine this is:

$$\theta = 1.22\frac{\lambda}{D} \tag{6.2.6.5}$$

in this equation, θ is the angle the objects are separated by (in radians), λ is the wavelength of light, and D is the aperture diameter. Consequently, with optical microscopy the ability to resolve two closely spaced objects is limited by the wavelength of light.



Rayleigh Criterion: (a) This is a graph of intensity of the diffraction pattern for a circular aperture. Note that, similar to a single slit, the central maximum is wider and brighter than those to the sides. (b) Two point objects produce overlapping diffraction patterns. Shown here is the Rayleigh criterion for being just resolvable. The central maximum of one pattern lies on the first minimum of the other.

Key Points

- Diffraction is the concept that is explained using Huygens's Principle, and is defined as the bending of a wave around the edges of an opening or an obstacle.
- This principle can be used to define reflection, as shown in the figure. It can also be used to explain refraction and interference. Anything that experiences this phenomenon is a wave. By applying this theory to light passing through a slit, we can prove it is a wave.

- The principle can be shown with the equation below: s=vt s – distance v – propagation speed t – time Each point on the wavefront emits a wave at speed, v. The emitted waves are semicircular, and occur at t, time later. The new wavefront is tangent to the wavelets.
- The wave characteristics of light cause the light to pass through the slits and interfere with each other, producing the light and dark areas on the wall behind the slits. The light that appears on the wall behind the slits is partially absorbed by the wall, a characteristic of a particle.
- Constructive interference occurs when waves interfere with each other crest-to-crest and the waves are exactly in phase with each other. Destructive interference occurs when waves interfere with each other crest-to-trough (peak-to-valley) and are exactly out of phase with each other.
- Each point on the wall has a different distance to each slit; a different number of wavelengths fit in those two paths. If the two path lengths differ by a half a wavelength, the waves will interfere destructively. If the path length differs by a whole wavelength the waves interfere constructively.
- The directions of the diffracted beams depend on the spacing of the grating and the wavelength of the light so that the grating acts as the dispersive element.
- Gratings are commonly used in monochromators, spectrometers, wavelength division multiplexing devices, optical pulse compressing devices, and other optical instruments.
- Diffraction of X-ray is used in crystallography to produce the three-dimensional picture of the density of electrons within the crystal.
- The Huygens's Principle states that every point on a wavefront is a source of wavelets. These wavelets spread out in the forward direction, at the same speed as the source wave. The new wavefront is a line tangent to all of the wavelets.
- If a slit is longer than a single wavelength, think of it instead as a number of point sources spaced evenly across the width of the slit.
- Downstream from a slit that is longer than a single wavelength, the light at any given point is made up of contributions from each of these point sources. The resulting phase differences are caused by the different in path lengths that the contributing portions of the rays traveled from the slit.
- Diffraction plays a large part in the resolution at which we are able to see things. There is a point where two light sources can be so close to each other that we cannot distinguish them apart.
- When two light sources are close to each other, they can be: unresolved (i.e., not able to distinguish one from the other), just resolved (i.e., only able to distinguish them apart from each other), and a little well resolved (i.e., easy to tell apart from one another).
- In order for two light sources to be just resolved, the center of one diffraction pattern must directly overlap with the first minimum of the other diffraction pattern.

Key Terms

- **diffraction**: The bending of a wave around the edges of an opening or an obstacle.
- **constructive interference**: Occurs when waves interfere with each other crest to crest and the waves are exactly in phase with each other.
- **destructive interference**: Occurs when waves interfere with each other crest to trough (peak to valley) and are exactly out of phase with each other.
- **interference**: An effect caused by the superposition of two systems of waves, such as a distortion on a broadcast signal due to atmospheric or other effects.
- **iridescence**: The condition or state of being iridescent; exhibition of colors like those of the rainbow; a prismatic play of color.
- **diffraction**: The bending of a wave around the edges of an opening or an obstacle.
- **monochromatic**: Describes a beam of light with a single wavelength (i.e., of one specific color or frequency).
- **resolution**: The degree of fineness with which an image can be recorded or produced, often expressed as the number of pixels per unit of length (typically an inch).

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6.2.7: Polarization

Learning Objectives

By the end of this section, you will be able to:

- Explain the change in intensity as polarized light passes through a polarizing filter
- Calculate the effect of polarization by reflection and Brewster's angle
- Describe the effect of polarization by scattering
- Explain the use of polarizing materials in devices such as LCDs

Polarizing sunglasses are familiar to most of us. They have a special ability to cut the glare of light reflected from water or glass (Figure **6.2.7.1**). They have this ability because of a wave characteristic of light called polarization. What is polarization? How is it produced? What are some of its uses? The answers to these questions are related to the wave character of light.



(a) (b)

Figure **6.2.7.1**: These two photographs of a river show the effect of a polarizing filter in reducing glare in light reflected from the surface of water. Part (b) of this figure was taken with a polarizing filter and part (a) was not. As a result, the reflection of clouds and sky observed in part (a) is not observed in part (b). Polarizing sunglasses are particularly useful on snow and water. (credit a and credit b: modifications of work by "Amithshs"/Wikimedia Commons)

Malus's Law

Light is one type of electromagnetic (EM) wave. EM waves are *transverse waves* consisting of varying electric and magnetic fields that oscillate perpendicular to the direction of propagation (Figure **6.2.7.2**). However, in general, there are no specific directions for the oscillations of the electric and magnetic fields; they vibrate in any randomly oriented plane perpendicular to the direction of propagation. Polarization is the attribute that a wave's oscillations do have a definite direction relative to the direction of propagation of the wave. (This is not the same type of polarization as that discussed for the separation of charges.) Waves having such a direction are said to be polarized. For an EM wave, we define the direction of polarization to be the direction parallel to the electric field. Thus, we can think of the electric field arrows as showing the direction of polarization, as in Figure **6.2.7.2**.

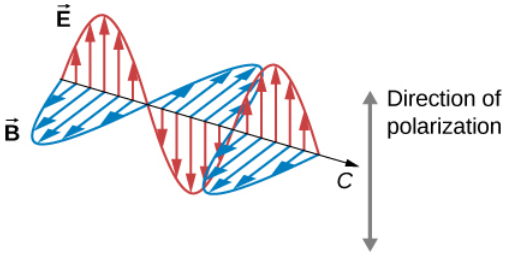


Figure **6.2.7.2**: An EM wave, such as light, is a transverse wave. The electric $\vec{E}$ and magnetic $\vec{B}$ fields are perpendicular to the direction of propagation. The direction of polarization of the wave is the direction of the electric field.

To examine this further, consider the transverse waves in the ropes shown in Figure **6.2.7.3**. The oscillations in one rope are in a vertical plane and are said to be vertically polarized. Those in the other rope are in a horizontal plane and are horizontally polarized. If a vertical slit is placed on the first rope, the waves pass through. However, a vertical slit blocks the horizontally polarized waves. For EM waves, the direction of the electric field is analogous to the disturbances on the ropes.

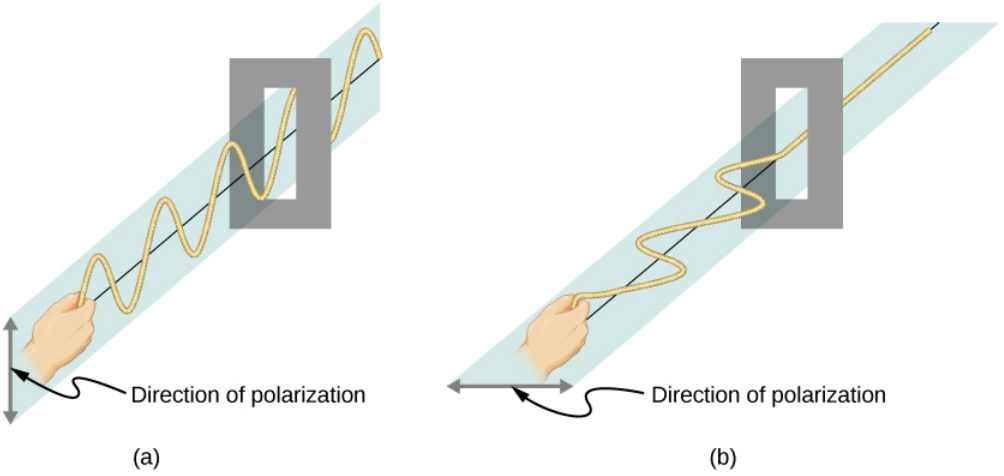


Figure **6.2.7.3**: The transverse oscillations in one rope (a) are in a vertical plane, and those in the other rope (b) are in a horizontal plane. The first is said to be vertically polarized, and the other is said to be horizontally polarized. Vertical slits pass vertically polarized waves and block horizontally polarized waves.

The Sun and many other light sources produce waves that have the electric fields in random directions (Figure **6.2.7.1***a*). Such light is said to be unpolarized, because it is composed of many waves with all possible directions of polarization. Polaroid materials—which were invented by the founder of the Polaroid Corporation, Edwin Land—act as a polarizing slit for light, allowing only polarization in one direction to pass through. Polarizing filters are composed of long molecules aligned in one direction. If we think of the molecules as many slits, analogous to those for the oscillating ropes, we can understand why only light with a specific polarization can get through. The axis of a polarizing filter is the direction along which the filter passes the electric field of an EM wave.

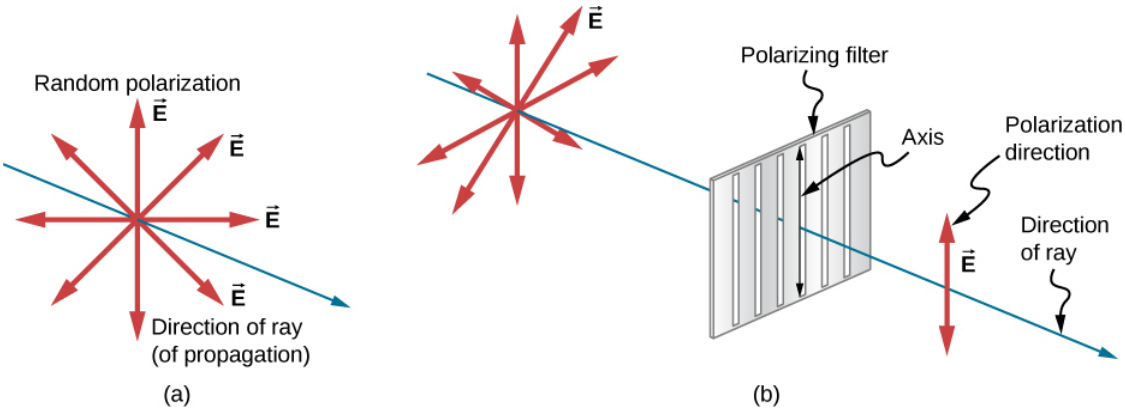


Figure **6.2.7.4**: The slender arrow represents a ray of unpolarized light. The bold arrows represent the direction of polarization of the individual waves composing the ray. (a) If the light is unpolarized, the arrows point in all directions. (b) A polarizing filter has a polarization axis that acts as a slit passing through electric fields parallel to its direction. The direction of polarization of an EM wave is defined to be the direction of its electric field.

Figure **6.2.7.5** shows the effect of two polarizing filters on originally unpolarized light. The first filter polarizes the light along its axis. When the axes of the first and second filters are aligned (parallel), then all of the polarized light passed by the first filter is also passed by the second filter. If the second polarizing filter is rotated, only the component of the light parallel to the second filter's axis is passed. When the axes are perpendicular, no light is passed by the second filter.

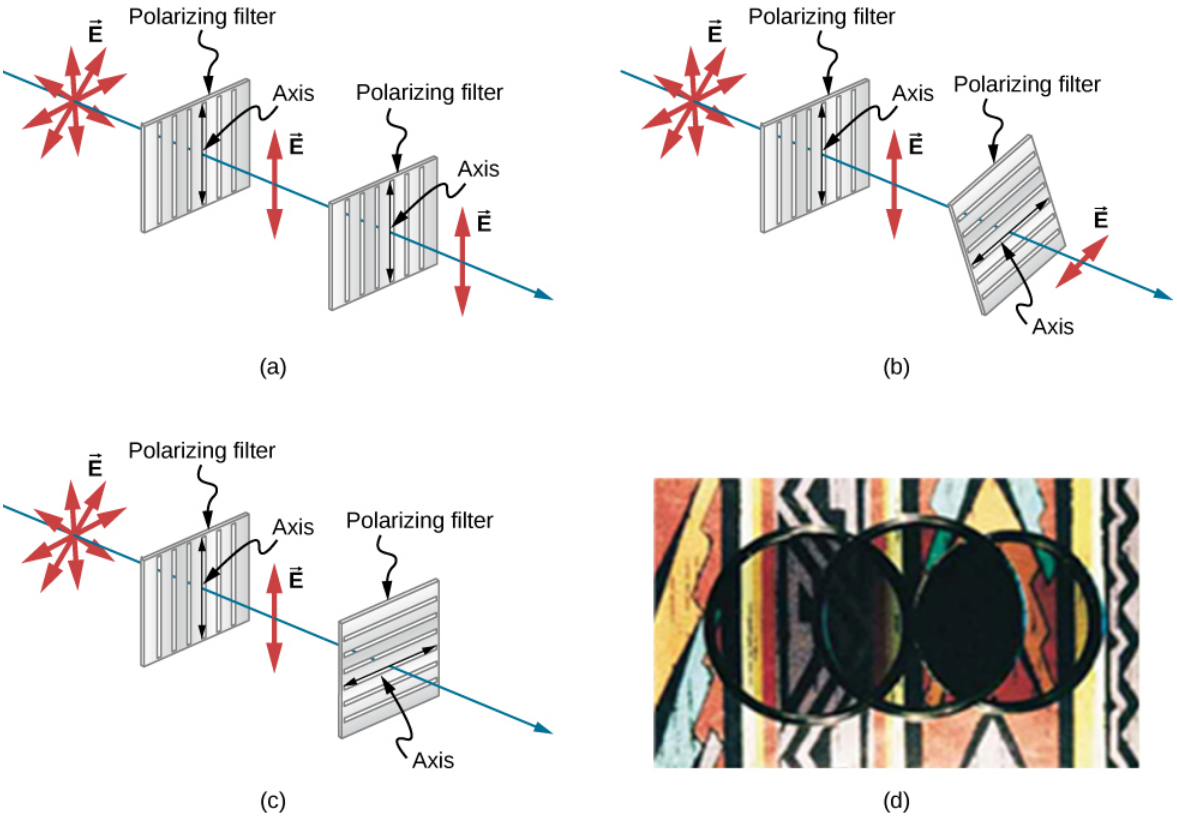


Figure 6.2.7.5: The effect of rotating two polarizing filters, where the first polarizes the light. (a) All of the polarized light is passed by the second polarizing filter, because its axis is parallel to the first. (b) As the second filter is rotated, only part of the light is passed. (c) When the second filter is perpendicular to the first, no light is passed. (d) In this photograph, a polarizing filter is placed above two others. Its axis is perpendicular to the filter on the right (dark area) and parallel to the filter on the left (lighter area). (credit d: modification of work by P.P. Urone)

Only the component of the EM wave parallel to the axis of a filter is passed. Let us call the angle between the direction of polarization and the axis of a filter θ. If the electric field has an amplitude **E**, then the transmitted part of the wave has an amplitude $E\cos\theta$ (Figure 6.2.7.6). Since the intensity of a wave is proportional to its amplitude squared, the intensity **I** of the transmitted wave is related to the incident wave by

$$I = I_0 \cos^2 \theta$$

where I_0 is the intensity of the polarized wave before passing through the filter. This equation is known as *Malus's law*.

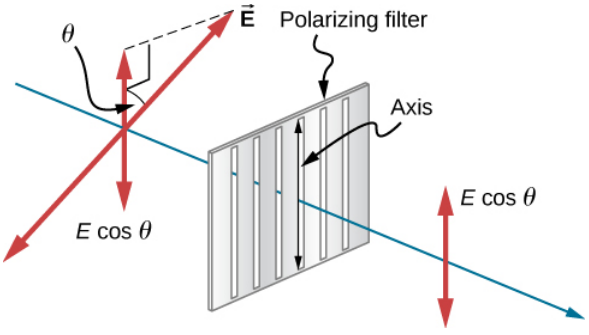


Figure 6.2.7.6: A polarizing filter transmits only the component of the wave parallel to its axis, reducing the intensity of any light not polarized parallel to its axis.

This Open Source Physics animation helps you visualize the electric field vectors as light encounters a polarizing filter. You can rotate the filter—note that the angle displayed is in radians. You can also rotate the animation for 3D visualization.

Example 6.2.7.1: Calculating Intensity Reduction by a Polarizing Filter

What angle is needed between the direction of polarized light and the axis of a polarizing filter to reduce its intensity by **90.0%**?

Strategy

When the intensity is reduced by **90.0%**, it is **10.0%** or 0.100 times its original value. That is, $\mathbf{I=0.100I_0}$. Using this information, the equation $\mathbf{I=I_0\cos^2\theta}$ can be used to solve for the needed angle.

Solution

Solving Malus's law (Equation ???) for $\cos\theta$ and substituting with the relationship between **I** and $\mathbf{I_0}$ gives

$$\cos\theta = \frac{I}{I_0} = \frac{0.100I_0}{I_0} = 0.3162.$$

Solving for θ yields

$$\theta = \cos^{-1} 0.3162 = 71.6°.$$

Significance

A fairly large angle between the direction of polarization and the filter axis is needed to reduce the intensity to 10.0% of its original value. This seems reasonable based on experimenting with polarizing films. It is interesting that at an angle of 45°, the intensity is reduced to 50% of its original value. Note that 71.6° is 18.4° from reducing the intensity to zero, and that at an angle of 18.4°, the intensity is reduced to 90.0% of its original value, giving evidence of symmetry.

? Exercise 6.2.7.1

Although we did not specify the direction in Example **6.2.7.1**, let's say the polarizing filter was rotated clockwise by 71.6° to reduce the light intensity by 90.0%. What would be the intensity reduction if the polarizing filter were rotated counterclockwise by 71.6°?

Answer

also 90.0%

Polarization by Reflection

By now, you can probably guess that polarizing sunglasses cut the glare in reflected light, because that light is polarized. You can check this for yourself by holding polarizing sunglasses in front of you and rotating them while looking at light reflected from water or glass. As you rotate the sunglasses, you will notice the light gets bright and dim, but not completely black. This implies the reflected light is partially polarized and cannot be completely blocked by a polarizing filter.

Figure **6.2.7.7** illustrates what happens when unpolarized light is reflected from a surface. Vertically polarized light is preferentially refracted at the surface, so the reflected light is left more horizontally polarized. The reasons for this phenomenon are beyond the scope of this text, but a convenient mnemonic for remembering this is to imagine the polarization direction to be like an arrow. Vertical polarization is like an arrow perpendicular to the surface and is more likely to stick and not be reflected. Horizontal polarization is like an arrow bouncing on its side and is more likely to be reflected. Sunglasses with vertical axes thus block more reflected light than unpolarized light from other sources.

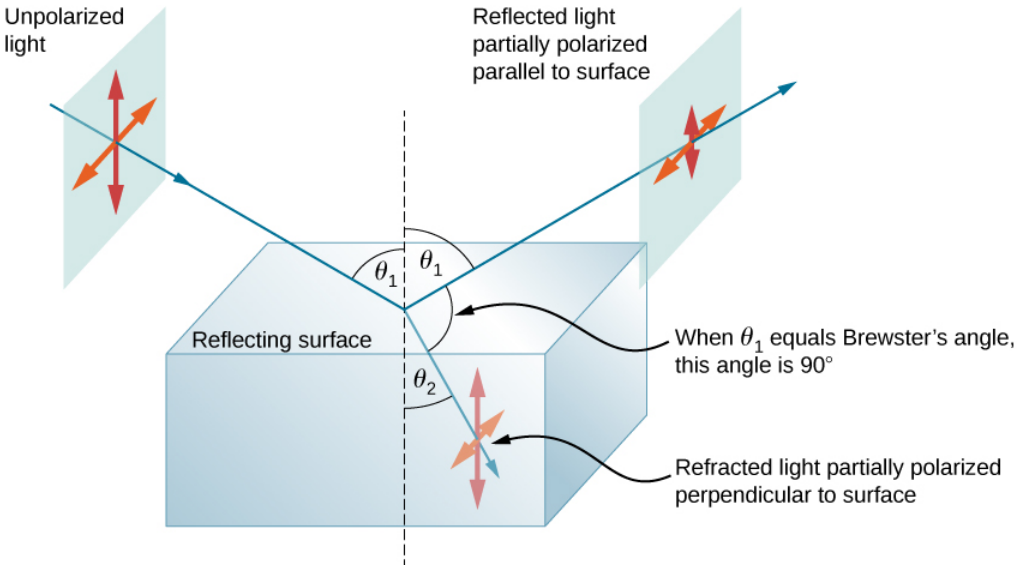


Figure **6.2.7.7**: Polarization by reflection. Unpolarized light has equal amounts of vertical and horizontal polarization. After interaction with a surface, the vertical components are preferentially absorbed or refracted, leaving the reflected light more horizontally polarized. This is akin to arrows striking on their sides and bouncing off, whereas arrows striking on their tips go into the surface.

Since the part of the light that is not reflected is refracted, the amount of polarization depends on the indices of refraction of the media involved. It can be shown that reflected light is completely polarized at an angle of reflection θ_b given by

$$tan\,\theta_b = \frac{n_2}{n_1}$$

where n_1 is the medium in which the incident and reflected light travel and n_2 is the index of refraction of the medium that forms the interface that reflects the light. This equation is known as Brewster's law and θ_b is known as Brewster's angle, named after the nineteenth-century Scottish physicist who discovered them.

This Open Source Physics animation shows incident, reflected, and refracted light as rays and EM waves. Try rotating the animation for 3D visualization and also change the angle of incidence. Near Brewster's angle, the reflected light becomes highly polarized.

Example 6.2.7.2: Calculating Polarization by Reflection

(a) At what angle will light traveling in air be completely polarized horizontally when reflected from water? (b) From glass?

Strategy

All we need to solve these problems are the indices of refraction. Air has n_1=1.00, water has n_2=1.333, and crown glass has n'_2=1.520. The equation $tan\,\theta_b = \frac{n_2}{n_1}$ can be directly applied to find θ_b in each case.

Solution

a. Putting the known quantities into the equation

$$\tan\,\theta_b = \frac{n_2}{n_1}$$

gives

$$\tan\,\theta_b = \frac{n_2}{n_1} = \frac{1.333}{1.00} = 1.333.$$

Solving for the angle θ_b yields

$$\theta_b = tan^{-1}1.333 = 53.1°.$$

b. Similarly, for crown glass and air,

$$\tan\theta'_b = \frac{n'_2}{n_1} = \frac{1.520}{1.00} = 1.52.$$

Thus,

$$\theta'_b = \tan^{-1}1.52 = 56.7°.$$

Significance

Light reflected at these angles could be completely blocked by a good polarizing filter held with its axis vertical. Brewster's angle for water and air are similar to those for glass and air, so that sunglasses are equally effective for light reflected from either water or glass under similar circumstances. Light that is not reflected is refracted into these media. Therefore, at an incident angle equal to Brewster's angle, the refracted light is slightly polarized vertically. It is not completely polarized vertically, because only a small fraction of the incident light is reflected, so a significant amount of horizontally polarized light is refracted.

? Exercise 6.2.7.2

What happens at Brewster's angle if the original incident light is already 100% vertically polarized?

Answer

There will be only refraction but no reflection.

Atomic Explanation of Polarizing Filters

Polarizing filters have a polarization axis that acts as a slit. This slit passes EM waves (often visible light) that have an electric field parallel to the axis. This is accomplished with long molecules aligned perpendicular to the axis, as shown in Figure **6.2.7.8**.

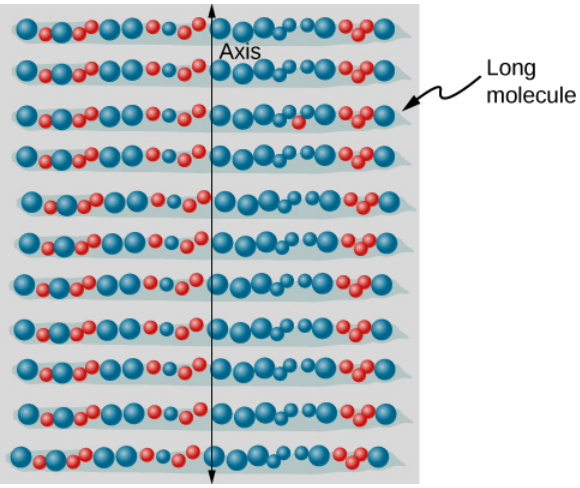


Figure **6.2.7.8**: Long molecules are aligned perpendicular to the axis of a polarizing filter. In an EM wave, the component of the electric field perpendicular to these molecules passes through the filter, whereas the component parallel to the molecules is absorbed.

Figure **6.2.7.9** illustrates how the component of the electric field parallel to the long molecules is absorbed. An EM wave is composed of oscillating electric and magnetic fields. The electric field is strong compared with the magnetic field and is more effective in exerting force on charges in the molecules. The most affected charged particles are the electrons, since electron masses are small. If an electron is forced to oscillate, it can absorb energy from the EM wave. This reduces the field in the wave and, hence, reduces its intensity. In long molecules, electrons can more easily oscillate parallel to the molecule than in the perpendicular direction. The electrons are bound to the molecule and are more restricted in their movement perpendicular to the molecule. Thus, the electrons can absorb EM waves that have a component of their electric field parallel to the molecule. The electrons are much less responsive to electric fields perpendicular to the molecule and allow these fields to pass. Thus, the axis of the polarizing filter is perpendicular to the length of the molecule.

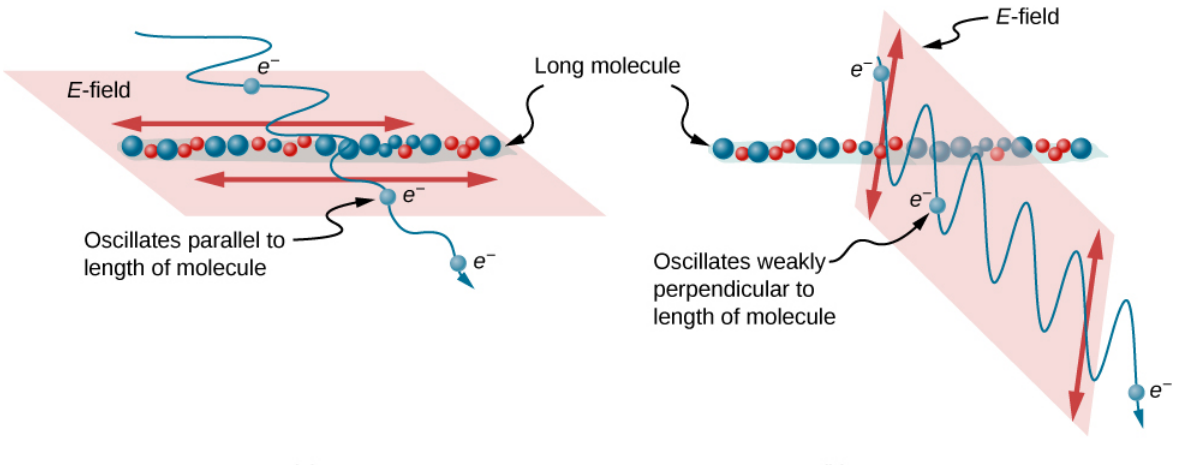


Figure 6.2.7.9: Diagram of an electron in a long molecule oscillating parallel to the molecule. The oscillation of the electron absorbs energy and reduces the intensity of the component of the EM wave that is parallel to the molecule.

Polarization by Scattering

If you hold your polarizing sunglasses in front of you and rotate them while looking at blue sky, you will see the sky get bright and dim. This is a clear indication that light scattered by air is partially polarized. Figure 6.2.7.10 helps illustrate how this happens. Since light is a transverse EM wave, it vibrates the electrons of air molecules perpendicular to the direction that it is traveling. The electrons then radiate like small antennae. Since they are oscillating perpendicular to the direction of the light ray, they produce EM radiation that is polarized perpendicular to the direction of the ray. When viewing the light along a line perpendicular to the original ray, as in the figure, there can be no polarization in the scattered light parallel to the original ray, because that would require the original ray to be a longitudinal wave. Along other directions, a component of the other polarization can be projected along the line of sight, and the scattered light is only partially polarized. Furthermore, multiple scattering can bring light to your eyes from other directions and can contain different polarizations.

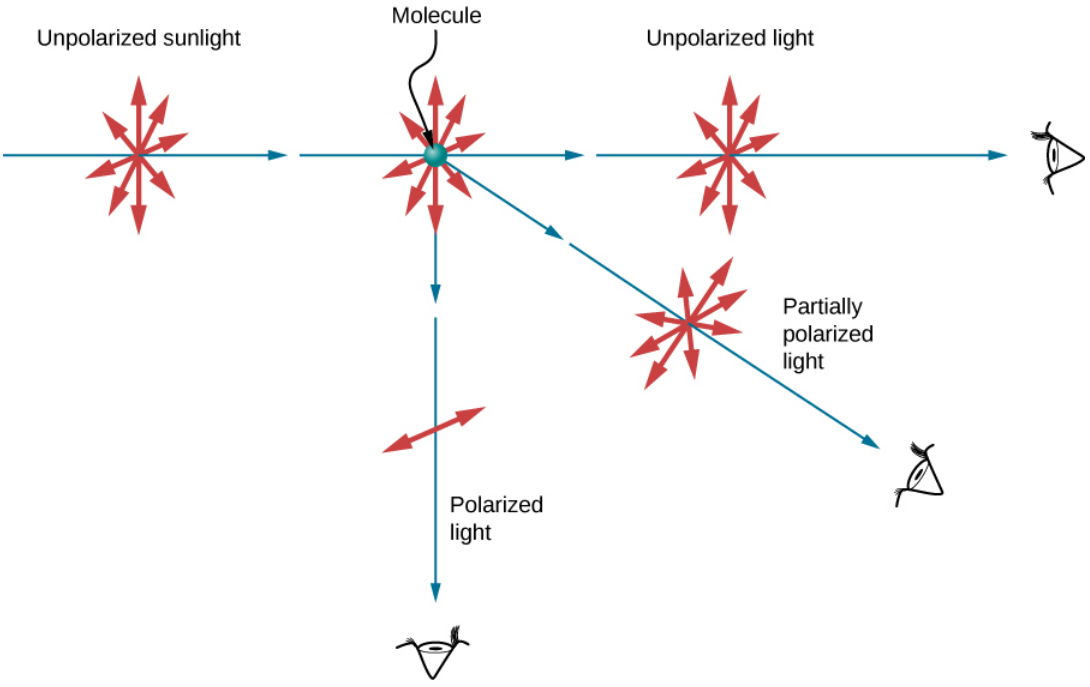


Figure 6.2.7.10: Polarization by scattering. Unpolarized light scattering from air molecules shakes their electrons perpendicular to the direction of the original ray. The scattered light therefore has a polarization perpendicular to the original direction and none parallel to the original direction.

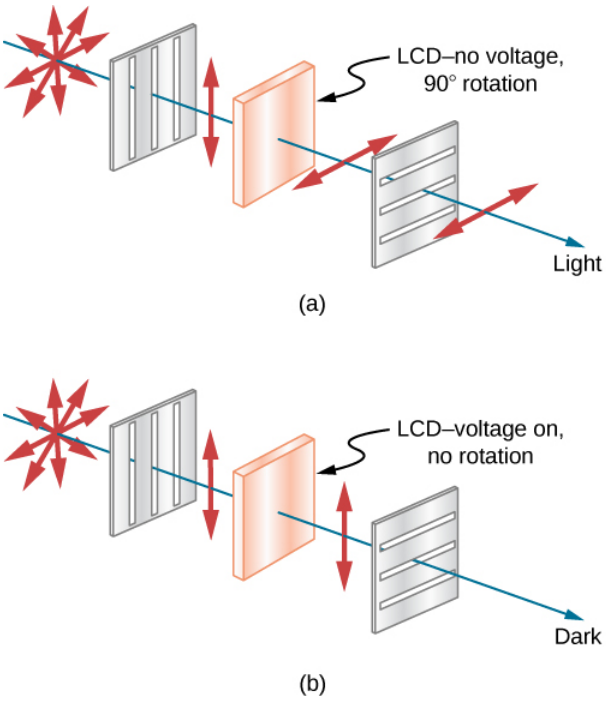
Photographs of the sky can be darkened by polarizing filters, a trick used by many photographers to make clouds brighter by contrast. Scattering from other particles, such as smoke or dust, can also polarize light. Detecting polarization in scattered EM waves can be a useful analytical tool in determining the scattering source.

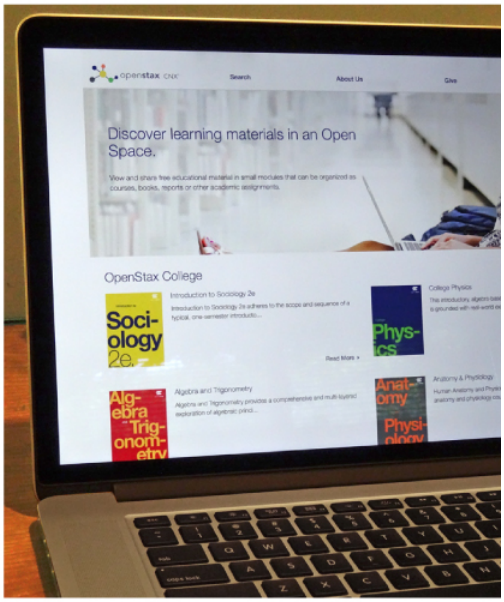
A range of optical effects are used in sunglasses. Besides being polarizing, sunglasses may have colored pigments embedded in them, whereas others use either a nonreflective or reflective coating. A recent development is photochromic lenses, which darken in the sunlight and become clear indoors. Photochromic lenses are embedded with organic microcrystalline molecules that change their properties when exposed to UV in sunlight, but become clear in artificial lighting with no UV.

Liquid Crystals and Other Polarization Effects in Materials

Although you are undoubtedly aware of liquid crystal displays (LCDs) found in watches, calculators, computer screens, cellphones, flat screen televisions, and many other places, you may not be aware that they are based on polarization. Liquid crystals are so named because their molecules can be aligned even though they are in a liquid. Liquid crystals have the property that they can rotate the polarization of light passing through them by 90°. Furthermore, this property can be turned off by the application of a voltage, as illustrated in Figure **6.2.7.11**. It is possible to manipulate this characteristic quickly and in small, well-defined regions to create the contrast patterns we see in so many LCD devices.

In flat screen LCD televisions, a large light is generated at the back of the TV. The light travels to the front screen through millions of tiny units called pixels (picture elements). One of these is shown in Figure **6.2.7.11**. Each unit has three cells, with red, blue, or green filters, each controlled independently. When the voltage across a liquid crystal is switched off, the liquid crystal passes the light through the particular filter. We can vary the picture contrast by varying the strength of the voltage applied to the liquid crystal.





(c)

Figure **6.2.7.11**: (a) Polarized light is rotated 90° by a liquid crystal and then passed by a polarizing filter that has its axis perpendicular to the direction of the original polarization. (b) When a voltage is applied to the liquid crystal, the polarized light is not rotated and is blocked by the filter, making the region dark in comparison with its surroundings. (c) LCDs can be made color specific, small, and fast enough to use in laptop computers and TVs.

Many crystals and solutions rotate the plane of polarization of light passing through them. Such substances are said to be optically active. Examples include sugar water, insulin, and collagen (Figure **6.2.7.11**). In addition to depending on the type of substance, the amount and direction of rotation depend on several other factors. Among these is the concentration of the substance, the distance the light travels through it, and the wavelength of light. Optical activity is due to the asymmetrical shape of molecules in the substance, such as being helical. Measurements of the rotation of polarized light passing through substances can thus be used to

measure concentrations, a standard technique for sugars. It can also give information on the shapes of molecules, such as proteins, and factors that affect their shapes, such as temperature and pH.

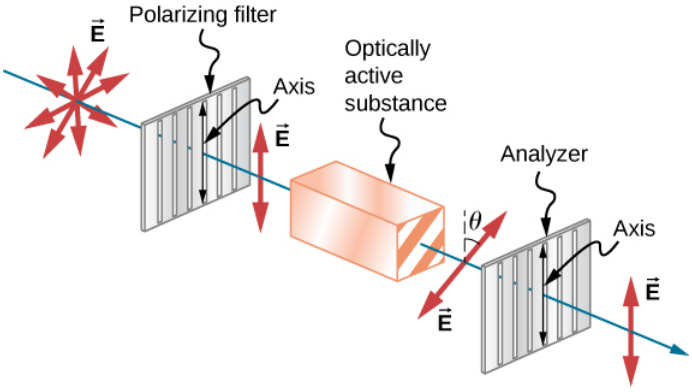


Figure **6.2.7.11**. Optical activity is the ability of some substances to rotate the plane of polarization of light passing through them. The rotation is detected with a polarizing filter or analyzer.

Glass and plastic become optically active when stressed: the greater the stress, the greater the effect. Optical stress analysis on complicated shapes can be performed by making plastic models of them and observing them through crossed filters, as seen in Figure **6.2.7.12**. It is apparent that the effect depends on wavelength as well as stress. The wavelength dependence is sometimes also used for artistic purposes.

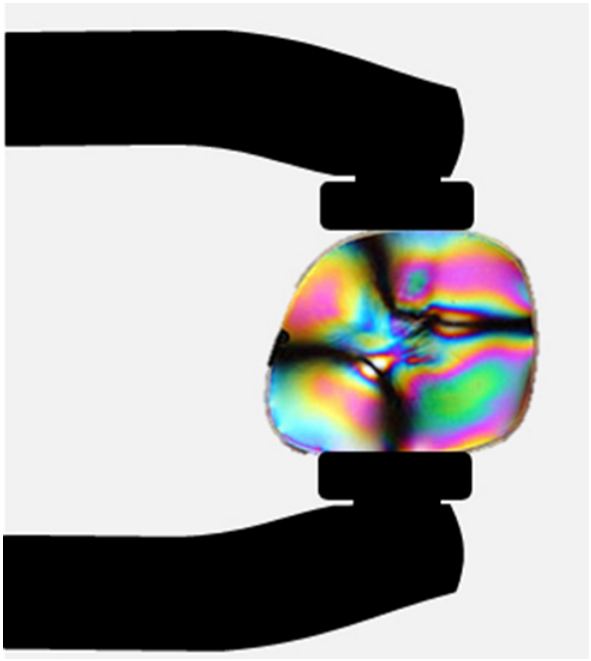


Figure **6.2.7.13**: Optical stress analysis of a plastic lens placed between crossed polarizers. (credit: “Infopro”/Wikimedia Commons)

Another interesting phenomenon associated with polarized light is the ability of some crystals to split an unpolarized beam of light into two polarized beams. This occurs because the crystal has one value for the index of refraction of polarized light but a different value for the index of refraction of light polarized in the perpendicular direction, so that each component has its own angle of refraction. Such crystals are said to be birefringent, and, when aligned properly, two perpendicularly polarized beams will emerge from the crystal (Figure **6.2.7.14**). Birefringent crystals can be used to produce polarized beams from unpolarized light. Some birefringent materials preferentially absorb one of the polarizations. These materials are called dichroic and can produce polarization by this preferential absorption. This is fundamentally how polarizing filters and other polarizers work.

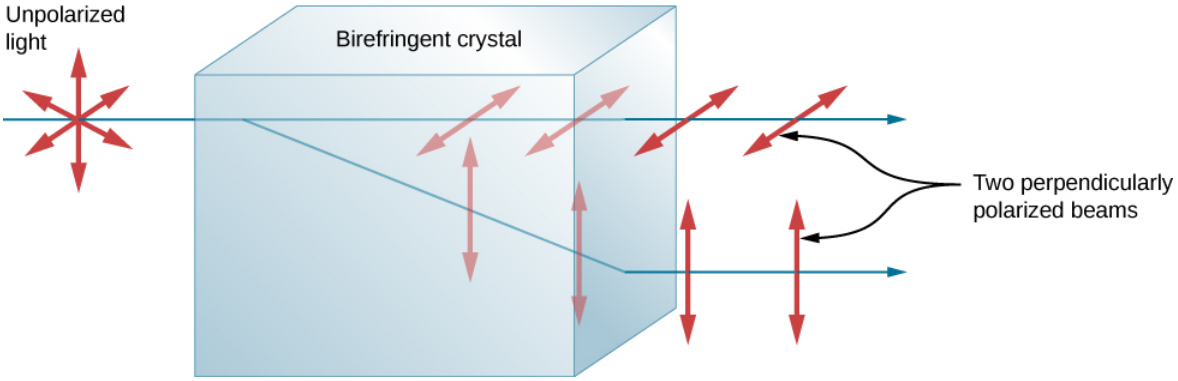


Figure **6.2.7.14**: Birefringent materials, such as the common mineral calcite, split unpolarized beams of light into two with two different values of index of refraction.

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6.3: Light as a Particle

Learning Objectives

- To understand how energy is quantized.

By the late 19th century, many physicists thought their discipline was well on the way to explaining most natural phenomena. They could calculate the motions of material objects using Newton's laws of classical mechanics, and they could describe the properties of radiant energy using mathematical relationships known as Maxwell's equations, developed in 1873 by James Clerk Maxwell, a Scottish physicist. The universe appeared to be a simple and orderly place, containing matter, which consisted of particles that had mass and whose location and motion could be accurately described, and electromagnetic radiation, which was viewed as having no mass and whose exact position in space could not be fixed. Thus matter and energy were considered distinct and unrelated phenomena. Soon, however, scientists began to look more closely at a few inconvenient phenomena that could *not* be explained by the theories available at the time.

Blackbody Radiation

One phenomenon that seemed to contradict the theories of classical physics was blackbody radiation, which is electromagnetic radiation given off by a hot object. The wavelength (i.e. color) of radiant energy emitted by a blackbody depends on only its temperature, not its surface or composition. Hence an electric stove burner or the filament of a space heater glows dull red or orange when heated, whereas the much hotter tungsten wire in an incandescent light bulb gives off a yellowish light.



Figure **6.3.1**: Blackbody Radiation. When heated, all objects emit electromagnetic radiation whose wavelength (and color) depends on the temperature of the object. A relatively low-temperature object, such as a horseshoe forged by a blacksmith, appears red, whereas a higher-temperature object, such as the surface of the sun, appears yellow or white. Images used with permission from Wikipedia.

The *intensity* of radiation is a measure of the energy emitted per unit area. A plot of the intensity of blackbody radiation as a function of wavelength for an object at various temperatures is shown in Figure **6.3.2**. One of the major assumptions of classical physics was that energy increased or decreased in a smooth, continuous manner. For example, classical physics predicted that as wavelength decreased, the intensity of the radiation an object emits should increase in a smooth curve without limit at *all* temperatures, as shown by the broken line for 6000 K in Figure **6.3.2**. Thus classical physics could not explain the sharp *decrease* in the intensity of radiation emitted at shorter wavelengths (primarily in the ultraviolet region of the spectrum), which was referred to as the "ultraviolet catastrophe." In 1900, however, the German physicist Max Planck (1858–1947) explained the ultraviolet catastrophe by proposing (in what he called "an act of despair") that the energy of electromagnetic waves is *quantized* rather than continuous. This means that for each temperature, there is a maximum intensity of radiation that is emitted in a blackbody object, corresponding to the peaks in Figure **6.3.2**, so the intensity does not follow a smooth curve as the temperature increases, as predicted by classical physics. Thus energy could be gained or lost only in integral multiples of some smallest unit of energy, a **quantum**.

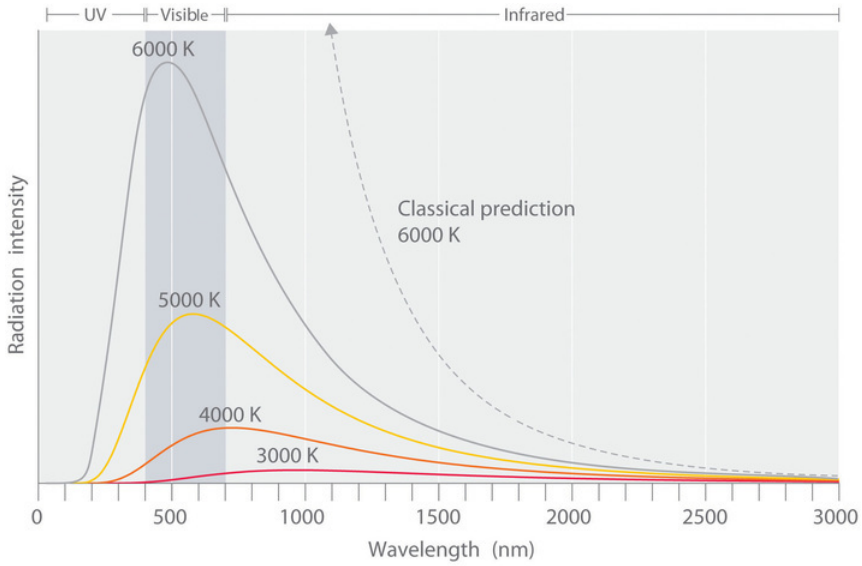
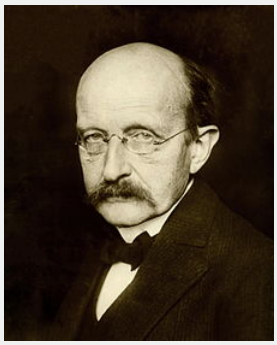


Figure **6.3.2**: Relationship between the Temperature of an Object and the Spectrum of Blackbody Radiation it Emits. At relatively low temperatures, most radiation is emitted at wavelengths longer than 700 nm, which is in the infrared portion of the spectrum. The dull red glow of the electric stove element in Figure **6.3.1** is due to the small amount of radiation emitted at wavelengths less than 700 nm, which the eye can detect. As the temperature of the object increases, the maximum intensity shifts to shorter wavelengths, successively resulting in orange, yellow, and finally white light. At high temperatures, all wavelengths of visible light are emitted with approximately equal intensities. The white light spectrum shown for an object at 6000 K closely approximates the spectrum of light emitted by the sun (Figure **6.3.1**). Note the sharp decrease in the intensity of radiation emitted at wavelengths below 400 nm, which constituted the ultraviolet catastrophe. The classical prediction fails to fit the experimental curves entirely and does not have a maximum intensity.

Max Planck (1858–1947)

In addition to being a physicist, Planck was a gifted pianist, who at one time considered music as a career. During the 1930s, Planck felt it was his duty to remain in Germany, despite his open opposition to the policies of the Nazi government.



One of his sons was executed in 1944 for his part in an unsuccessful attempt to assassinate Hitler, and bombing during the last weeks of World War II destroyed Planck's home. After WWII, the major German scientific research organization was renamed the Max Planck Society.

Although quantization may seem to be an unfamiliar concept, we encounter it frequently. For example, US money is integral multiples of pennies. Similarly, musical instruments like a piano or a trumpet can produce only certain musical notes, such as C or F sharp. Because these instruments cannot produce a continuous range of frequencies, their frequencies are quantized. Even electrical charge is quantized: an ion may have a charge of −1 or −2 but *not* −1.33 electron charges.

Planck postulated that the energy of a particular quantum of radiant energy could be described by the equation

$$E = hu \tag{6.3.1}$$

where the proportionality constant h is called Planck's constant, one of the most accurately known fundamental constants in science. For our purposes, its value to four significant figures is generally sufficient:

$$h = 6.626 \times 10^{-34}\ J \bullet s\ \text{(joule-seconds)}$$

As the frequency of electromagnetic radiation increases, the magnitude of the associated quantum of radiant energy increases. By assuming that energy can be emitted by an object only in integral multiples of $h\nu$, Planck devised an equation that fit the experimental data shown in Figure **6.3.2**. We can understand Planck's explanation of the ultraviolet catastrophe qualitatively as follows: At low temperatures, radiation with only relatively low frequencies is emitted, corresponding to low-energy quanta. As the temperature of an object increases, there is an increased probability of emitting radiation with higher frequencies, corresponding to higher-energy quanta. At any temperature, however, it is simply more probable for an object to lose energy by emitting a large number of lower-energy quanta than a single very high-energy quantum that corresponds to ultraviolet radiation. The result is a maximum in the plot of intensity of emitted radiation versus wavelength, as shown in Figure **6.3.2**, and a shift in the position of the maximum to lower wavelength (higher frequency) with increasing temperature.

At the time he proposed his radical hypothesis, Planck could not explain *why* energies should be quantized. Initially, his hypothesis explained only one set of experimental data—blackbody radiation. If quantization were observed for a large number of different phenomena, then quantization would become a law. In time, a theory might be developed to explain that law. As things turned out, Planck's hypothesis was the seed from which modern physics grew.

The Photoelectric Effect

Only five years after he proposed it, Planck's quantization hypothesis was used to explain a second phenomenon that conflicted with the accepted laws of classical physics. When certain metals are exposed to light, electrons are ejected from their surface (Figure **6.3.3**). Classical physics predicted that the number of electrons emitted and their kinetic energy should depend on only the intensity of the light, not its frequency. In fact, however, each metal was found to have a characteristic threshold frequency of light; below that frequency, no electrons are emitted regardless of the light's intensity. Above the threshold frequency, the number of electrons emitted was found to be proportional to the intensity of the light, and their kinetic energy was proportional to the frequency. This phenomenon was called the photoelectric effect (A phenomenon in which electrons are ejected from the surface of a metal that has been exposed to light).

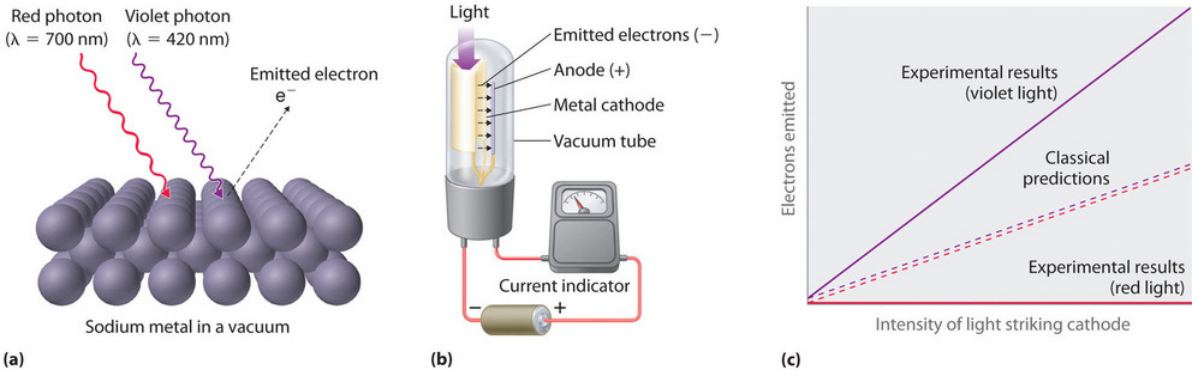


Figure **6.3.3**: The Photoelectric Effect (a) Irradiating a metal surface with photons of sufficiently high energy causes electrons to be ejected from the metal. (b) A photocell that uses the photoelectric effect, similar to those found in automatic door openers. When light strikes the metal cathode, electrons are emitted and attracted to the anode, resulting in a flow of electrical current. If the incoming light is interrupted by, for example, a passing person, the current drops to zero. (c) In contrast to predictions using classical physics, no electrons are emitted when photons of light with energy less than E_o, such as red light, strike the cathode. The energy of violet light is above the threshold frequency, so the number of emitted photons is proportional to the light's intensity.

Albert Einstein (1879–1955; Nobel Prize in Physics, 1921) quickly realized that Planck's hypothesis about the quantization of radiant energy could also explain the photoelectric effect. The key feature of Einstein's hypothesis was the assumption that radiant energy arrives at the metal surface in particles that we now call **photons** (a quantum of radiant energy, each of which possesses a particular energy energy E given by Equation 6.3.1 Einstein postulated that each metal has a particular electrostatic attraction for its electrons that must be overcome before an electron can be emitted from its surface ($E_o = u_o$). If photons of light with energy less than E_o strike a metal surface, no single photon has enough energy to eject an electron, so no electrons are emitted regardless of the

intensity of the light. If a photon with energy greater than E_o strikes the metal, then part of its energy is used to overcome the forces that hold the electron to the metal surface, and the excess energy appears as the kinetic energy of the ejected electron:

$$\begin{aligned} \textbf{kinetic energy of ejected electron} &= E - E_o \\ &= hu - hu_o \\ &= h(u - u_o) \end{aligned} \tag{6.3.2}$$

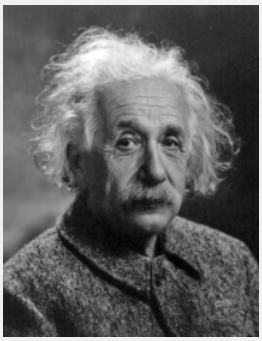
When a metal is struck by light with energy above the threshold energy E_o, the *number* of emitted electrons is proportional to the *intensity* of the light beam, which corresponds to the *number* of photons per square centimeter, but the *kinetic energy* of the emitted electrons is proportional to the *frequency* of the light. Thus Einstein showed that the energy of the emitted electrons depended on the frequency of the light, contrary to the prediction of classical physics. Moreover, the idea that light could behave not only as a wave but as a particle in the form of photons suggested that matter and energy might not be such unrelated phenomena after all.



Figure **6.3.4**: A Beam of Red Light Emitted by a Helium Neon laser reads a bar code. Originally Helium neon lasers, which emit red light at a wavelength of 632.8 nm, were used to read bar codes. Today, smaller, inexpensive diode lasers are used.

Albert Einstein (1879–1955)

In 1900, Einstein was working in the Swiss patent office in Bern. He was born in Germany and throughout his childhood his parents and teachers had worried that he might be developmentally disabled. The patent office job was a low-level civil service position that was not very demanding, but it did allow Einstein to spend a great deal of time reading and thinking about physics.



In 1905, his "miracle year" he published four papers that revolutionized physics. One was on the special theory of relativity, a second on the equivalence of mass and energy, a third on Brownian motion, and the fourth on the photoelectric effect, for which he received the Nobel Prize in 1921, the theory of relativity and energy-matter equivalence being still controversial at the time

Planck's and Einstein's postulate that energy is quantized is in many ways similar to Dalton's description of atoms. Both theories are based on the existence of simple building blocks, atoms in one case and quanta of energy in the other. The work of Planck and Einstein thus suggested a connection between the quantized nature of energy and the properties of individual atoms.

Example 6.3.1

A ruby laser, a device that produces light in a narrow range of wavelengths emits red light at a wavelength of 694.3 nm (Figure **6.3.4**). What is the energy in joules of a single photon?

Given: wavelength

Asked for: energy of single photon.

Strategy:

A. Use Equation 6.3.1 and the relationship between wavelength and frequency to calculate the energy in joules.

Solution:

The energy of a single photon is given by

$$E = h\nu = \frac{hc}{\lambda}. \tag{6.3.3}$$

Exercise 6.3.1

An x-ray generator, such as those used in hospitals, emits radiation with a wavelength of 1.544 Å.

a. What is the energy in joules of a single photon?
b. How many times more energetic is a single x-ray photon of this wavelength than a photon emitted by a ruby laser?

Answer a

$1.287 \times 10^{-15}\ J/photon$

Answer a

4497 times

External Videos and Examples

- Calculating Energy of a Mole of Photons_- Johnny Cantrell
- .Photons - ViaScience, an advanced explanation of the Planck radiation law and the photoelectric effect (below) as well as biological interactions with UV light.and the nature of light and quantum weirdness. Probably the first 6 minutes and the last 3 (from 12:00 on) as an introduction to wave particle duality.are useful to a beginning student.
- Quantum Chemistry - Ohio State
- Quantum Chemistry Quizzes - mhe education
- AP Chemistry Chapter 7 Review - Science Geek
- Quantum Theory of the Atom Practice Quiz - Northrup

Summary

The fundamental building blocks of energy are quanta and of matter are atoms. The properties of **blackbody radiation**, the radiation emitted by hot objects, could not be explained with classical physics. Max Planck postulated that energy was quantized and could be emitted or absorbed only in integral multiples of a small unit of energy, known as a **quantum**. The energy of a quantum is proportional to the frequency of the radiation; the proportionality constant h is a fundamental constant (Planck's constant). Albert Einstein used Planck's concept of the quantization of energy to explain the **photoelectric effect**, the ejection of electrons from certain metals when exposed to light. Einstein postulated the existence of what today we call **photons**, particles of light with a particular energy, $E = hu$. Both energy and matter have fundamental building blocks: quanta and atoms, respectively.

Contributors and Attributions

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6.4: The Nature of Light (Exercises)

Conceptual Questions

1.1 The Propagation of Light

1. Under what conditions can light be modeled like a ray? Like a wave?

2. Why is the index of refraction always greater than or equal to 1?

3. Does the fact that the light flash from lightning reaches you before its sound prove that the speed of light is extremely large or simply that it is greater than the speed of sound? Discuss how you could use this effect to get an estimate of the speed of light.

4. Speculate as to what physical process might be responsible for light traveling more slowly in a medium than in a vacuum.

1.2 The Law of Reflection

5. Using the law of reflection, explain how powder takes the shine off of a person's nose. What is the name of the optical effect?

1.3 Refraction

6. Diffusion by reflection from a rough surface is described in this chapter. Light can also be diffused by refraction. Describe how this occurs in a specific situation, such as light interacting with crushed ice.

7. Will light change direction toward or away from the perpendicular when it goes from air to water? Water to glass? Glass to air?

8. Explain why an object in water always appears to be at a depth shallower than it actually is?

9. Explain why a person's legs appear very short when wading in a pool. Justify your explanation with a ray diagram showing the path of rays from the feet to the eye of an observer who is out of the water.

10. Explain why an oar that is partially submerged in water appears bent.

1.4 Total Internal Reflection

11. A ring with a colorless gemstone is dropped into water. The gemstone becomes invisible when submerged. Can it be a diamond? Explain.

12. The most common type of mirage is an illusion that light from faraway objects is reflected by a pool of water that is not really there. Mirages are generally observed in deserts, when there is a hot layer of air near the ground. Given that the refractive index of air is lower for air at higher temperatures, explain how mirages can be formed.

13. How can you use total internal reflection to estimate the index of refraction of a medium?

1.5 Dispersion

14. Is it possible that total internal reflection plays a role in rainbows? Explain in terms of indices of refraction and angles, perhaps referring to that shown below. Some of us have seen the formation of a double rainbow; is it physically possible to observe a triple rainbow? A photograph of a double rainbow.



15. A high-quality diamond may be quite clear and colorless, transmitting all visible wavelengths with little absorption. Explain how it can sparkle with flashes of brilliant color when illuminated by white light.

1.6 Huygens's Principle

16. How do wave effects depend on the size of the object with which the wave interacts? For example, why does sound bend around the corner of a building while light does not?

17. Does Huygens's principle apply to all types of waves?

18. If diffraction is observed for some phenomenon, it is evidence that the phenomenon is a wave. Does the reverse hold true? That is, if diffraction is not observed, does that mean the phenomenon is not a wave?

1.7 Polarization

19. Can a sound wave in air be polarized? Explain.

20. No light passes through two perfect polarizing filters with perpendicular axes. However, if a third polarizing filter is placed between the original two, some light can pass. Why is this? Under what circumstances does most of the light pass?

21. Explain what happens to the energy carried by light that it is dimmed by passing it through two crossed polarizing filters.

22. When particles scattering light are much smaller than its wavelength, the amount of scattering is proportional to $\frac{1}{\lambda}$. Does this mean there is more scattering for small λ than large λ? How does this relate to the fact that the sky is blue?

23. Using the information given in the preceding question, explain why sunsets are red.

24. When light is reflected at Brewster's angle from a smooth surface, it is 100 polarized parallel to the surface. Part of the light will be refracted into the surface. Describe how you would do an experiment to determine the polarization of the refracted light. What direction would you expect the polarization to have and would you expect it to be 100?

25. If you lie on a beach looking at the water with your head tipped slightly sideways, your polarized sunglasses do not work very well. Why not?

Problems

1.1 The Propagation of Light

26. What is the speed of light in water? In glycerine?

27. What is the speed of light in air? In crown glass?

28. Calculate the index of refraction for a medium in which the speed of light is $2.012 \times 10^8 m/s$, and identify the most likely substance based on Table 6.2.1.1.

29. In what substance in Table 6.2.1.1 is the speed of light $2.290 \times 10^8 m/s$?

30. There was a major collision of an asteroid with the Moon in medieval times. It was described by monks at Canterbury Cathedral in England as a red glow on and around the Moon. How long after the asteroid hit the Moon, which is $3.84 \times 10^5 km$ away, would the light first arrive on Earth?

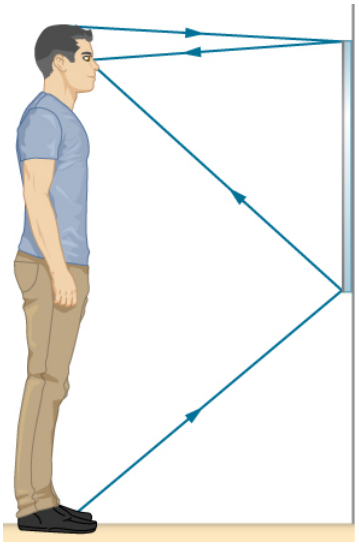
31. Components of some computers communicate with each other through optical fibers having an index of refraction $n = 1.55$. What time in nanoseconds is required for a signal to travel 0.200 m through such a fiber?

32. Compare the time it takes for light to travel 1000 m on the surface of Earth and in outer space.

33. How far does light travel underwater during a time interval of $1.50 \times 10^{-6} s$?

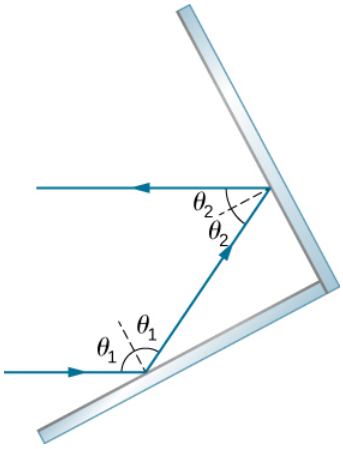
1.2 The Law of Reflection

34. Suppose a man stands in front of a mirror as shown below. His eyes are 1.65 m above the floor and the top of his head is 0.13 m higher. Find the height above the floor of the top and bottom of the smallest mirror in which he can see both the top of his head and his feet. How is this distance related to the man's height?



The figure is a drawing of a man standing in front of a mirror and looking at his image. The mirror is about half as tall as the man, with the top of the mirror above his eyes but below the top of his head. The light rays from his feet reach the bottom of the mirror and reflect to his eyes. The rays from the top of his head reach the top of the mirror and reflect to his eyes.

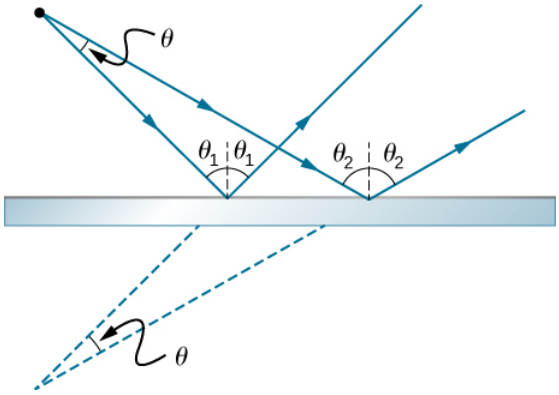
35. Show that when light reflects from two mirrors that meet each other at a right angle, the outgoing ray is parallel to the incoming ray, as illustrated below.



Two mirrors meet each other at a right angle. An incoming ray of light hits one mirror at an angle of theta one to the normal, is reflected at the same angle of theta one on the other side of the normal, then hits the other mirror at an angle of theta two to the normal and reflects at the same angle of theta two on the other side of the normal, such that the outgoing ray is parallel to the incoming ray.

36. On the Moon's surface, lunar astronauts placed a corner reflector, off which a laser beam is periodically reflected. The distance to the Moon is calculated from the round-trip time. What percent correction is needed to account for the delay in time due to the slowing of light in Earth's atmosphere? Assume the distance to the Moon is precisely $3.84 \times 10^8 m$ and Earth's atmosphere (which varies in density with altitude) is equivalent to a layer 30.0 km thick with a constant index of refraction $n = 1.000293$.

37. A flat mirror is neither converging nor diverging. To prove this, consider two rays originating from the same point and diverging at an angle θ (see below). Show that after striking a plane mirror, the angle between their directions remains θ.



Light rays diverging from a point at an angle theta are incident on a mirror at two different places and their reflected rays diverge. One ray hits at an angle theta one from the normal, and reflects at the same angle theta one on the other side of the normal. The other ray hits at a larger angle theta two from the normal, and reflects at the same angle theta two on the other side of the normal. When the reflected rays are extended backwards from their points of reflection, they meet at a point behind the mirror, at the same angle theta with which they left the source.

1.3 Refraction

Unless otherwise specified, for problems 1 through 10, the indices of refraction of glass and water should be taken to be 1.50 and 1.333, respectively.

38. A light beam in air has an angle of incidence of $35°$ at the surface of a glass plate. What are the angles of reflection and refraction?

39. A light beam in air is incident on the surface of a pond, making an angle of $20°$20° with respect to the surface. What are the angles of reflection and refraction?

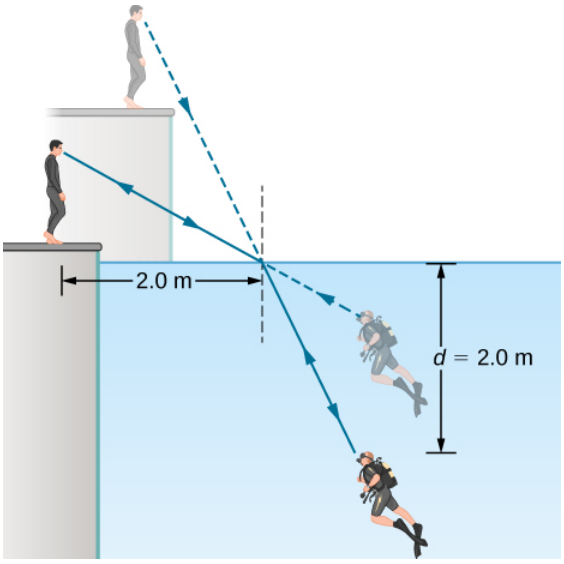
40. When a light ray crosses from water into glass, it emerges at an angle of $30°$ with respect to the normal of the interface. What is its angle of incidence?

41. A pencil flashlight submerged in water sends a light beam toward the surface at an angle of incidence of $30°$. What is the angle of refraction in air?

42. Light rays from the Sun make a $30°$ angle to the vertical when seen from below the surface of a body of water. At what angle above the horizon is the Sun?

43. The path of a light beam in air goes from an angle of incidence of $35°$ to an angle of refraction of $22°$ when it enters a rectangular block of plastic. What is the index of refraction of the plastic?

44. A scuba diver training in a pool looks at his instructor as shown below. What angle does the ray from the instructor's face make with the perpendicular to the water at the point where the ray enters? The angle between the ray in the water and the perpendicular to the water is $25.0°$.



A scuba diver and his trainer look at each other. They see each other at the locations given by straight line extrapolations of the rays reaching their eyes. To the trainer, the scuba diver appears less deep than he actually is, and to the diver, the trainer appears higher than he actually is. To the trainer, the scuba diver's feet appear to be at a depth of two point zero meters. The incident ray from the trainer strikes the water surface at a horizontal distance of two point zero meters from the trainer. The diver's head is a vertical distance of d equal to two point zero meters below the surface of the water.

45. (a) Using information in the preceding problem, find the height of the instructor's head above the water, noting that you will first have to calculate the angle of incidence.

(b) Find the apparent depth of the diver's head below water as seen by the instructor.

1.4 Total Internal Reflection

46. Verify that the critical angle for light going from water to air is $48.6°$, as discussed at the end of Example 1.4, regarding the critical angle for light traveling in a polystyrene (a type of plastic) pipe surrounded by air.

47. (a) At the end of Example 1.4, it was stated that the critical angle for light going from diamond to air is $24.4°$. Verify this.

(b) What is the critical angle for light going from zircon to air?

48. An optical fiber uses flint glass clad with crown glass. What is the critical angle?

49. At what minimum angle will you get total internal reflection of light traveling in water and reflected from ice?

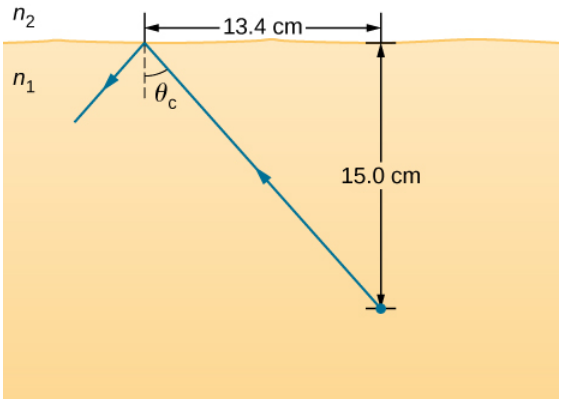
50. Suppose you are using total internal reflection to make an efficient corner reflector. If there is air outside and the incident angle is $45.0°$, what must be the minimum index of refraction of the material from which the reflector is made?

51. You can determine the index of refraction of a substance by determining its critical angle.

(a) What is the index of refraction of a substance that has a critical angle of $68.4°$ when submerged in water? What is the substance, based on Table 6.2.1.1?

(b) What would the critical angle be for this substance in air?

52. A ray of light, emitted beneath the surface of an unknown liquid with air above it, undergoes total internal reflection as shown below. What is the index of refraction for the liquid and its likely identification?

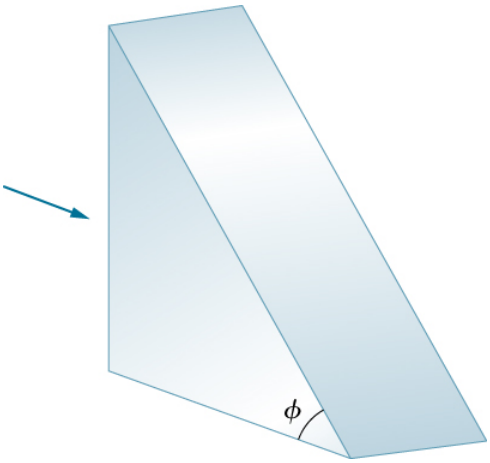


A light ray travels from an object placed in a medium n 1 at 15.0 centimeters below the horizontal interface with medium n 2. This ray gets totally internally reflected with theta c as critical angle. The horizontal distance between the object and the point of incidence is 13.4 centimeters.

53. Light rays fall normally on the vertical surface of the glass prism ($n = 1.50$ shown below.

(a) What is the largest value for ϕ such that the ray is totally reflected at the slanted face?

(b) Repeat the calculation of part (a) if the prism is immersed in water.



A right angle triangular prism has a horizontal base and a vertical side. The hypotenuse of the triangle makes an angle of phi with the horizontal base. A horizontal light rays is incident normally on the vertical surface of the prism.

1.5 Dispersion

54. (a) What is the ratio of the speed of red light to violet light in diamond, based on Table 6.2.4.1?

(b) What is this ratio in polystyrene?

(c) Which is more dispersive?

55. A beam of white light goes from air into water at an incident angle of $75.0°$. At what angles are the red (660 nm) and violet (410 nm) parts of the light refracted?

56. By how much do the critical angles for red (660 nm) and violet (410 nm) light differ in a diamond surrounded by air?

57. (a) A narrow beam of light containing yellow (580 nm) and green (550 nm) wavelengths goes from polystyrene to air, striking the surface at a $30.0°$ incident angle. What is the angle between the colors when they emerge?

(b) How far would they have to travel to be separated by 1.00 mm?

58. A parallel beam of light containing orange (610 nm) and violet (410 nm) wavelengths goes from fused quartz to water, striking the surface between them at a $60.0°$ incident angle. What is the angle between the two colors in water?

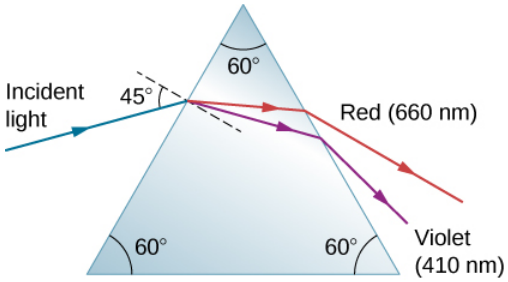
59. A ray of 610-nm light goes from air into fused quartz at an incident angle of $55.0°$. At what incident angle must 470 nm light enter flint glass to have the same angle of refraction?

60. A narrow beam of light containing red (660 nm) and blue (470 nm) wavelengths travels from air through a 1.00-cm-thick flat piece of crown glass and back to air again. The beam strikes at a $30.0°$ incident angle.

(a) At what angles do the two colors emerge?

(b) By what distance are the red and blue separated when they emerge?

61. A narrow beam of white light enters a prism made of crown glass at a $45.0°$ incident angle, as shown below. At what angles, θ_R and θ_V, do the red (660 nm) and violet (410 nm) components of the light emerge from the prism?



A blue incident light ray at an angle of incidence equal to 45 degrees to the normal falls on an equilateral triangular prism whose corners are all at angles equal to 60 degrees. At the first surface, the ray refracts and splits into red and violet rays. These rays hit the second surface and emerge from the prism. The red light with 660 nanometers bends less than the violet light with 410 nanometers.

1.7 Polarization

62. What angle is needed between the direction of polarized light and the axis of a polarizing filter to cut its intensity in half?

63. The angle between the axes of two polarizing filters is $45.0°$. By how much does the second filter reduce the intensity of the light coming through the first?

64. Two polarizing sheets P_1 and P_2 are placed together with their transmission axes oriented at an angle θ to each other. What is θ when only 25 of the maximum transmitted light intensity passes through them?

65. Suppose that in the preceding problem the light incident on P_1 is unpolarized. At the determined value of θ, what fraction of the incident light passes through the combination?

66. If you have completely polarized light of intensity $150 W/m^2$, what will its intensity be after passing through a polarizing filter with its axis at an $89.0°$ angle to the light's polarization direction?

67. What angle would the axis of a polarizing filter need to make with the direction of polarized light of intensity $1.00 kW/m^2$ to reduce the intensity to $10.0 W/m^2$?

68. At the end of Example 1.7, it was stated that the intensity of polarized light is reduced to 90.0 of its original value by passing through a polarizing filter with its axis at an angle of $18.4°$ to the direction of polarization. Verify this statement.

69. Show that if you have three polarizing filters, with the second at an angle of $45.0°$ to the first and the third at an angle of $90.0°$ to the first, the intensity of light passed by the first will be reduced to 25.0 of its value. (This is in contrast to having only the first and third, which reduces the intensity to zero, so that placing the second between them increases the intensity of the transmitted light.)

70. Three polarizing sheets are placed together such that the transmission axis of the second sheet is oriented at $25.0°$ to the axis of the first, whereas the transmission axis of the third sheet is oriented at $40.0°$ (in the same sense) to the axis of the first. What fraction of the intensity of an incident unpolarized beam is transmitted by the combination?

71. In order to rotate the polarization axis of a beam of linearly polarized light by $90.0°$, a student places sheets P_1 and P_2 with their transmission axes at $45.0°$ and $90.0°$, respectively, to the beam's axis of polarization.

(a) What fraction of the incident light passes through P_1 and

(b) through the combination?

(c) Repeat your calculations for part (b) for transmission-axis angles of $30.0°$ and $90.0°$, respectively.

72. It is found that when light traveling in water falls on a plastic block, Brewster's angle is $50.0°$. What is the refractive index of the plastic?

73. At what angle will light reflected from diamond be completely polarized?

74. What is Brewster's angle for light traveling in water that is reflected from crown glass?

75. A scuba diver sees light reflected from the water's surface. At what angle will this light be completely polarized?

Additional Problems

76. From his measurements, Roemer estimated that it took 22 min for light to travel a distance equal to the diameter of Earth's orbit around the Sun.

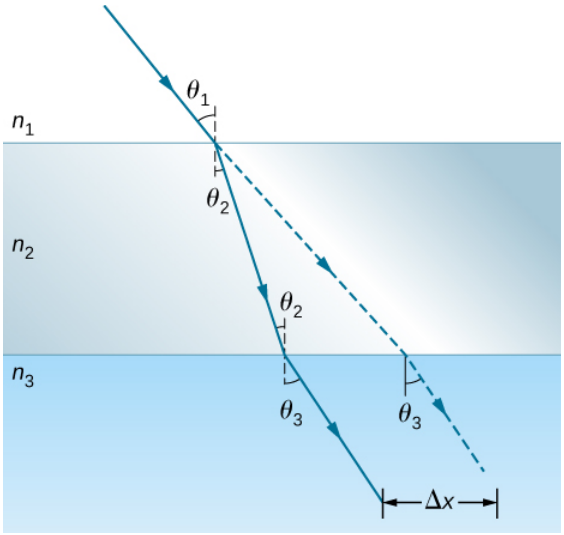
(a) Use this estimate along with the known diameter of Earth's orbit to obtain a rough value of the speed of light.

(b) Light actually takes 16.5 min to travel this distance. Use this time to calculate the speed of light.

77. Cornu performed Fizeau's measurement of the speed of light using a wheel of diameter 4.00 cm that contained 180 teeth. The distance from the wheel to the mirror was 22.9 km. Assuming he measured the speed of light accurately, what was the angular velocity of the wheel?

78. Suppose you have an unknown clear substance immersed in water, and you wish to identify it by finding its index of refraction. You arrange to have a beam of light enter it at an angle of $45.0°$, and you observe the angle of refraction to be $40.3°$. What is the index of refraction of the substance and its likely identity?

79. Shown below is a ray of light going from air through crown glass into water, such as going into a fish tank. Calculate the amount the ray is displaced by the glass (Δx), given that the incident angle is $40.0°$. and the glass is 1.00 cm thick.



The figure illustrates refraction occurring when light travels from medium n_1 to n_3 through an intermediate medium n_2. The incident ray makes an angle θ_1 with a perpendicular drawn at the point of incidence at the interface between n_1 and n_2. The light ray entering n_2 bends towards the perpendicular line making an angle θ_2 with it on the n_2 side. The ray arrives at the interface between n_2 and n_3 at an angle of θ_2 to a perpendicular drawn at the point of incidence at this interface, and the transmitted ray bends away from the perpendicular, making an angle of theta three to the perpendicular on the n_3 side. A straight line extrapolation of the original

incident ray is shown as a dotted line. This line is parallel to the refracted ray in the third medium, n_3, and is shifted a distance delta x from the refracted ray. The extrapolated ray is at the same angle theta three to the perpendicular in medium n_3 as the refracted ray.

80. Considering the previous problem, show that θ_3 is the same as it would be if the second medium were not present.

81. At what angle is light inside crown glass completely polarized when reflected from water, as in a fish tank?

82. Light reflected at $55.6°$ from a window is completely polarized. What is the window's index of refraction and the likely substance of which it is made?

83. (a) Light reflected at $62.5°$ from a gemstone in a ring is completely polarized. Can the gem be a diamond?

(b) At what angle would the light be completely polarized if the gem was in water?

84. If θ_b is Brewster's angle for light reflected from the top of an interface between two substances, and θ_b' is Brewster's angle for light reflected from below, prove that $\theta_b + \theta_b' = 90.0°$.

85. Unreasonable results Suppose light travels from water to another substance, with an angle of incidence of $10.0°$ and an angle of refraction of $14.9°$.

(a) What is the index of refraction of the other substance?

(b) What is unreasonable about this result?

(c) Which assumptions are unreasonable or inconsistent?

86. Unreasonable results Light traveling from water to a gemstone strikes the surface at an angle of $80.0°$ and has an angle of refraction of $15.2°$.

(a) What is the speed of light in the gemstone?

(b) What is unreasonable about this result?

(c) Which assumptions are unreasonable or inconsistent?

87. If a polarizing filter reduces the intensity of polarized light to 50.0 of its original value, by how much are the electric and magnetic fields reduced?

88. Suppose you put on two pairs of polarizing sunglasses with their axes at an angle of $15.0°$. How much longer will it take the light to deposit a given amount of energy in your eye compared with a single pair of sunglasses? Assume the lenses are clear except for their polarizing characteristics.

89. (a) On a day when the intensity of sunlight is $1.00 kW/m^2$, a circular lens 0.200 m in diameter focuses light onto water in a black beaker. Two polarizing sheets of plastic are placed in front of the lens with their axes at an angle of $20.0°$. Assuming the sunlight is unpolarized and the polarizers are 100 efficient, what is the initial rate of heating of the water in $°C/s$, assuming it is 80.0 absorbed? The aluminum beaker has a mass of 30.0 grams and contains 250 grams of water.

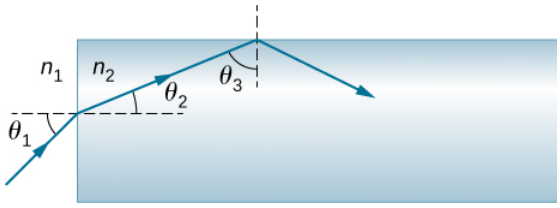
(b) Do the polarizing filters get hot? Explain.

Challenge Problems

90. Light shows staged with lasers use moving mirrors to swing beams and create colorful effects. Show that a light ray reflected from a mirror changes direction by 2θ when the mirror is rotated by an angle θ.

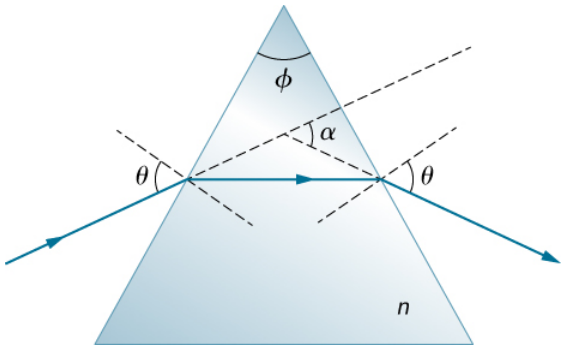
91. Consider sunlight entering Earth's atmosphere at sunrise and sunset—that is, at a $90.0°$. incident angle. Taking the boundary between nearly empty space and the atmosphere to be sudden, calculate the angle of refraction for sunlight. This lengthens the time the Sun appears to be above the horizon, both at sunrise and sunset. Now construct a problem in which you determine the angle of refraction for different models of the atmosphere, such as various layers of varying density. Your instructor may wish to guide you on the level of complexity to consider and on how the index of refraction varies with air density.

92. A light ray entering an optical fiber surrounded by air is first refracted and then reflected as shown below. Show that if the fiber is made from crown glass, any incident ray will be totally internally reflected.



The figure shows light traveling from n_1 and incident onto the left face of a rectangular block of material n_2. The ray is incident at an angle of incidence θ_1, measured relative to the normal to the surface where the ray enters. The angle of refraction is θ_2, again, relative to the normal to the surface. The refracted ray falls onto the upper face of the block and gets totally internally reflected with θ_3 as the angle of incidence.

93. A light ray falls on the left face of a prism (see below) at the angle of incidence θ for which the emerging beam has an angle of refraction θ at the right face. Show that the index of refraction n of the glass prism is given by $n = \frac{sin\frac{1}{2}(\alpha+\phi)}{sin\frac{1}{2}\phi}$ where ϕ is the vertex angle of the prism and α is the angle through which the beam has been deviated. If $\alpha = 37.0°$ and the base angles of the prism are each $50.0°$, what is **n**?



A light ray falls on the left face of a triangular prism whose upper vertex has an angle of phi and whose index of refraction is n. The angle of incidence of the ray relative to the normal to the left face is theta. The ray refracts in the prism. The refracted ray is horizontal, parallel to the base of the prism. The refracted ray reaches the right face of the prism and refracts as it emerges out of the prism. The emerging ray makes an angle of theta with the normal to the right face.

94. If the apex angle ϕ in the previous problem is $20.0°$ and $n = 1.50$, what is the value of α?

95. The light incident on polarizing sheet P_1 is linearly polarized at an angle of $30.0°$ with respect to the transmission axis of P_1. Sheet P_2 is placed so that its axis is parallel to the polarization axis of the incident light, that is, also at $30.0°$ with respect to P_1.

(a) What fraction of the incident light passes through P_1?

(b) What fraction of the incident light is passed by the combination?

(c) By rotating P_2, a maximum in transmitted intensity is obtained. What is the ratio of this maximum intensity to the intensity of transmitted light when P_2 is at $30.0°$ with respect to P_1?

96. Prove that if **I** is the intensity of light transmitted by two polarizing filters with axes at an angle θ and I' is the intensity when the axes are at an angle $90.0°-\theta$, then $I + I' = I_0$, the original intensity. (Hint: Use the trigonometric identities $cos90.0°-\theta = sin\theta$ and $cos^2\theta + sin^2\theta = 1$.)

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6.4.1: The Nature of Light (Answers)

Check Your Understanding

1.1. 2.1% (to two significant figures)

1.2. 15.1°

1.3. air to water, because the condition that the second medium must have a smaller index of refraction is not satisfied

1.4. 9.3 cm

1.5. AA' becomes longer, $A'B'$ tilts further away from the surface, and the refracted ray tilts away from the normal.

1.6. also 90.0

1.7. There will be only refraction but no reflection.

Conceptual Questions

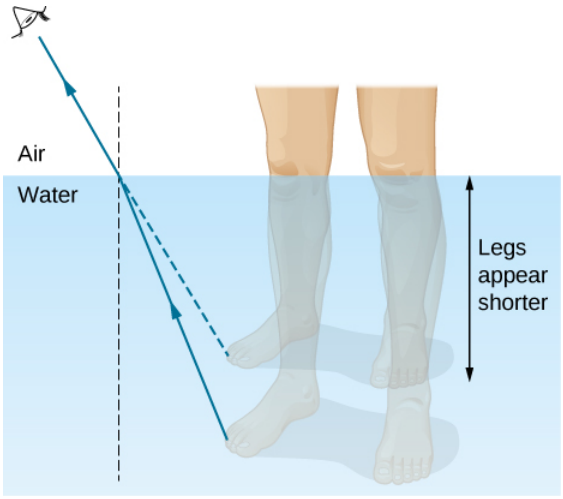
1. model as a ray when devices are large compared to wavelength, as a wave when devices are comparable or small compared to wavelength

3. This fact simply proves that the speed of light is greater than that of sound. If one knows the distance to the location of the lightning and the speed of sound, one could, in principle, determine the speed of light from the data. In practice, because the speed of light is so great, the data would have to be known to impractically high precision.

5. Powder consists of many small particles with randomly oriented surfaces. This leads to diffuse reflection, reducing shine.

7. "toward" when increasing **n** (air to water, water to glass); "away" when decreasing **n** (glass to air)

9. A ray from a leg emerges from water after refraction. The observer in air perceives an apparent location for the source, as if a ray traveled in a straight line. See the dashed ray below.



The figure is illustration of the formation of the image of a leg under water, as seen by a viewer in the air above the water. A ray is shown leaving the leg and refracting at the water air interface. The refracted ray bends away from the normal. Extrapolating the refracted ray back into the water, the extrapolated ray is above the actual ray so that the image of the leg is above the actual leg and the leg appears shorter.

11. The gemstone becomes invisible when its index of refraction is the same, or at least similar to, the water surrounding it. Because diamond has a particularly high index of refraction, it can still sparkle as a result of total internal reflection, not invisible.

13. One can measure the critical angle by looking for the onset of total internal reflection as the angle of incidence is varied. Equation 1.5 can then be applied to compute the index of refraction.

15. In addition to total internal reflection, rays that refract into and out of diamond crystals are subject to dispersion due to varying values of n across the spectrum, resulting in a sparkling display of colors.

17. yes

19. No. Sound waves are not transverse waves.

21. Energy is absorbed into the filters.

23. Sunsets are viewed with light traveling straight from the Sun toward us. When blue light is scattered out of this path, the remaining red light dominates the overall appearance of the setting Sun.

25. The axis of polarization for the sunglasses has been rotated $90°$.

Problems

27. $2.99705 \times 10^8 m/s; 1.97 \times 10^8 m/s$

29. ice at $0°C$

31. 1.03 ns

33. 337 m

35. proof

37. proof

39. reflection, $70°$; refraction, $45°$

41. $42°$

43. 1.53

45. a. 2.9 m;

b. 1.4 m

47. a. $24.42°$;

b. $31.33°$

49. $79.11°$

51. a. 1.43, fluorite;

b. $44.2°$

53. a. $48.2°$;

b. $27.3°$

55. $46.5°$ for red, $46.0°$ for violet

57. a. $0.04°$;

b. 1.3 m

59. $72.8°$

61. $53.5°$ for red, $55.2°$ for violet

63. 0.500

65. 0.125 or 1/8

67. $84.3°$

69. $0.250 I_0$

71. a. 0.500;

b. 0.250;

c. 0.187

73. $67.54°$

75. $53.1°$

Additional Problems

77. 114 radian/s

79. 3.72 mm

81. $41.2°$

83. a. 1.92. The gem is not a diamond (it is zircon).

b. $55.2°$

85. a. 0.898;

b. We cannot have $n < 1.00$, since this would imply a speed greater than **c**.

c. The refracted angle is too big relative to the angle of incidence.

87. $0.707B_1$

89. a. $1.69 \times 10^{-2} °C/s$;

b. yes

Challenge Problems

91. First part: $88.6°$. The remainder depends on the complexity of the solution the reader constructs.

93. proof; 1.33

95. a. 0.750;

b. 0.563;

c. 1.33

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CHAPTER OVERVIEW

7: Components of Optical Instruments for Molecular Spectroscopy in the UV and Visible

7.1: General Instrument Designs

7.2: Sources of Radiation

7.3: Wavelength Selectors

7.4: Sample Containers

7.5: Radiation Transducers

7.6: Signal Processors and Readouts

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7.1: General Instrument Designs

The two most important spectroscopic methods in analytical chemistry are based on the absorption of emission of light.

Instruments for absorption spectroscopy are of two types: single beam and double beam. A shown in Figure 1(a), in a single beam instrument light passes through the sample from the source to the photoelectric transducer (detector). Absorbance values are based on first measuring the transmittance of light through a sample containing an absorbing species, then switching the sample for a reference sample (a blank), and then measuring the transmittance through the reference sample. In a double beam instrument and as shown in Figure 1 (b), the light path alternates between passing through the sample with absorbing species and a reference sample.

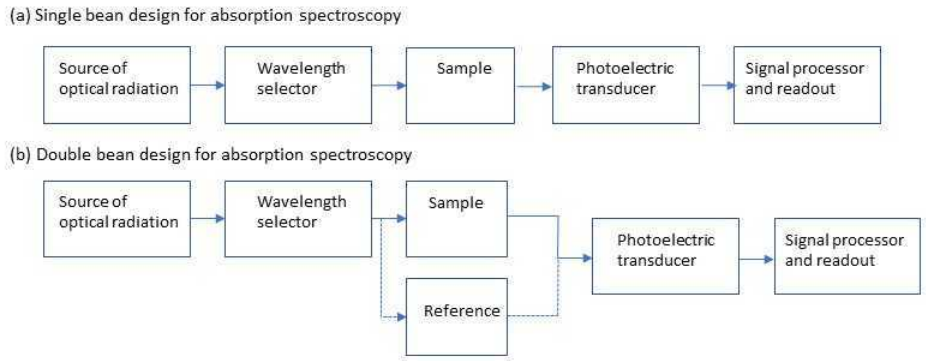


Figure 7.1.1:Block diagrams for (a) a single beam instrument and (b) a double beam instrument for absorption spectroscopy

Instruments for fluorescence spectroscopy are based on measurements of the intensity of emitted light following excitation of the sample with light of a shorter wavelength. As shown in Figure 2, the emitted light is collected at an angle perpendicular to the path of the excitation light.

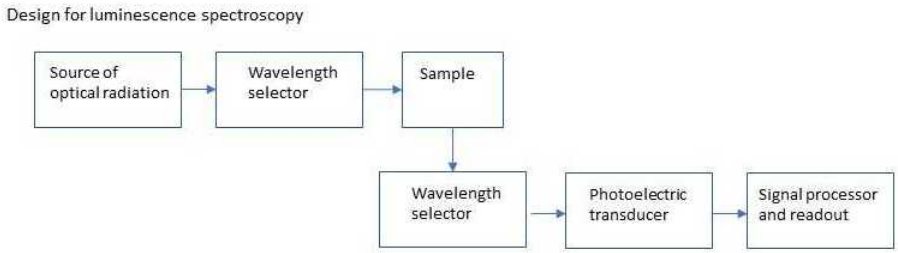


Figure 7.1.2:Block diagram for an instrument for fluorescence spectroscopy.

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7.2: Sources of Radiation

There are four major optical sources used in benchtop instruments for molecular spectroscopy in the UV and visible. These sources are (1) the tungsten or tungsten-halogen lam, (2) the deuterium lamp, (3) the xenon arc lamp. All of these sources are broadband sources that emit significant intensities of light over a wide range of wavelengths. At the end of this section light emitting diodes and lasers were will be described because of their use in optical spectroscopy and as examples of discrete wavelength sources.

The Tungsten Lamp

The tungsten lamp or tungsten halogen lamp is a blackbody emitter that produces useful radiation over the range from 320 nm to 2400 nm. A picture of a tungsten lamp is shown in Figure 7.2.1 and an example of the spectral output of this lamp is shown in Figure 7.2.2. The lamp consists of a tungsten filament in a evacuated glass or quartz envelope that contains a small amount of iodine vapor to increase the lifetime of the filament. The light from a tungsten lamp is randomly polarized and incoherent. This low cost optical source is the most common source for absorption spectroscopy in the visible region of the spectrum.



Figure **7.2.1**: Picture is a (dead) tungsten halogen from a Spec20 spectrophotomter.

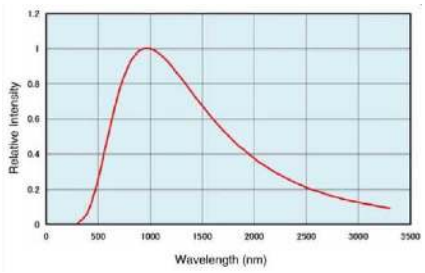


Figure **7.2.2**: The output spectrum from a tungsten halogen lamp.

The Deuterium Lamp

The deuterium lamp is a high pressure gas discharge lamp that produces useful radiation over the range from 160 nm to 380 nm. A picture of a deuterium lamp is shown in Figure 7.2.3 and the spectral output of this lamp is shown in Figures 7.2.4. The light from a deuterium lamp is randomly polarized and incoherent. This optical source considerably more costly and has a shorter lifetime that the tungsten lamp but is the most common source for absorption spectroscopy in the UV region of the spectrum.

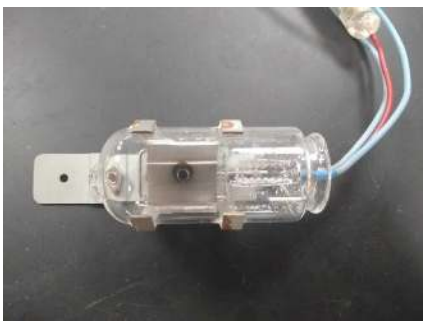


Figure 7.2.3: Pictured is a (dead) deuterium lamp from a Varian absorbance detector for liquid chromatography.

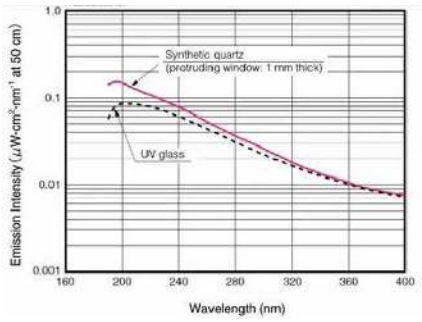


Figure 7.2.4: The output spectrum of a deuterium lamp with either a UV-glass or quartz envelope.

The Xenon Arc Lamp

The xenon arc lamp is a gas discharge lamp the produces useful radiation of the range from 190 nm to 1100 nm. The light from a xenon lamp is randomly polarized and incoherent. Pictured in Figure 7.2.5 is the xenon flicker lamp found in the Cary 50 and Cary 60 absorption spectrometers sold by the Agilent Corp, in Figure 7.2.6 is a high pressure, how power xenon arc lamp typically found in instruments for fluorescence spectroscopy and in Figure 7.2.7 is the output spectrum of a 150 W xenon lamp with the characteristic peak at 467 nm.. In general as the pressure of xenon inside the lamp the broad background increases in intensity and intensity the discrete atomic lines becomes less apparent.



Figure 7.2.5: Xenon flicker lamp.



Figure 7.2.6: High power xenon arc lamp.

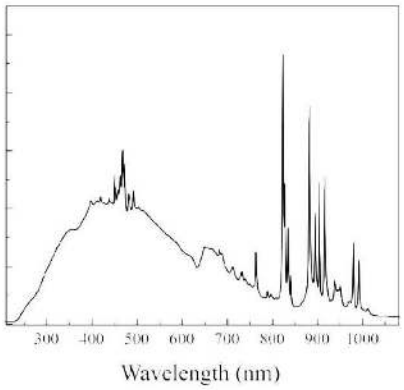


Figure 7.2.7: The output spectrum form a 150 W high pressure xenon arc lamp.

Lasers

Lasers have been dependable and commercially available light sources since the early 1970's. The word laser stands for light amplification by stimulated emission. As shown in Figure 7.2.8 a laser consists of a lasing medium contained within a resonant cavity from by a high reflector (100% reflector) and an output coupler from which the laser light leaks out. Energy must be pumped into the lasing medium which could come from a current, a discharge or a flashlamp.

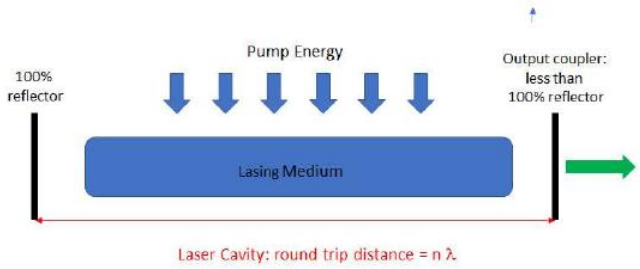


Figure 7.2.8: The basic design of a laser.

As shown in Figure 7.2.9 for the case of a Nd^{3+} laser, energy pumped into the system is absorbed and the energy is quickly transferred among the excited Nd^{3+} ions placing them in the upper lasing electronic state, $^4F_{3/2}$. The resonant cavity is set to have a round trip distance equal to an integral number of wavelengths of the light emitted corresponding to the energy difference between the upper and lower lasing states. Because of the resonant condition the emission process is stimulated and the emitted light is both very monochromatic and coherent. Often, but not for all lasers, the emitted light is linearly polarized.

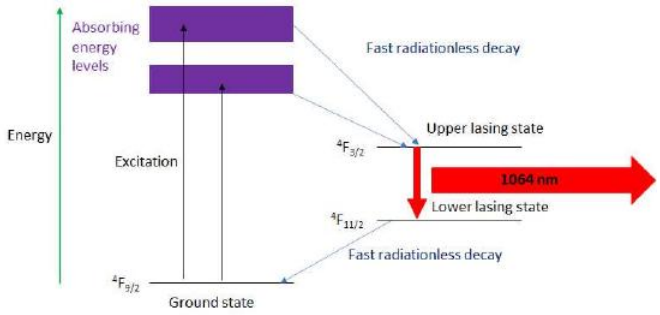


Figure 7.2.9: The energy level diagram for a Nd^{3+}: Yag laser.

A list of lasers commonly found in chemistry labs and some of their characteristics is shown in the table below:

Laser	Wavelength(s)	Pulsed of CW	common use
Helium Neon (HeNe)	632 nm	CW	Alignment, thermal lensing
Krypton ion	406.7 nm, 413.1 nm, 415.4 nm, 468.0 nm, 476.2 nm, 482.5 nm, 520.8 nm, 530.9 nm, 568.2 nm, 647.1 nm, and 676.4 nm	CW	Emission spectroscopy
Argon Ion	351.1 nm, 363.8 nm, 454.6 nm, 457.9 nm, 465.8 nm, 476.5 nm, 488.0 nm, 496.5 nm, 501.7 nm, 514.5 nm, 528.7 nm, and 1092.3 nm	CW	Emission spectroscopy
Neodynium: YAG	1064 nm (also 532 nm, 355 nm, 266 nm)	Pulsed (10 ns)	Emission spectroscopy, photoionization, MALDI
Nitrogen	337 nm	Pulsed (4 ns)	MALDI
Titanium: Saphire	700 - 900 nm	Pulsed (100 fs)	Dynamic

Lasers are discrete wavelength sources and while they might emit light at a few wavelengths, they are generally not tunable light sources. When combined with a dye laser or an optical parametric oscillator the light can be tuned over a narrow range of wavelengths, especially relative to a broadband, blackbody, light source. Despite the lack of tunablity, lasers are widely used as sources for emission spectroscopy and because of the very high peak power pulsed lasers are used for photoionization and matrix assisted laser desorption (MALDI) sources in mass spectrometry.

Light Emitting Diodes (LEDs)

The use of small, low-cost, and rugged light emitting diodes (LED's) has expanded greatly in the past few years, well beyond the red LEDs commonly seen on display panels. Now available with emission wavelengths ranging from the UV (365 nm), through the visible, to the near infrared (990nm) single LED's or banks of LEDs can be used as light sources for both emission and absorption spectroscopy.

As shown in Figure 7.2.10, light is emitted from a forward biased LED when the holes in the p-type semiconductor material and carriers in the adjacent n-type semiconductor recombine and release energy in an amount equal to the band gap. The typical bandwidth of the light emitted is on the order of 25 nm and light light is incoherent and randomly polarized.

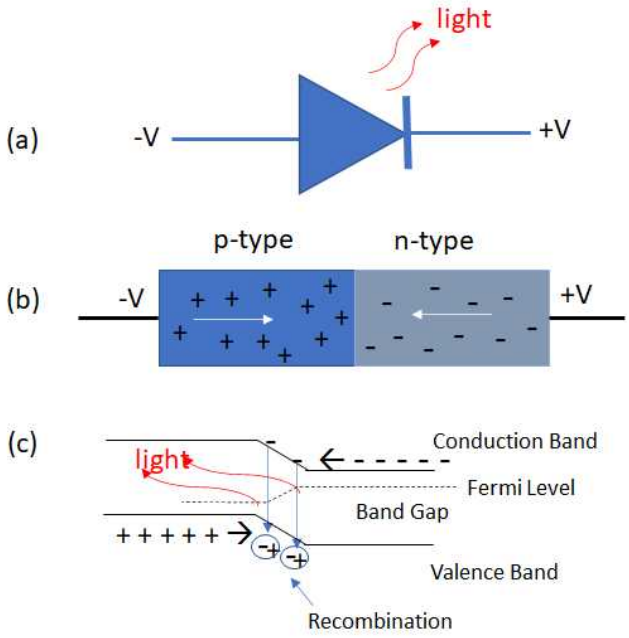


Figure **7.2.10**: A light emitting diode shown in (a) using the diode symbol, (b) with the carriers and holes in the *n*-type and *p*-type semiconductor materials shown combining, in (c) the band description for an LED.

LEDs of a wide variety of wavelengths find uses as sources for emission spectroscopy, especially in small portable instruments and fluorescence microscopes.

For absorption spectroscopy, especially in small portable instruments such as the Red Tide Spectrometer from Ocean Optics or the SpectroVis spectrometer from Vernier, a white light LED is required. In a white light LED the emission from a blue light emitting LED at 450 nm is used to excite a Ce^{3+} doped YAG phosphor producing yellow light over the range of 500 nm - 700 nm. The combination of blue excitation light and yellow phosphorescence, shown in Figure 7.2.11 produce "white" light spanning the range of 425 nm - 700 nm. Similar LED lights are available today as low energy, long-life, replacements for incandescent and fluorescent light bulbs in your home.

undefined

Figure Runaway argument for \eatSpaces?: A white light LED.

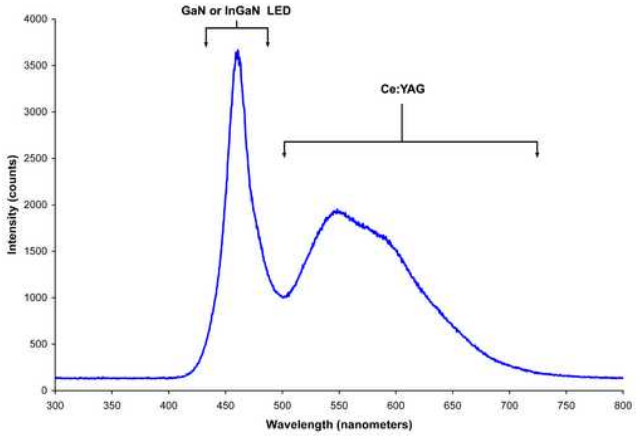


Figure Runaway argument for \eatSpaces?: The spectrum emanating from a white light LED.

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7.3: Wavelength Selectors

Wavelength Selectors

A wavelength selector is a instrument component that either selects and transmits a narrow band of wavelengths emanating from a broad band optical source of transmits one or more lines from a discrete wavelength source. Wavelength selectors come in two types; fixed wavelength or scanning. In either case the main quality characteristics of a wavelength selector are the effective bandwidth and the %transmittance. As shown in Figure 7.3.1, the effective bandwidth is the spread in the wavelengths transmitted around the central wavelength, specifically those wavelengths transmitted in intensities > 50% of the maximum transmittance.

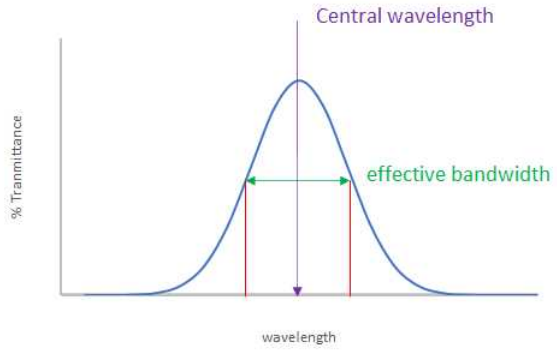


Figure**7.3.1**: An illustration of the meaning of the term "bandwidth" for a wavelength selector.

Monochromators

A monochromator is a scanning type of wavelength selector. Originally based on glass or quartz prisms, current monochromators are constructed with holographic diffraction gratings that are produced using the kinds of lithographic techniques used to created computer chips. These holographic diffraction gratings are superior in terms groover regularity and lower amounts of scattered light and to the ruled master and replica gratings of the past.

As shown in Figure 7.3.2, a monochromator consists of an entrance and exit slits, a diffraction grating mirrors to first expand the incoming light to fill the area of the grating and subsequently focus the diffracted light on the exit slit. A grating for the UV and visible regions of the spectrum will between 300 - 2000 grooves per mm (most commonly 1200 to 1400 grooves/mm) and be blazed at an angle where the transmittance is best for the wavelengths of interest. Gratings for the IR where the wavelengths are longer that in the UV and visible will have less grooves /mm.

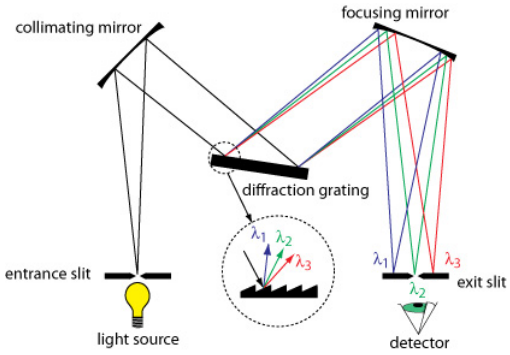


Figure**7.3.2**: An illustration of the meaning of the term "bandwidth" for a wavelength selector. Image from the image and video exchange hosted by community.asdlib.org

As shown in Figure 7.3.3, the light diffracted off each facet of the grating can be considered to be emanating from a point source. If we consider two beams of light incident at an angle α on adjacent facets spaced at a distance **d** and the results diffracted beams reflected at angle β, the difference in distance traveled can be shown to be $\mathbf{d}(\sin\alpha+\sin\beta)$. If this difference in distance traveled is

equal to an integral number of wavelengths, **nλ**, the beams will be in phase with one another and constructively interfere. If the distance is anything other than an integral number of wavelength the beams with destructively interfere with annihilation occurring when the beams are 180° out of phase. For a beam of white light incident at angle α, constructive interference will occur for different wavelengths different angles of reflection, β. Consequently the light is dispersed along the plane of the exit slit. In practice, a monochromator is scanned by the rotating the grating so that both angle α and angle β are changing and different wavelengths are passed through the exit slit.

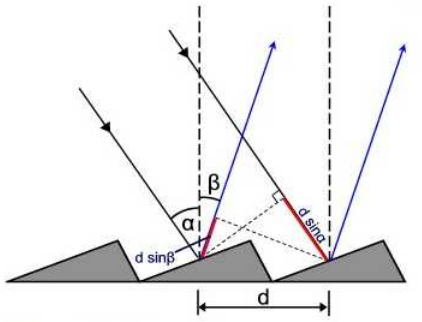


Figure**7.3.3**: Each facet of the grating behaves like a point source and for constructive interference the difference in path traveled needs to me an integral number of wavelenghts. Image from the physics.stackexchange.com

The grating described above is called an echellette grating and typically these are used in first order (**n**=1). Echelle gratings which have many less grooves per mm but are operated in much higher orders are used in instruments for atomic spectroscopy.

For grating based monochromators used at small angle *r* the linear dispersion of wavelengths is a constant meaning the that distance along the exit slit between where 300 nm light and 400 nm light strikes is the same distance as between 600 nm and 700 nm. The linear dispersion from prism based monochromators is not a constant.

The resolving power or resolution for a monochromator is the ability to separate images that have a slight difference in wavelength and the resolving power improves as the number of grooves of the grating are illuminated, hence expanding the light to fill the area of the grating is advantageous.

The effective bandwidth for a monochromator is the product of the reciprocal linear dispersion, D^{-1} and the slit with, W. D^{-1} has units of nm/mm and describes the spread of wavelengths in nm per mm of linear distance along the exit plane of the slit. For most benchtop instruments the slit width, W, is adjustable.

For a 0.5 m focal length monochromator with a 1200 groove/mm grating operated at small angle r and in first order the resolving power at 30 nm is about 60,000 and the D^{-1} is about 2 nm/mm. Both metrics would improve for a larger monochromator with longer focal length.

Interference Filters

Interference filters are fixed wavelength selectors based on the principle of constructive and destructive interference. Consider the two rays, 1 and 2, shown in Figure 7.3.4. When these two rays strike the first semi-reflective surface some of the light is reflected and some of the light enters the transparent dielectric. The light entering the dielectric is diffracted and travels to the second semi-reflective surface. At this second interface some of hte light is reflected and some of the light is transmitted. Focusing on the internal reflected beam or ray 1, if when this beam combines in phase with beam 2 at the point circled in purple then constructive interference will occur. This condition for constructive interference will be met when the path highlighted in yellow is a distance equal to an integral number of wavelengths of the light in the dielectric material, $n\lambda = 2d\eta/cos\theta$. Thus the central wavelength transmitted by an interference filter is dependent on the thickness and composition of the dielectric layer.

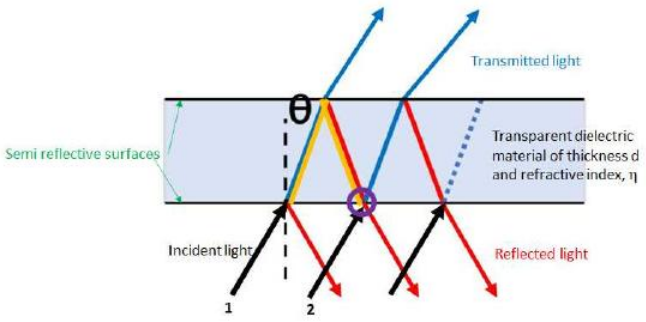


Figure**7.3.4**: A sketch of an interference filter where d is the thickness of the dielectric material, η is the refractive indez and θ. is the angle of incidence.

For light incident along the surface normal the angle θ will be zero and the equation simplifies to $n\lambda = 2d\eta$.

Depicted in Figure 7.3.5 are the transmission profiles of a series of interference filters. As evidenced by figure 7.3.5 the typical bandwidth is on the order of 10 nm or less with the smaller bandwidths, 1 nm, produced by multilayer filters.

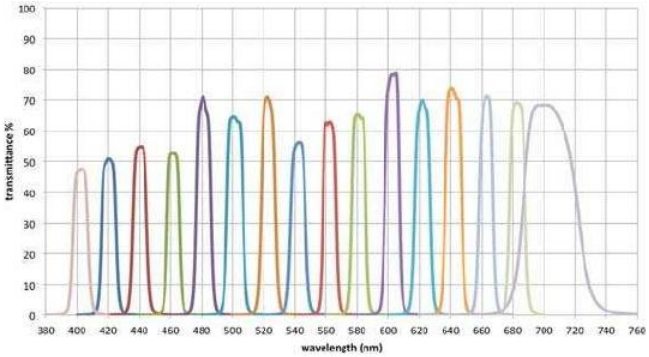


Figure**7.3.5**: The transmittance profiles of a series of interference filters covering the visible region of the spectrum. Image taken from Flückiger, Barbara & Pfluger, David & Trumpy, Giorgio & Aydin, Tunç & Smolic, Aljosa. (2018). Film Material-Scanner Interaction.

Shortpass, Longpass and Band Pass Filters

Other filters that find use in analytical experiments include shortpass, longpass and band pass filters. As shown in Figure 7.3.6 these types of filters absorb or reflect large portions of UV or visible regions of the spectrum.

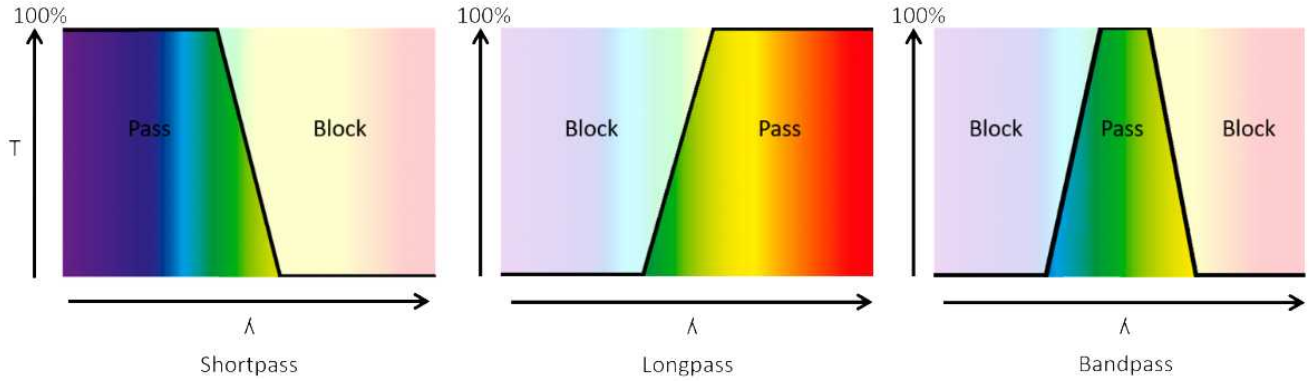


Figure**7.3.6**: A series of sketches illustrating the difference between a short pass, long pass and bandpass filter. Image taken from https://www.photometrics.com/learn/microscopy.

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7.4: Sample Containers

Sample Containers

Most experiments using absorption of emissions spectroscopy interrogate samples that are gases, liquids or solutions. The exception to these are solid samples that can be mounted in the spectrometer. Spectroscopy experiments with gases are generally accomplished with long-path cells that are either sealed or through which the gas flows. For liquids and solutions cuvettes are the most common sample containers.

The key characteristics for sample containers are:

1) the window material or cuvette material is transparent in the spectral region of the experiment

2) the window, cell or cuvette material does not reactive with the sample

3) the path length of the cell is matched to the experiment and instrument

4) the cell volume is matched to the sample.

Pictured in Figure 7.4.1 is a 10 cm pathlength demountable cell useful for absorption experiments with gases such as iodine vapor. It is often difficult to fit a longer pathlengh cell in the sample compartment of a benchtop UV- Vis spectrophotometer. If a longer pathlength is required, multipath cell with pathlenghts up to 100 m are commercially available.



Figure **7.4.1**: A 10 cm pathlength demountable cell for absorption experiments with gases. Demountable means the cell can be disassembled, say for cleaning or changing the windows, and then reassembeled.

Cuvettes for experiments for liquids and solutions can be purchased with pathlengths ranging from 0.1 cm to 10 cm and the pathlenghts are precise to +/- 0.05 mm. The volume of sample held can be between 1.4 and 35 milliliters (macro), between 0.7 ml and 1.4 ml (semi micro) and between .35 and 0.7 (micro). Cuvettes are constructed with two polished sides for absorption spectroscopy and with four polished sides for emission spectroscopy. Cuvettes can be purchased individualy or in matched sets of 2 or 4. Shown in Figure 7.4.2a is a quartz macro cuvette. for absorption spectroscopy and Figure 7.4.2b is a semi micro cuvette for emission spectroscopy.

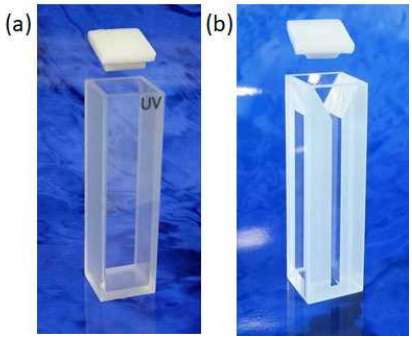


Figure **7.4.2**: (a) A 1.0 cm pathlength macro cuvette absorption experiments (b) A 1.0 cm pathlength semimicro cuvette for emission experiments.

The most common materials for windows and cuvettes for experiments in the UV and visible regions are shown in Table 7.4.1.

Material	Useful spectral range
Glass (BK-7)	340 - 2500 nm
Fused Silica (IR grade)	240 - 3500 nm
Fused Silica (UV grade)	190 - 2500 nm
Polystyrene (PS)	340 - 800 nm
Polymethylmethacrylate (PMMA)	280 - 900 nm
Saphire	250 - 5000 nm

Note: Brand-UV cuvettes are disposable plastic cuvettes with a short wavelength cutoff reported to be 230 nm

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7.5: Radiation Transducers

Radiation Transducers

Simply described, a radiation transducer converts an optical signal, light, into an electrical signal, current or voltage. Quality characteristics instrument makers consider when designing instruments for absorption or emission spectroscopy include:

1. The sensitivity
2. The signal to noise ratio
3. The temporal response response
4. The response of the transducer as a function wavelengths
5. The signal coming from the transducer in the absence of light (dark current)
6. The response of the transducer to different intensities of light (linear or not ?)

A common metric for sensitivity is D* or D-star. D* tells you a detector's sensitivity for a fixed active detector area (because not all detectors are the same size) and at a specific optical wavelength (because detectors respond differently according to the nature of the incident radiation). The formal definition of D* is the square root of the active area (A, in cm^2) divided by the noise equivalent power (NEP) where the NEP is intensity of light that would produce the same amount of signal as the inherent electrical noise coming from the transducer. A sensitive transducer would have a large D* and a low NEP.

The Vacuum Phototube

The vacuum phototube is a small, low cost, optical transducer that works is based on the photoelectric effect. As shown in Figure 7.5.1, a vacuum phototube consists of a large area photocathode with an anode contained in an evacuated quartz envelope. As shown in the circuit in Figure 7.5.2 the photocathode is biased -V and the anode -V by 50 to 90 V. Photons of sufficient energy strike the photocathode surface releasing photoelectrons that are repelled by the cathode and collected at the anode. The current produced is proportional to the intensity of the light. The wavelength response of the vacuum phototube depends on the material coating the photocathode.



Figure **7.5.1**: A photograph of a vacuum phototube.

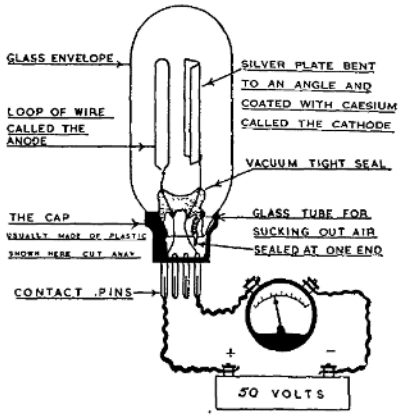


Figure 7.5.2: This old image from the 1954 Bulletin on Narcotics from United Nations Office on Drugs and Crime shows how a vacuum phototube is biased for the collection of photoelectron current.

Coatings fall into one of four categories: (1) highly sensitive, (2) red sensitive, (3) UV sensitive, and (4) flat response . The most sensitive coatings work well in the UV and visible regions are of the bialkali type composed of K, Cs or Sb. A Ga/As (128) coating offers a relatively flat wavelength response for wavelengths 200 - 900 nm. Red sensitive coatings are multi-alkali types such as Na/K/Cs/Sb or Ag/O/CS (S-11) while coatings such as Ga/In/As (S-12) extends the response to 1100 nm but with a loss of sensitivity.

The Photomultiplier Tube

The photomultipler is also based on the photoelectric effect and the same photoemissive surface coating described for the vacuum phototube are also found in photomultiplier tubes. As shown in Figure 7.5.3, the big difference between the phototube and the photomultiplier tube is the series of dynodes between the cathode and anode, all of which are contained in an evacuated quartz envelope. When a photon of sufficient energy strikes the photocathode surface the resulting photoelectron is accelerated towards the first dynode. When the electron strikes the dynode it produces 2 - 3 secondary electrons each of which is accelerated towards the second dynode where they each create 2 - 3 secondary electrons. Figure 7.5.4 shows the potential bias arrangemtns for the photocathod, dynaodes and anode that ensures photoelectrons produced at the cathode are directed towards the anode. For a photomultiplier tube with 9 dynodes, 10^6 to 10^7 electrons are produced and collected at the anode for each incident photon. The gain is the number of electrons produced per incident photon.

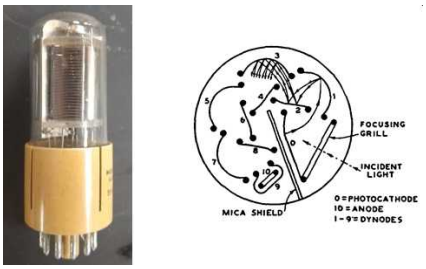


Figure 7.5.3: A photograph of a small, low cost 1p28 photomultipler tube manufactured by the RCA Corp and diagram of the photocathode and dynode structure contained within.

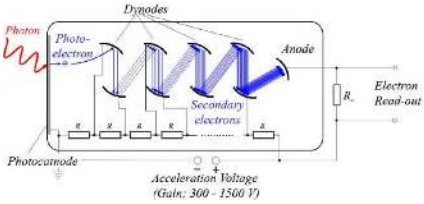


Figure 7.5.4: A sketch illustrating the gain in electrons following creation of the first photelectron in a photomultiplier tube. Image taken from the physicsopenlab.org

Photomultiplier tubes are among the most sensitive and fast optical transducers for UV and visible light. The sensitivity is limited by the dark-current emission the main source of which is thermal emission. Consequently the sensitivity can be improved if need by cooling. Photomultiplier tubes are limited to measuring low-power radiation because intense light can permanently damage the photocathode.

The Silicon Photodiode

The silicon photodiode is a small, rugged and low cost optical transducer that works well over the range of 200 - 1200 nm with a D* better than a vacuum phototube but four orders of magnitude smaller than a photomultiplier tube. As shown in Figure 7.5.5a

the diode is reverse biased so that the nominal dark current is small. As illustrated in Figure 7.5.5b, when a light of sufficient energy strikes the *p*-*n* junction a new charge pair is generated and the movement of the carrier and holes is measured as a current. The current produced is proportional to the number of photons striking the active region. In Figure 7.5.5c is a photograph of a single photodiode.

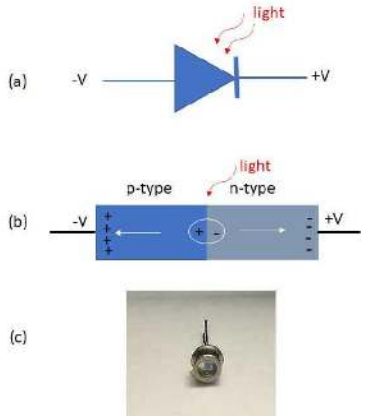


Figure **7.5.5**: (a) the voltage arrangement for reversing biasing a photodiode, (b) an illustration of the direction of movement of a new charge pair producing a photocurrent and (c) a photodiode.

Photodiode Arrays

A photodiode array (PDA) is a linear series of small individual silicon photodiodes each one reverse biased and part of a larger integrated circuit. Each individual diode is abut 2.5 mm by 0.025 mm in size and the arrays typically consist of 64 to 4096 diodes. An old PDA with 64 elements is shown in Figure 7.5.6. The most common PDAs have 1024 diodes in a package with a dimension of 2.5 cm. The integrated circuit contains storage capacitors for each photodiode and a circuit for sequentially reading the charge stored for each photodiode. When coupled with a wavelength dispersing device, such as a optical grating, a PDA allows for recording a nm resolution absorption spectrum in a single measurement without the need to scan the wavelength range (change the angle for the grating).

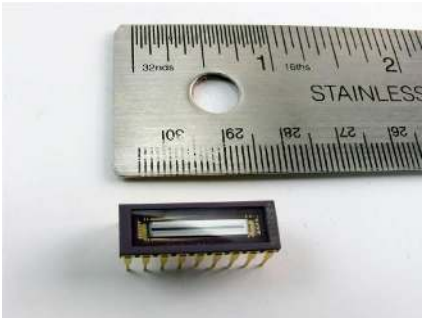


Figure **7.5.6**: A 64 element photdiode array manufactured by the EG&G Corp.

Charge - Transfer Devices

Charger- transfer devices, charge injection devices (CIDs) or charge coupled devices (CCDs) are two dimensional arrays of small solid state phototransducers. The D* for these devices is far superior to silicon diode based PDAs and rivals that of photomultiplier tubes . In concept these devices are similar to the camera element in your smartphone although the CMOS technology in your cell phone is superior in terms of low power consumption.

As shown in Figure 7.5.6, when light strikes the active region of a charge transfer device an new charge pair is generated. In a CID the positive holes are collected in a potential well in *n*-type material created by a negatively biased electrode while in a CCD the electrons are collected in a potential well *p*-type material created by a positively biased electrode. Each charge collection point is a pixel and today a 1 cm x 1 cm CCD will have at least 1 million pixels.

For both CIDs and CCDs, an integrated circuit allows for a sequential read of the charge stored in each pixel by moving the charge packet within the main silicon substrate by adjusting the potential of the electrodes for each pixel. CID's can be read in a non destructive mode so that the information stored can be read while integration continues. CCD's are more sensitive to low light levels but the readout is destructive of the information.

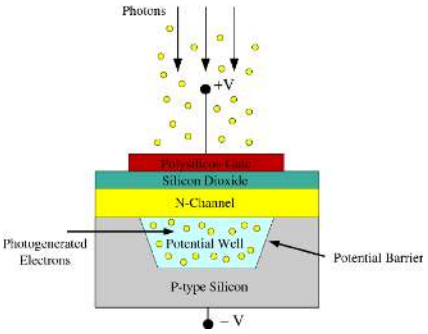


Figure 7.5.7: An sketch of a single pixel of a CCD shown the creation of photoelectrons and their capture in a potential well within the main p-type silicon substrate by the potential on the gate electrode.

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7.6: Signal Processors and Readouts

While older instruments such as the Milton Roy Spectronic 20 pictured on the left in Figure 7.6.1 was a purely analog instrument all laboratory grade instruments are digital. In an analog instrument such as the Spectronic 20 the current from the vacuum phototube is amplified and used to drive the front panel meter. Today if you purchased the equivalent instrument pictured on the right in figure 7.6.1, the Spectronic 20 Model D, you will note that the meter has been replaced by a digital display.



Figure 7.6.1: Two Spectronic 20 or Spec 20 instruments for absorption spectroscopy in the visible region of the spectrum. teh instrumetn on the left is an older analog spectrophotometer and the right on the right is a newer spectrophotometer

In both cases, the small current produced by the vacuum phototube is amplified close to the source using a circuit like that shown in Figure 7.6.2, in this case for a photodiode. The difference is in an analog instrument the voltage coming from the current to voltage converter is further amplifier and used to drive the meter in an analog instrument or directly converted to a number by an analog to digital converter.

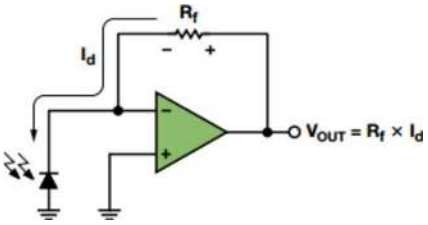


Figure 7.6.2: An illustration of a the circuit used for current to voltage conversion and signal amplification.

Analog to Digital (A/D) converters are common electronic components in all modern instrumentation. 16 to 20 bit A/Ds are readily available at low cost and introduce a digitization error of 1 part in 2^{16} (better than 1 part in 65,000) for a 16-bit A/D.

For low light level experiments using photomultiplier tubes, such as in emission spectroscopy, a similar signal train of amplification and digitization is possible. An alternative signal processing method is photon counting. As shown in Figure 7.6.3, the signal from the photomultiplier tube is amplified and converted to a voltage pulse by a circuit similar to that shown in Figure 7.6.2. In photon counting, the voltage pulse is shaped into a standard form, compared to a voltage threshold in the discriminator and if larger than the set voltage value in the discriminator counted. The number of counts for a specified period of time will be proportional to the intensity of light striking the detector.

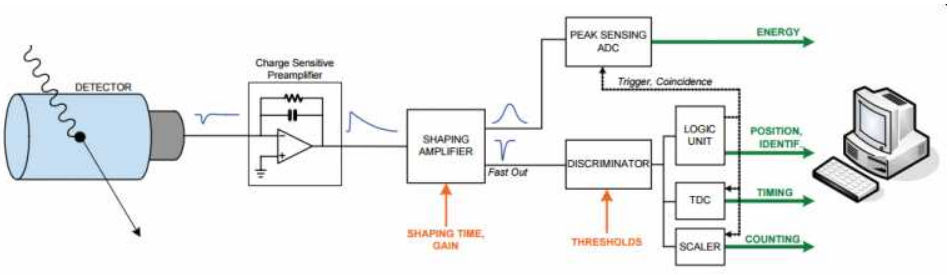


Figure 7.6.3: An illustration of a data processing scheme for photon counting. Not discussed in this section is the peak height analysis involving the peak sensing ADC that is useful in X-ray fluorescence spectroscopy for determining the energy of the photon. This image was copied from the physicsopenlab.org

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CHAPTER OVERVIEW

8: An Introduction to Ultraviolet-Visible Absorption Spectrometry

8.1: Measurement of Transmittance and Absorbance

8.2: Beer's Law

8.3: The Effects of Instumental Noise on Spectrophotometric Analyses

8.4: Instrumentation

8.4.1: Single Beam Instruments for Absorption Spectroscopy - The Spec 20 and the Cary 50

8.4.2: Instruments for Absorption Spectroscopy with Multichannel Detectors

8.4.3: Double Beam Instruments for Absorption Spectroscopy

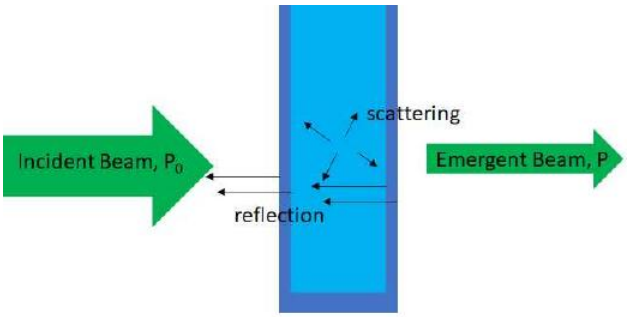
8.4.4: UV - Vis (and Near IR) Instruments with Double Dispersion

Thumbnail: Light dispersion of a mercury-vapor lamp with a flint glass prism/ Image used with permission (CC BY-SA 3.0; D-Kuru).

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8.1: Measurement of Transmittance and Absorbance

In absorption spectroscopy a beam of light is incident on a sample and most of the light passes through the sample. The intensity of the light emerging from the sample is attenuated by reflections losses at each of four interfaces where the refractive index of the media change (air/container wall, container wall/solution, solution/container wall and container wall/air), possibly attenuated by particles scattering the light in the sample, and, most importantly, by the absorption of the light by the sample. In order for the sample to absorb the light two conditions need be met; (1) there must be a mechanism by which a component of the sample can interact with the electric or magnetic field components of the light and (2) the wavelength or energy of the light must be resonant (match) the difference in energy between two of the quantized energy levels of the absorbing component.



$$T = \frac{P_{solution}}{P_{solvent}} = \frac{P}{P_0}$$

$$A = -log\left(\frac{P_{solution}}{P_{solvent}}\right) \approx -log\left(\frac{P}{P_0}\right) = -log\left(\frac{P_0}{P}\right)$$

Figure **8.1.1**: An illustration of the passage of light through a sample (light blue) and the container (dark blue) also showing reflections losses at the four interfaces where the refractive index changes and light loses due to scattering.

The transmittance, T, is simply the fraction of light intensity passing through the sample and the absorbance, A, is the - $\log_{10}$ of the intensity of the light passing though the solvent relative to the intensity of light passing though the sample. For a non-absorbing solvent $A = -\log(P_0/P)$

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8.2: Beer's Law

Absorbance and Concentration: Beer's Law

When monochromatic electromagnetic radiation passes through an infinitesimally thin layer of sample of thickness *dx*, it experiences a decrease in its power of *dP* (Figure 8.2.8).

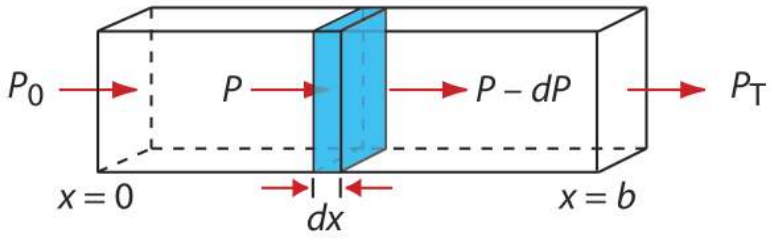


Figure 8.2.1. Factors used to derive the Beer's law.

This fractional decrease in power is proportional to the sample's thickness and to the analyte's concentration, *C*; thus

$$-\frac{dP}{P} = \alpha C dx \tag{8.2.1}$$

where *P* is the power incident on the thin layer of sample and α is a proportionality constant. Integrating the left side of equation 8.2.1 over the sample's full thickness

$$-\int_{P=P_0}^{P=P_T} \frac{dP}{P} = \alpha C \int_{x=0}^{x=b} dx \qquad \text{Equation 8.2.2a}$$

gives

$$\ln\left(\frac{P_0}{P_T}\right) = \alpha b C \qquad \text{Equation 8.2.2b}$$

converting from ln to log, and substituting into equation 8.2.2b, gives

$$A = abC \tag{8.2.2}$$

where *a* is the analyte's ***absorptivity*** with units of cm^{-1} $conc^{-1}$. If we express the concentration using molarity, then we replace *a* with the ***molar absorptivity***, ε, which has units of cm^{-1} M^{-1}.

$$A = \varepsilon b C \tag{8.2.3}$$

The absorptivity and the molar absorptivity are proportional to the probability that the analyte absorbs a photon of a given energy. As a result, values for both *a* and ε depend on the wavelength of the absorbed photon.

Example 8.2.2

A 5.00×10^{-4} M solution of analyte is placed in a sample cell that has a pathlength of 1.00 cm. At a wavelength of 490 nm, the solution's absorbance is 0.338. What is the analyte's molar absorptivity at this wavelength?

Solution

Solving equation 8.2.3 for ϵ and making appropriate substitutions gives

$$\varepsilon = \frac{A}{bC} = \frac{0.338}{(1.00\ \text{cm})\left(5.00 \times 10^{-4}\ \text{M}\right)} = 676\ \text{cm}^{-1}\ \text{M}^{-1}$$

Exercise 8.2.2

A solution of the analyte from Example 8.2.2 has an absorbance of 0.228 in a 1.00-cm sample cell. What is the analyte's concentration?

Answer

Making appropriate substitutions into Beer's law

$$A = 0.228 = \varepsilon bC = \left(676\ \text{M}^{-1}\ \text{cm}^{-1}\right)(1\ \text{cm})C$$

and solving for C gives a concentration of 3.37×10^{-4} M.

Equation 8.2.3 and equation 8.2.4, which establish the linear relationship between absorbance and concentration, are known as Beer's law. Calibration curves based on Beer's law are common in quantitative analyses.

As is often the case, the formulation of a law is more complicated than its name suggests. This is the case, for example, with Beer's law, which also is known as the Beer-Lambert law or the Beer-Lambert-Bouguer law. Pierre Bouguer, in 1729, and Johann Lambert, in 1760, noted that the transmittance of light decreases exponentially with an increase in the sample's thickness.

$$T \propto e^{-b}$$

Later, in 1852, August Beer noted that the transmittance of light decreases exponentially as the concentration of the absorbing species increases.

$$T \propto e^{-C}$$

Together, and when written in terms of absorbance instead of transmittance, these two relationships make up what we know as Beer's law.

Beer's Law and Multicomponent Samples

We can extend Beer's law to a sample that contains several absorbing components. If there are no interactions between the components, then the individual absorbances, A_i, are additive. For a two-component mixture of analyte's X and Y, the total absorbance, A_{tot}, is

$$A_{tot} = A_X + A_Y = \varepsilon_X b C_X + \varepsilon_Y b C_Y$$

Generalizing, the absorbance for a mixture of n components, A_{mix}, is

$$A_{mix} = \sum_{i=1}^{n} A_i = \sum_{i=1}^{n} \varepsilon_i b C_i \tag{8.2.4}$$

Limitations to Beer's Law

Beer's law suggests that a plot of absorbance vs. concentration—we will call this a Beer's law plot—is a straight line with a y-intercept of zero and a slope of ab or εb. In some cases a Beer's law plot deviates from this ideal behavior (see Figure 8.2.9), and such deviations from linearity are divided into three categories: fundamental, chemical, and instrumental.

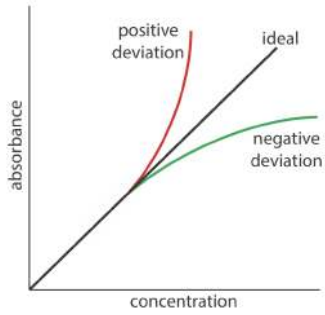


Figure 8.2.2. Plots of absorbance vs. concentration showing positive and negative deviations from the ideal Beer's law relationship, which is a straight line.

Fundamental Limitations to Beer's Law

Beer's law is a limiting law that is valid only for low concentrations of analyte. There are two contributions to this fundamental limitation to Beer's law. At higher concentrations the individual particles of analyte no longer are independent of each other. The

resulting interaction between particles of analyte may change the analyte's absorptivity. A second contribution is that an analyte's absorptivity depends on the solution's refractive index. Because a solution's refractive index varies with the analyte's concentration, values of a and ε may change. For sufficiently low concentrations of analyte, the refractive index essentially is constant and a Beer's law plot is linear.

Chemical Limitations to Beer's Law

A chemical deviation from Beer's law may occur if the analyte is involved in an equilibrium reaction. Consider, for example, the weak acid, HA. To construct a Beer's law plot we prepare a series of standard solutions—each of which contains a known total concentration of HA—and then measure each solution's absorbance at the same wavelength. Because HA is a weak acid, it is in equilibrium with its conjugate weak base, A^-.

> In the equations that follow, the conjugate weak base A^- is written as A as it is easy to mistake the symbol for anionic charge as a minus sign.

$$\mathrm{HA}(aq) + \mathrm{H_2O}(l) \rightleftharpoons \mathrm{H_3O^+}(aq) + \mathrm{A^-}(aq)$$

If both HA and A^- absorb at the selected wavelength, then Beer's law is

$$A = \varepsilon_{\mathrm{HA}} b C_{\mathrm{HA}} + \varepsilon_{\mathrm{A}} b C_{\mathrm{A}} \tag{8.2.5}$$

Because the weak acid's total concentration, C_{total}, is

$$C_{\text{total}} = C_{\mathrm{HA}} + C_{\mathrm{A}}$$

we can write the concentrations of HA and A^- as

$$C_{\mathrm{HA}} = \alpha_{\mathrm{HA}} C_{\text{total}} \tag{8.2.6}$$

$$C_{\mathrm{A}} = (1 - \alpha_{\mathrm{HA}}) C_{\text{total}} \tag{8.2.7}$$

where α_{HA} is the fraction of weak acid present as HA. Substituting equation 8.2.6 and equation 8.2.7 into equation 8.2.5 and rearranging, gives

$$A = \left(\varepsilon_{\mathrm{HA}} \alpha_{\mathrm{HA}} + \varepsilon_{\mathrm{A}} - \varepsilon_{\mathrm{A}} \alpha_{\mathrm{A}}\right) b C_{\text{total}} \tag{8.2.8}$$

To obtain a linear Beer's law plot, we must satisfy one of two conditions. If $\varepsilon_{\mathrm{HA}}$ and ε_{A} have the same value at the selected wavelength, then equation 8.2.8 simplifies to

$$A = \varepsilon_{\mathrm{A}} b C_{\text{total}} = \varepsilon_{\mathrm{HA}} b C_{\text{total}}$$

Alternatively, if α_{HA} has the same value for all standard solutions, then each term within the parentheses of equation 8.2.8 is constant—which we replace with k—and a linear calibration curve is obtained at any wavelength.

$$A = k b C_{\text{total}}$$

Because HA is a weak acid, the value of α_{HA} varies with pH. To hold α_{HA} constant we buffer each standard solution to the same pH. Depending on the relative values of α_{HA} and α_{A}, the calibration curve has a positive or a negative deviation from Beer's law if we do not buffer the standards to the same pH.

Instrumental Limitations to Beer's Law

There are two principal instrumental limitations to Beer's law. The first limitation is that Beer's law assumes that radiation reaching the sample is of a single wavelength—that is, it assumes a purely monochromatic source of radiation. As shown in Figure 10.1.10, even the best wavelength selector passes radiation with a small, but finite effective bandwidth. Polychromatic radiation always gives a negative deviation from Beer's law, but the effect is smaller if the value of ε essentially is constant over the wavelength range passed by the wavelength selector. For this reason, as shown in Figure 8.2.10, it is better to make absorbance measurements at the top of a broad absorption peak. In addition, the deviation from Beer's law is less serious if the source's effective bandwidth is less than one-tenth of the absorbing species' natural bandwidth [(a) Strong, F. C., III *Anal. Chem.* **1984**, *56*, 16A–34A; Gilbert, D.

D. *J. Chem. Educ.* **1991**, *68*, A278–A281]. When measurements must be made on a slope, linearity is improved by using a narrower effective bandwidth.

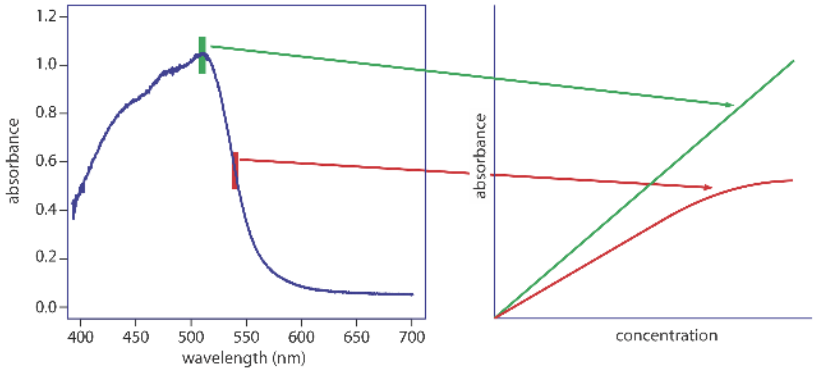


Figure 8.2.3. Effect of wavelength selection on the linearity of a Beer's law plot. Another reason for measuring absorbance at the top of an absorbance peak is that it provides for a more sensitive analysis. Note that the green Beer's law plot has a steeper slope—and, therefore, a greater sensitivity—than the red Beer's law plot. A Beer's law plot, of course, is equivalent to a calibration curve.

Stray radiation is the second contribution to instrumental deviations from Beer's law. Stray radiation arises from imperfections in the wavelength selector that allow light to enter the instrument and to reach the detector without passing through the sample. As shown in Figure 8.2.4, stray radiation adds an additional contribution, P_{stray}, to the radiant power that reaches the detector

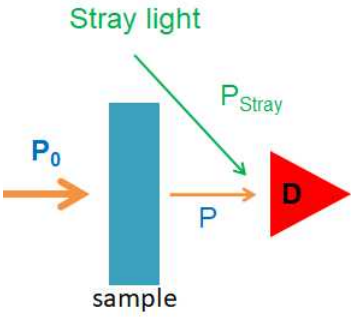


Figure**8.2.4**: A simple illustration showing stray light, whether not of the correct wavelength or not passing though the sample striking the detector.

Thus

$$A = -\log \frac{P_T + P_{stray}}{P_0 + P_{stray}}$$

For a small concentration of analyte, P_{stray} is significantly smaller than P_0 and P_T, and the absorbance is unaffected by the stray radiation. For higher concentrations of analyte, less light passes through the sample and P_T and P_{stray} become similar in magnitude. This results is an absorbance that is smaller than expected, and a negative deviation from Beer's law.

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8.3: The Effects of Instumental Noise on Spectrophotometric Analyses

The Relationship Between the Uncertainty in c and the Uncertainty in T

The accuracy and precision of quantitative spectrochemical analysis with Beer's Law are often limited by the uncertainties or electrical noise associated with the instrument and its components (source, detector, amplifiers, etc.).

Starting with Beer's Law we can write the following expressions for c

$$c = -\frac{1}{\varepsilon b}\log T = -\frac{0.434}{\varepsilon b}\ln T$$

taking the partial derivative of c with respect to T and holding b and [\epsilon} constant yeilds

$$\frac{\partial c}{\partial T} = -\frac{0.434}{\varepsilon b T}$$

converting from standard deviations to variances yeilds

$$\sigma_c^2 = \left(\frac{\partial c}{\partial T}\right)^2 \sigma_T^2 = \left(\frac{-0.434}{\delta D T}\right)^2 \sigma_T^2$$

and dividing the square of the first expression for c results in the equation below involving the variance in c and the variance in T

$$\left(\frac{\sigma_c}{c}\right)^2 = \left(\frac{\sigma_T}{T \ln T}\right)^2$$

Taking the square root of both sides yields the equation below for the relative uncertainty in c

$$\frac{\sigma_c}{c} = \frac{\sigma_T}{T \ln T} = \frac{0.434 \sigma_T}{T \log T}$$

For a limited number of measurements one replaces the standard deviations of the population, σ, with the standard deviations of the sample, s, and obtain

$$\frac{s_c}{c} = \frac{0.434 s_T}{T \log T}$$

This last equation relates the relative standard deviation (relative uncertainty) of c to the absolute standard deviation of T, s_T. Experimentally s_T can be evaluated by repeatedly measuring T for the same sample. The last equation also shows that the relative uncertainty in c varies nonlinearly with the magnitude of T.

It should be noted that the last equation for the relative uncertainty in c understates the complexity of this problem as the uncertainty in T, s_T is is many circumstances dependent on the magnitude of T.

Sources of Instrumental Noise

In a detailed theoretical and experimental study Rothman, Crouch, and Ingle (Anal. Chem 47, 1226 (1975)) described several sources of instrumental uncertainties and showed their net effect on the precision of transmittance measurements. As shown in the table below, these uncertainties fall into three categories depending on how affected they are by the magnitude of T

Category	Characterized by s_T =	Typical sources for UV/Vis experiments	Likely to be important in
1	k_1	limited readout resolution Dark current and amplifier noise	low cost instruments with meters or few digits in display regions where source intensity and detector sensitivity is low
2	$k_2\sqrt{T^2+T}$	Photon detector shot noise	High quality instruments with photomultiplier tubes
3	k_3T	cell positioning uncertainty Source flick noise	High quality instruments low cost instruments

Category 1: $s_T = k_1$

Low cost instruments which in the past might have had analog meter movements or possibly available today 4 digits (reporting %T with values ranging from 0.0 to 100.0) are instruments where the readout error is on the order of 0.2 %T. The magnitude of this

error is larger that should be expected from detector and amplifier noise except under the condition where the source intensity or detector sensitivity is low (little light being measured by the detector).

Substitution of s_T = k1 yields

$$\frac{s_c}{c} = \frac{0.434k_1}{T \log T}$$

and evaluating this equation over the range of T = 0.95 to 0.001 (corresponding A values 0.022 to 3.00) and using a reasonable value for k_1 such as 0.003 or 0.3 %T is shown as the red curve in Figure 8.3.1. Looking carefully at the red curve the relative uncertainty is at best on the order of 1.0% and gets considerably larger as the T increases (A decreases) or T decreases (A increases). These ends of the "U" shaped curve correspond to situations where not much light is absorbed and P is very close to P_0 or when most of the light is absorbed and P is very small).

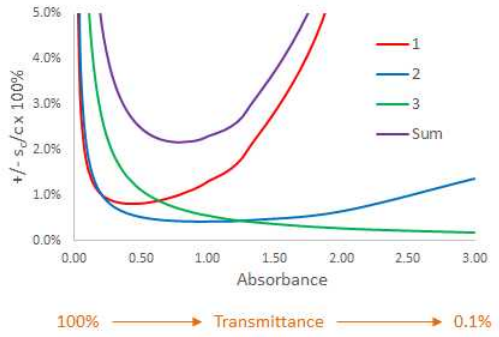


Figure **8.3.1**: The relative uncertainty in c for different values of T or A in accord with the three Categories of instrument noise affect measurements of T.

Category 2: $s_T = k_2\sqrt{T^2 + T}$

The type of uncertainty described by Category 2 is associated with the highest quality instruments containing the most sensitive light detector s. Photon shot noise is observed when ever an electron moves across a junction such as the movement of an electron from the photocathode to the first dynode in a photomultiplier tube. In these cases the current results from a series of discrete events the number of which per unit time is distributed in a random way about a mean value. The magnitude of the current fluctuations is proportional to the square root of the current. Therefore for Category 2 $s_T = k_2\sqrt{T^2 + T}$ and

$$\frac{s_c}{c} = \frac{0.434 \times k_2}{\log T}\sqrt{\frac{1}{T} + 1}$$

An evaluation of s_c/c for Category 2, as done for Category 1, is shown in the blue curve in figure 8.3.1 where k_2 = 0.003. The contribution of the uncertainty associated with Category 2 is largest when the T is large and the A is low. Under these conditions the photocurrent in the detector is large as a great deal of light is striking the detector.

Category 3: $s_T = k_3$

The most common phenomena associated with the Category 3 uncertainty in the measurement of T is flicker noise. Flicker noise, or 1/f noise, is often associated with the slow drift in source intensity, P_0. Instrument makers address flicker noise by either regulating the power to the optical source, designing a double beam spectrophotometer where the light path of a single source alternates between passing through the sample or a reference, or both.

Another widely encountered noise associated with Category 3 is the failure to position the sample and or reference cells is exactly the same place each time. Even the highest quality cuvettes will have some scattering imperfections in the cuvette material and other scattering sources can be dust particles on the cuvette surface and finger smudges. One method to reduce the problems associated with cuvette positioning is to leave the cuvettes in place and carefully change the samples with rinsing using disposable pipets. In their paper mentioned earlier, Rothman, Crouch and Ingle argue that this is the largest source of error for experiments with high quality spectrophotometer.

The effect of the Category 3 uncertainty where $s_T = k_3T$ is revealed by green curve in Figure 8.2.1.

$$\frac{s_c}{c} = \frac{0.434 \times k_3}{\log T}$$

In this case k_3 = 0.0013 and like Category 2 the contribution of Category 3 uncertainty to the relative uncertainty in c is largest when T is large and A is small.

The purple curve in Figure 8.2.1 is the sum of the contribution of Categories 1, 2 and 3. The shape of the curve is very much like the Category 1 curve shown in red. The relative uncertainty is largest when T is large (small A) or when T is small (large A). The curve has a minimum near T = 0.2 (or % T = 20%) or A = 0.7 and most analytical chemists will try to get there calibration curve standards to have A values between 0.1 and 1.

The Effect of Slit Width on Absorbance Measurements

As presented in Section 7.3, the bandwidth of a wavelength selector is the product of the reciprocal linear dispersion and the slit width, D^{-1} W. It can be readily observed that increasing the bandwidth of the light passing through the wavelength selector result in a loss of detail, fine structure, in the absorbance spectrum provided the sample has revealable fine structure. Most molecules and complex ions in solution do not have observable fine structure but gas phase analytes, some rigid aromatic molecules and species containing lanthanides or actinides do.

For systems with observable fine structure the bandwdith set through the slit width, W, must be matched to the system. However, a decrease in slit width is accompanied by a second order decrease in the radiant power emanating from the wavelength selector and available to interrogate the sample. At very narrow slit widths spectral detail can be lost due to a decrease in signal to noise, especially at wavelengths when the source intensity or the detector response in low.

In general it is a good practice to decrease the slit width and decrease the bandwidth no more that necessary. In most benchtop instruments the slit width is an adjustable parameter and the slits can be sequentially narrowed until the absorbance peaks heights become constant.

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8.4: Instrumentation

In this section, designs of commonly encountered commercial instruments for absorption spectroscopy in the UV and Visible regions of the spectrum will be presented.

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8.4.1: Single Beam Instruments for Absorption Spectroscopy - The Spec 20 and the Cary 50

All single beam instruments require either separate calibration at a single wavelength or separate recording of base line spectrum for a reference solution that usually only contains the solvent. Single beam instruments are more susceptible to 1//f noice (drift)

The Spectonic 20

The **Spectronic 20** is a brand single-beam spectophotometer, designed to operate in the visible region of the spectrum across a wavelength range of 340 nm to 950 nm, with a spectral bandpass of 20 nm. It is designed for quantitative absorption measurement at single wavelengths. Because it measures the transmittance or absorption of visible light through a solution, it is sometimes referred to as a colorimeter.The name of the instrument is a trademark of the manufacturer.

Developed by Bausch and Lomb and launched in 1953, the Spectronic 20 was the first low-cost spectrophotometer (less than $1,000). It rapidly became an industry standard due to its low cost, durability and ease of use, and has been referred to as an "iconic lab spectrophotometer". Approximately 600,000 units were sold over its nearly 60 year production run. It has been the most widely used spectrophotometer worldwide. Production was discontinued in 2011 when it was replaced by the Spectronic 200,but the Spectronic 20 is still in common use. It is sometimes referred to as the "Spec 20" and I have kept 10 working units at Providence (8 with analog meters and 2 with digital readouts).

Pictured in Figure 8.4.1.1 is a vintage Spec 20. The only difference between this vintage instrument and the last version of the Spec 20 is the replacement of the analog meter readout by a digital meter readout.

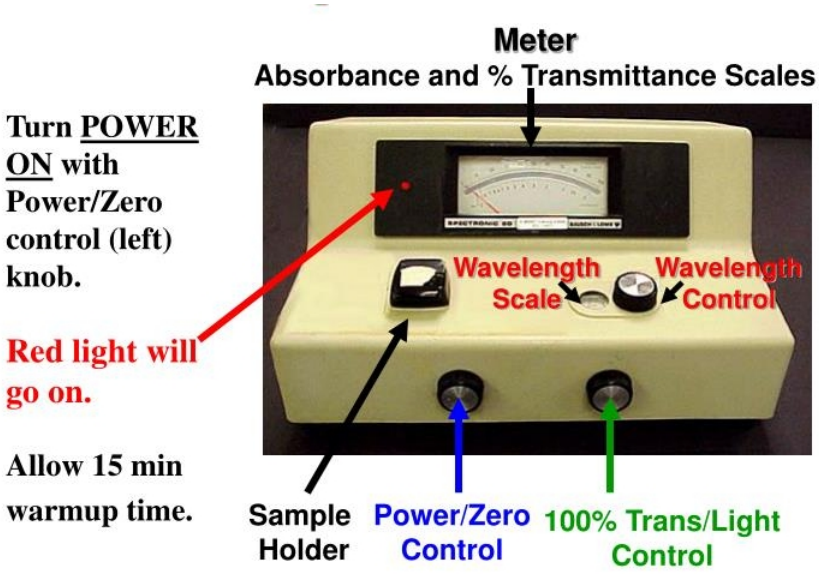


Figure 8.4.1.1: A vintage Bausch and Lomb Spec 20 with all controls and display items identified.

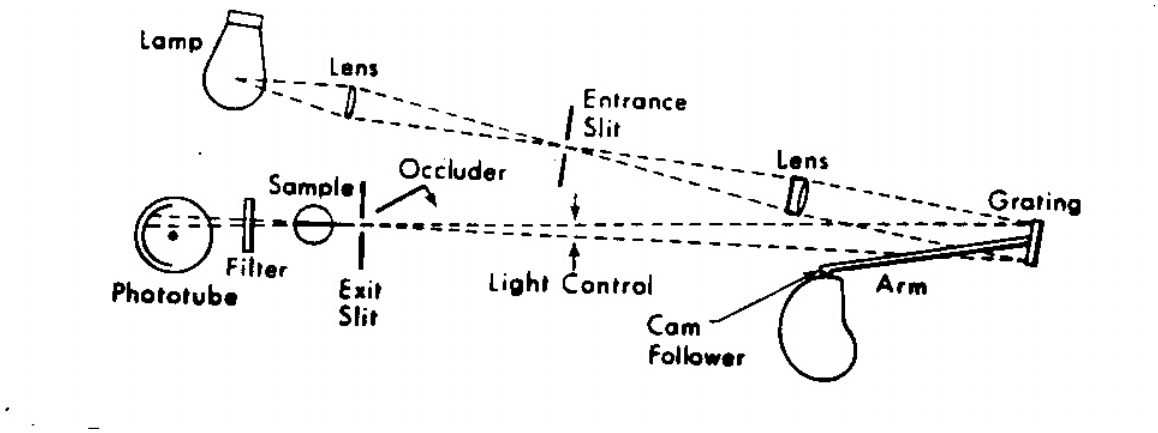


Figure 8.4.1.2: The schematic diagram of a Spec 20.

Pictured in Figure 8.4.1.2 is the schematic diagram for the Spec 20. The optical source in a Spec 20 is a tungsten halogen lamp the intensity of which is controlled by the Power/Zero Control in a manner similar to a common light dimmer. The instrument employs a phototube detector that can be easily changed for work either in the blue or red regions of the visible spectrum. The wavelength selector is a grating the angle of which is controlled by the Wavelgth Control dial attached to the cam follower. Samples are contained in round cuvettes that resemble common test tubes. The light controller is a "V" shaped occluder that blocks a fraction of the light passing through the reference and sample by using the 100% Trans/Light Control knob to insert the occulder more or less into the path of the light beam.

It is best to work with two cuvettes with the spec 20, one containing your solvent and the other your solvent (your reference) and sample. The general procedure to make a a transmittance (0 - 100 %) is as follows.

Step 1. Set the wavelength

Step 2. With nothing in the sample compartment and with the sample compartment closed, adjust the Power/Zero Control knob so that the meter shows 0 % T.

Step 3. With your reference in the sample compartment and with the sample compartment closed, adjust the 100% Trans/Light Control knob so that the meter shows 100 %T.

Repeat steps 2 and 3 as needed for consistency.

Step 4. Insert you sample in the sample compartment, close the lid, and record the % T.

Again, the procedure outlined above needs to be followed each time the wavelength is changed and even if the wavelength is not changed it is good practice to reset the 0 %T and 100% T every 20 minutes of so to account for instrument drift.

The Varian Cary 50 (now Agilent Cary 60)

A single beam instrument that has been a popular choice in research laboratories for the past 20 years is the Varian Cary 50, pictured below in Figure 8.4.1.3. The Cary 50 is a lower cost instrument that competes well against more expensive double beam instrument for many applications. This instrument has been succeeded by the Agilent Cary 60.



Figure 8.4.1.3: The Varian Cary 50

The Cary 50 gets all its power from a computer and it an especially versatile instrument with many accessories that are especially useful in biological chemistry laboratories. A picture of the Cary 50 layout is shown in Figure 8.4.1.4

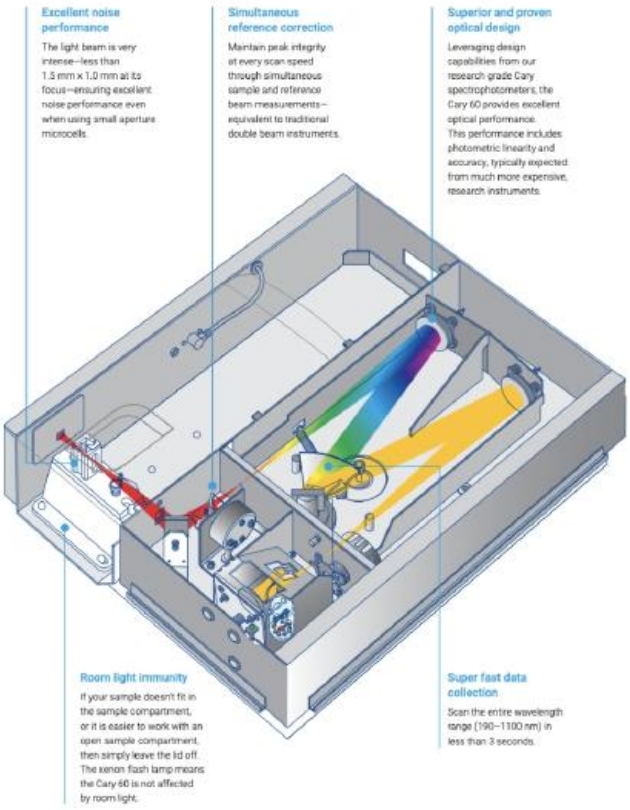


Figure 8.4.1.4: An annotated picture of the Cary 50 optical layout from the sales brochure.

A key feature of the Cary 50 is the Xenon flicker lamp used as the single optical source in this instrument. Unlike tungsten halogen lamps ($50) and the much more expensive deuterium lamps ($600) which have to be replaced every 2000 - 3000 hours, the Xenon flicker lamp last for a very long time (at least the life of the instrument). The lamp flickers on and off at 80 Hz. Consequently, the modulation of the source means that, unlike most absorption instruments, measurements can be made with the sample compartment open to the ambient light in the lab because the detector signal is sampled at the same frequency. In addition and very attractive, the 80 Hz repetition rate of the optical source makes this instrument well suited for kinetic measurements on the millisecond time scale as absorption measurements can be made every 0.0125 s.

The instrument employs two Si photodiode detectors and has a Beer's Law limit of linearity of 3.3 absorbance units.

The internal wavelength selector is based on a Czerny Turner monochromator design covering wavelengths from 190 mnm to 1100 nm and with a fixed spectral bandpass of 1.5 nm. The scan speed for the entire wavelength range can be as fast as 24,000 nm/min so an entire spectrum can be measured in about 3 seconds.

8.4.1: Single Beam Instruments for Absorption Spectroscopy - The Spec 20 and the Cary 50 is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

8.4.2: Instruments for Absorption Spectroscopy with Multichannel Detectors

Spectrophotometric instruments with multichannel detectors have no moving parts and are both more rugged and easier to makes small and portable relative to scanning wavelength instruments.

The Hewlett Packard 8453 Diode Array UV - Vis

Historically the first widely used spectrophotometers were produced by Hewlett Packard in the late 1980's (now sold under the Agilent Cary label) such as the HP 8453 UV-Visible instrument pictured below in Figure **8.4.2.1**.



Figure **8.4.2.1**: The HP 8453 UV - Vis

The optical layout of this instrument is shown in Figure **8.4.2.2**.

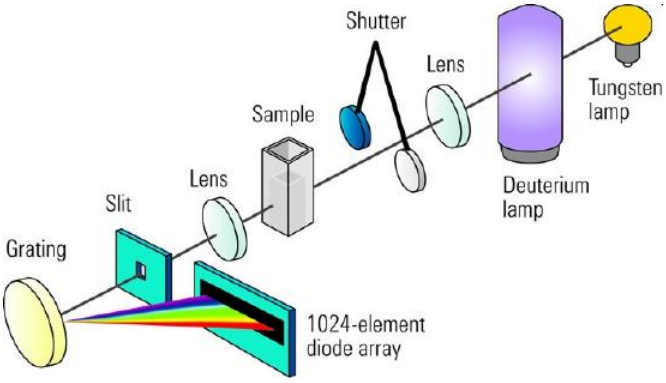


Figure **8.4.2.2**: The optical layout of a HP photodiode array spectrophotmeter.

An instrument such as the HP 8453 simultaneously uses a tungsten halogen lam and a deuterium lamp to cover the 190 nm to 1100 nm spectral range. The spectral bandwidth of 1 nm is limited by the size of the photodiode array and the fixed number of array elements. Based on Si photdiodes the Beer's Law linearity is limited to less than 2 absorbance units. Spectra can be acquired in a matter of only a a second or two second and the elements of this spectrophotometer has been incorporated into HPLC instruments.

Because to the digital nature of the instrument and the need to read the diode array, diode array spectrometers require either an internal or external computer. However, by recording the entire absorption spectrum in each measurement, quantitative multi-component analyses can easily be overdetermined (completed at more than the minimum number of wavelengths - 2 to two components, 3 for three components) allowing for uncertainties to be calculated.

The Red Tide Spectrophotometer

Today almost all low cost spectrophotometers are based on multichannel detectors with CCD type detectors. Such detectors are widely available at a low cost due to their similarity to the photodetectors in cell phones and other small camera systems. An

important example of a low cost spectrophotometer with a multichannel detector is the Red Tide instrument from Ocean Optics pictured in Figure 8.4.2.3.



Figure 8.4.2.3: A small and portable Red Tide spectrometer configured for standard 1 cm sq. cuvettes.

The Red Tide family of instruments use a USB interface and can be configured with a tungsten halogen lamp for work in the visible region ($1500) or a Xenon lamp for work in the UV and Visible regions ($4500). The optical sources are contained in the smaller section of the instrument with the cuvette. The optical layout inside the dispersing and detecting elements of the Red Tide spectophotometer are pictured in Figure 8.4.2.4 and contained in the larger section of the instrument underneath the colorful label.

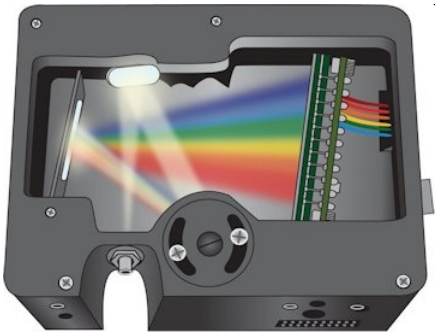


Figure 8.4.2.4: A cartoon sketch of the optical layout of the Red Tide spectrophotometer.

Light passing through a reference solution or a sample solution enters the spectrophotometer through the fiber optic assemble at the bottom of Figure 8.4.2.4. The light is collimated by the reflector at the top of the picture then dispersed by the reflective grating under the rotatable (fixed in this picture by two Phillips head screws). The dispersed light is reflected by the mirror on the left before striking the multichannel CCD detector on the right.

As in the HP photodiode array instrument the spectral band pass, 2 nm in this instrument, is limited by the size of the instrument and the number of detector elements in the CCD array. The Beer's Law linearity is also limited to about 2 absorbance units.

The Vernier SpectoVis Plus Spectrophotometer

It is likely that you are already used a spectrophotometer with a multichannel detector in your high school or first year chemistry laboratory course. Because of it's low cost ($400) the Vernier SpectroVis Plus, pictured in Figure 8.4.2.5, has been widely adopted in many instructional laboratories.



Figure 8.4.2.5: A Vernier SpectroVis Plus instrument.

The SpectroVis Plus also uses a USB interface to connect to either a Vernier LabQuest or a computer for instrument control and data processing.

As shown in Figure 8.4.2.6 the SpectroVis Plus can be used for absorption and luminescence spectroscopy.

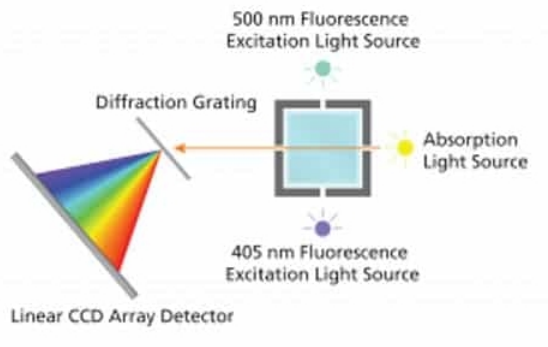


Figure 8.4.2.6: The optical layout of the Vernier SpectroVis Plus.

The SpectroVis Plus uses a small tungsten halogen lamp for absorption spectroscopy only in the visible region of the spectrum (380 - 950 nm) and is equipped with both a green LED and a Blue LED for luminescence measurements of compounds like compounds such as fluorescein, chlorophyll, or GFP. Dispersion of the white light or emission is accomplished with a transmission grating with a resulting spectral band pass of 4 nm. The photodetector in the Spectrovis Plus is a linear CCD array detetor.

8.4.2: Instruments for Absorption Spectroscopy with Multichannel Detectors is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

8.4.3: Double Beam Instruments for Absorption Spectroscopy

Most research grade instruments found in academic and industrial labs are double beam instruments. An important advantage of a double-beam spectrophotometer over a single-beam spectrophotometer is that a double beam instrument permits compensation for source power fluctuations greatly improving S/N and extension to dilute solution samples and measurements with gases.

n a double beam configuration, the beam from the light source is split in two. One beam illuminates the reference standard and the other illuminates the sample. The beams may be recombined before they reach a single monochromator and a single detector. In some cases two monochromators and two monchromators may be used although detector matching problems can arise.. The splitting of the beam is normally accomplished in one of two manners: statically, with a partially-transmitting mirror or similar device, or by attenuating the beams using moving optical and mechanical devices (a chopper wheel). Double beam instruments became quite popular in the early days of spectrophotometry due to the instability of light sources, detectors, and the associated electronics. Figure **8.4.3.1** below illustrates the general configuration of a double beam spectrophotometer.

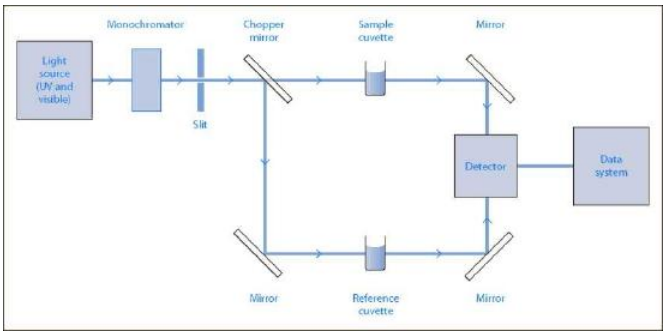


Figure **8.4.3.1**: The general layout of a double beam instrument for absorption spectroscopy.

The double beam UV-Vis in our instrument room is the Shimadzu UV 2600i purchased in Spring 2020 and shown in Figure **8.4.3.2**.



Figure **8.4.3.2**: Th Shimadzu UV 2600i instruemt with computer.

The Shimadzu 2600i is a double beam, single 0.278 m monochromator, single detector covering the wavelength range of 185 nm to 900 nm. The instruments both a 50 W tungsten halogen lamp for the visible and a deuterium lamp for the UV. A mirror on a turret selects the light from either lamp and the wavelength at which the switch occurs is adjustable. The instrument contains a photomultiplier detector (PMT). A cartoon of the optical layout is shown in Figure **8.4.3.3**.

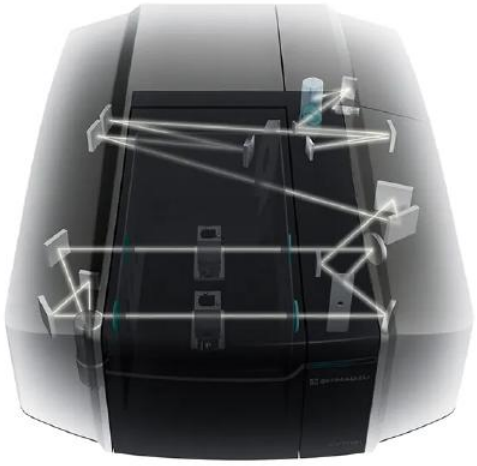


Figure 8.4.3.3: The beam path in the Shimadzu UV 2600i.

The wavelength selector slits are adjustable yielding spectral band with of 0.1, 0.2, 0.5, 1, 2, or 5 nm. The scanning speed of the monochromator can be adjusted from 4000 to 0.5 nm/min. The UV-2600 is also equipped with **Shimadzu's** proprietary Lo-Ray-Ligh grade diffraction grating, which achieves high efficiency and low stray light levels allowing the Beer's Law limit of linearity to approach 5 absorbance units.

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8.4.4: UV - Vis (and Near IR) Instruments with Double Dispersion

The most expensive instruments for absorption spectroscopy in the UV and visible regions of the spectrum contain two monochromators to better reduce stray light. These instruments which cost approximately $60k are coveted by researchers studying highly absorbing materials such as Heme containing proteins. Also, because of the cost of these instruments, manufacturers often extend their usefulness further into the near IR (3200 nm) by incorporating a lead sulfide detector along with the photomultiplier detector (PMT) .

A general layout of a UV-vis spectrophotometer is shown below in Figure **8.4.4.1**.

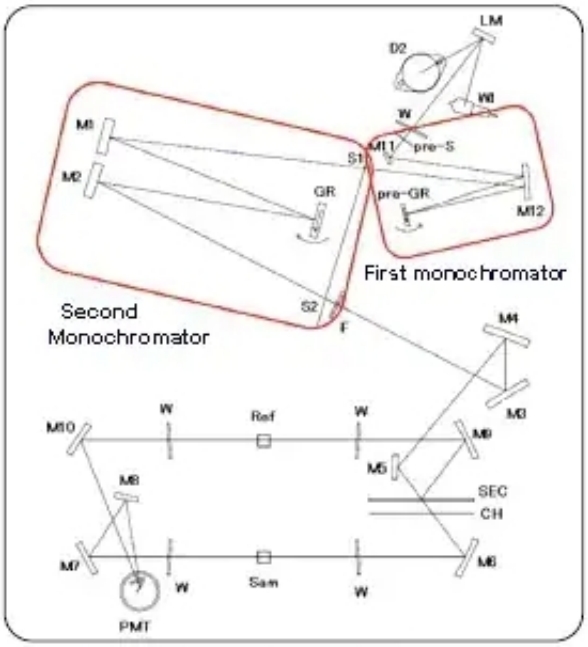


Figure **8.4.4.1**. The general optical layout of an instrument with double dispersion.

As shown in Figure **8.4.4.1**, light captured from the source is filtered to select a narrow band of wavelengths by the first 0.140 m monochromator. The light in the narrow band of wavelengths is dispersed again in the second, 0.278 m, monochromator. This double dispersion helps to halve the contributions from stray light to the detector signal and extend the Beer's Law limit of linearity to greater than 6 absorbance units.

8.4.4: UV - Vis (and Near IR) Instruments with Double Dispersion is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

9: Applications of Ultraviolet-Visable Molecular Absorption Spectrometry

9.1: The Magnitude of Molar Absorptivities

9.2: Absorbing Species

9.2.1: Electronic Spectra - Ultraviolet and Visible Spectroscopy - Organics

9.2.2: Electronic Spectra - Ultraviolet and Visible Spectroscopy - Transition Metal Compounds and Complexes

9.2.3: Electronic Spectra - Ultraviolet and Visible Spectroscopy - Metal to Ligand and Ligand to Metal Charge Transfer Bands

9.2.4: Electronic Spectra - Ultraviolet and Visible Spectroscopy - Lanthanides and Actinides

9.3: Qualitative Applications of Ultraviolet Visible Absorption Spectroscopy

9.4: Quantitative Analysis by Absorption Measurements

9.5: Photometric and Spectrophotometric Titrations

9.6: Spectrophotometric Kinetic Methods

9.6.1: Kinetic Techniques versus Equilibrium Techniques

9.6.2: Chemical Kinetics

9.7: Spectrophotometric Studies of Complex Ions

9: Applications of Ultraviolet-Visable Molecular Absorption Spectrometry is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

9.1: The Magnitude of Molar Absorptivities

Molar absorptivities,ϵ, have units of liters/(mole cm) and range in value from 0 to 10^5. The relationship between ϵ and the capture cross-section for a photon - chemical species interaction and the probability for an energy-absorbing transition was shown by Braude (J. Am Chem Soc, 379 (1950) to be

$\epsilon = 8.7 \times 10^{19}$ PA

where P is the transition probability and A is the section target area in units of cm^2. Typical organic molecules have been shown to have cross-sectional areas on the order of 10^{-15} cm^2 and transition probabilities range from 0 to 1.

Absorption bands with ϵ ranging from 10^4 to 10^5 are considered strong absorbers while absorption bands with ϵ less than or equal to 10^3 are considered weak absorbers and are likely the results of quantum mechanically forbidden transitions with $P < 0.01$.

9.1: The Magnitude of Molar Absorptivities is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

9.2: Absorbing Species

As shown in Figure 9.2.1, when a molecule, M, absorbs light it transitions to a higher energy, excited, electronic state, M*. The molecule resides in the excited state for a short time before dissipating the energy along one of three pathways. The most common relaxation pathway is radiationless relaxation where the excess energy is quickly transferred as heat through collisions with surrounding molecules in the bath. A small fraction of molecules will relax by releasing the energy as light through the processes of fluorescence or phosphorescence. Another relaxation pathway that is very much dependent on the nature of the electronic state leads to fragmentation through dissociation or predissociation. The timescales for absorption, radiationless relaxation and dissociation are short, 10^{-13} s - 10^{-15} s , while fluorecence, 10^{-9} s and phosphorescence, 10^{-6} s are much slower.

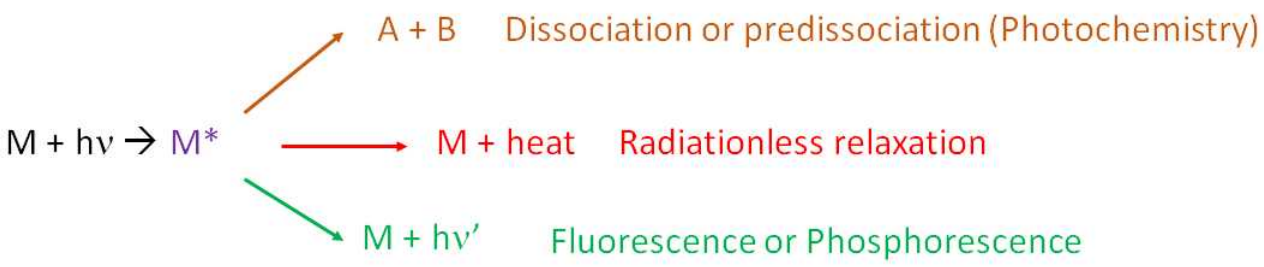


Figure 9.2.1: An illustration of the three major relaxation pathways for a molecule that has absorbed light in the UV - Vis.

In the next four subsections of section 9.2, the absorption characteristics of organic molecules, inorganic molecules, charge transfer systems and lanthanide containing species are presented.

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9.2.1: Electronic Spectra - Ultraviolet and Visible Spectroscopy - Organics

Objectives

After completing this section, you should be able to

1. identify the ultraviolet region of the electromagnetic spectrum which is of most use to organic chemists.
2. interpret the ultraviolet spectrum of 1,3-butadiene in terms of the molecular orbitals involved.
3. describe in general terms how the ultraviolet spectrum of a compound differs from its infrared and NMR spectra.

Key Terms

Make certain that you can define, and use in context, the key term below.

- ultraviolet (UV) spectroscopy
- Molar absorptivity

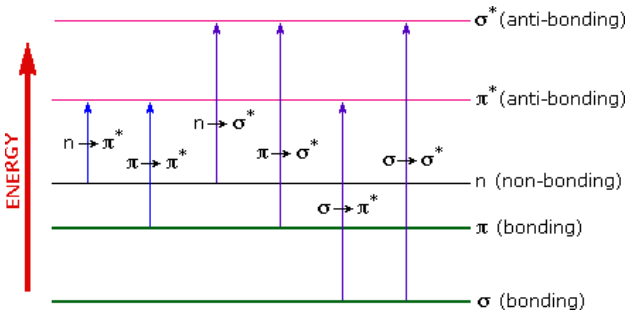
Study Notes

Ultraviolet spectroscopy provides much less information about the structure of molecules than do the spectroscopic techniques studied earlier (infrared spectroscopy, mass spectroscopy, and NMR spectroscopy). Thus, your study of this technique will be restricted to a brief overview. You should, however, note that for an organic chemist, the most useful ultraviolet region of the electromagnetic spectrum is that in which the radiation has a wavelength of between 200 and 400 nm.

UV-Visible Absorption Spectra

To understand why some compounds are colored and others are not, and to determine the relationship of conjugation to color, we must make accurate measurements of light absorption at different wavelengths in and near the visible part of the spectrum. Commercial optical spectrometers enable such experiments to be conducted with ease, and usually survey both the near ultraviolet and visible portions of the spectrum. Ultraviolet-visible absorption spectroscopy provides much less information about the structure of molecules than do the spectroscopic techniques studied earlier (infrared spectroscopy, mass spectroscopy, and NMR spectroscopy) and mainly provides information about conjugated pi systems. For an organic chemist the most useful ultraviolet region of the electromagnetic spectrum involves radiation with a wavelength between 200 and 400 nm. UV/Vis absorption spectra also involve radiation from the visible region of the electromagnetic spectrum with wavelengths between 400 and 800 nm.

A diagram highlighting the various kinds of electronic excitation that may occur in organic molecules is shown below. Of the six transitions outlined, only the two lowest energy ones, n to pi* and pi to pi* (colored blue) are achieved by the energies available in the 200 to 800 nm range of a UV/VIs spectrum. These energies are sufficient to promote or excite a molecular electron to a higher energy orbital in many conjugated compounds.



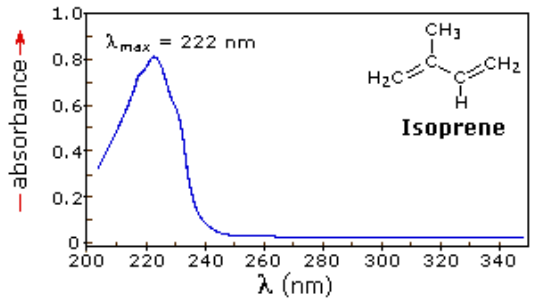
When sample molecules are exposed to light having an energy that matches a possible electronic transition within the molecule, some of the light energy will be absorbed as the electron is promoted to a higher energy orbital. A UV/Vis spectrometer records the wavelengths at which absorption occurs, together with the degree of absorption at each wavelength. A**bsorbance**, abbreviated 'A', is a unitless number which contains the same information as the 'percent transmittance' number used in IR spectroscopy. To

calculate absorbance at a given wavelength, the computer in the spectrophotometer simply takes the intensity of light at that wavelength *before* it passes through the sample (I_0), divides this value by the intensity of the same wavelength *after* it passes through the sample (I), then takes the $\log_{10}$ of that number:

$$A = \log\left(\frac{I_0}{I}\right)$$

The resulting spectrum is presented as a graph of absorbance (A) versus wavelength, as in the isoprene spectrum shown below. Since isoprene is colorless, it does not absorb in the visible part of the spectrum and this region is not displayed on the graph. Notice that the convention in UV-vis spectroscopy is to show the baseline at the bottom of the graph with the peaks pointing up. Wavelength values on the x-axis are generally measured in nanometers (nm) rather than in cm^{-1} as is the convention in IR spectroscopy.

Typically, there are two things that are noted and recorded from a UV-Vis spectrum. The first is λ_{max}, which is the wavelength at maximal light absorbance. As you can see, isoprene has λ_{max}, = 222 nm. The second valuable piece of data is the absorbance at the λ_{max}. In the isoprene spectrum the absorbance at the value λ_{max} of 222 nm is about 0.8.



Molar absorptivity

Molar absorptivity (*epsilon*) is a physical constant, characteristic of the particular substance being observed and thus characteristic of the particular electron system in the molecule. Molar absorptivities may be very large for strongly absorbing chromophores (>100,000) and very small if absorption is weak (10 to 100). The magnitude of *epsilon* reflects both the size of the chromophore and the probability that light of a given wavelength will be absorbed when it strikes the chromophore. Molar absorptivity (ϵ) is defined via Beer's law as:

$$\epsilon = \frac{A}{c\,l}$$

where

- A is the sample absorbance
- c is the sample concentration in moles/liter
- l is the length of light path through the sample in cm

If the isoprene spectrum show above was obtained from a dilute hexane solution (c = 4 * 10^{-5} moles per liter) in a 1 cm sample cuvette, a simple calculation using the above formula indicates a molar absorptivity of 20,000 at the maximum absorption wavelength, symbolized as λ_{max}.

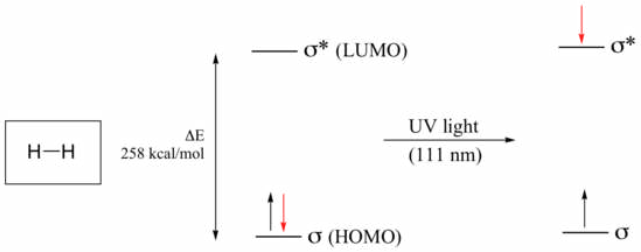
The only molecular moieties likely to absorb light in the 200 to 800 nm region are functional groups that contain pi-electrons and hetero atoms having non-bonding valence-shell electron pairs. Such light absorbing groups are referred to as **chromophores**. A list of some simple chromophores and their light absorption characteristics are provided below. The oxygen non-bonding electrons in alcohols and ethers do not give rise to absorption above 160 nm. Consequently, pure alcohol and ether solvents may be used for spectroscopic studies.

Electronic Transitions (cause of UV-Visible absorption)

As previously noted, electronic transitions in organic molecules lead to UV and visible absorption. As a rule, energetically favored electron promotion will be from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital

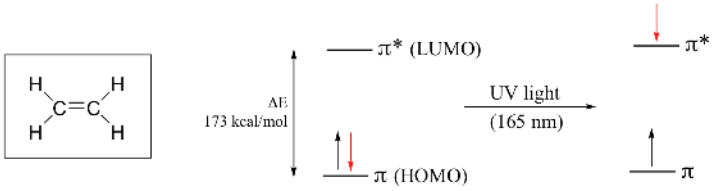
Chromophore	Example	Excitation	λmax, nm	ε @ λmax	Solvent
C=C	Ethene	π → π*	171	15,000	hexane
C≡C	1-Hexyne	π → π*	180	10,000	hexane
C=O	Ethanal	n → π* π → π*	290 180	15 10,000	hexane hexane
N=O	Nitromethane	n → π* π → π*	275 200	17 5,000	ethanol ethanol
C-X X=Br X=I	Methyl bromide Methyl Iodide	n → σ* n → σ*	205 255	200 360	hexane hexane

(LUMO), and the resulting species is called an **excited state.** The molecular orbital picture for the hydrogen molecule (H_2) consists of one bonding σ MO, and a higher energy antibonding σ* MO. When the molecule is in the ground state, both electrons are paired in the lower-energy bonding orbital – this is the Highest Occupied Molecular Orbital (HOMO). The antibonding σ* orbital, in turn, is the Lowest Unoccupied Molecular Orbital (LUMO).

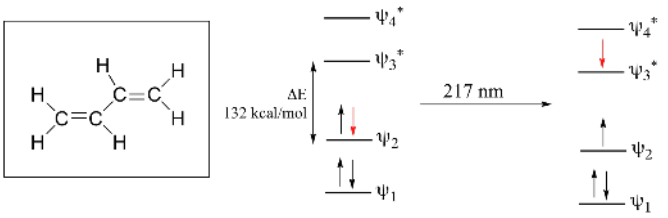


If the molecule is exposed to light of a wavelength with energy equal to ΔE, the HOMO-LUMO energy gap, this wavelength will be absorbed and the energy used to bump one of the electrons from the HOMO to the LUMO – in other words, from the σ to the σ* orbital. This is referred to as a **σ - σ* transition**. ΔE for this electronic transition is 258 kcal/mol, corresponding to light with a wavelength of 111 nm.

When a double-bonded molecule such as ethene (common name ethylene) absorbs light, it undergoes a **π - π* transition**. Because π- π* energy gaps are narrower than σ - σ* gaps, ethene absorbs light at 165 nm - a longer wavelength than molecular hydrogen.

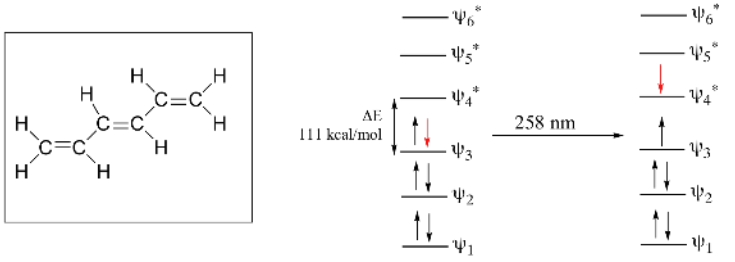


The electronic transitions of both molecular hydrogen and ethene are too energetic to be accurately recorded. Where electronic transition becomes useful to most organic and biological chemists is in the study of molecules with conjugated pi systems. In these groups, the energy gap for π -π* transitions is smaller than for isolated double bonds, and thus the wavelength absorbed is longer. The MO diagram for 1,3-butadiene, the simplest conjugated system. Recall that we can draw a diagram showing the four pi MO's that result from combining the four $2p_z$ atomic orbitals. The lower two orbitals are pi bonding, while the upper two are pi antibonding.

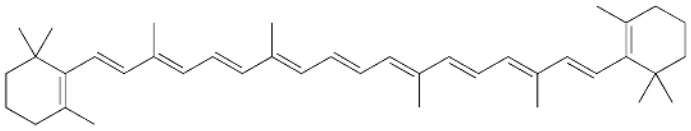


Comparing this MO picture to that of ethene, our isolated pi-bond example, we see that the HOMO-LUMO energy gap is indeed smaller for the conjugated system. 1,3-butadiene absorbs UV light with a wavelength of 217 nm.

As conjugated pi systems become larger, the energy gap for a π - π* transition becomes increasingly narrow, and the wavelength of light absorbed correspondingly becomes longer. The absorbance due to the π - π* transition in 1,3,5-hexatriene, for example, occurs at 258 nm, corresponding to a **Δ**E of 111 kcal/mol.

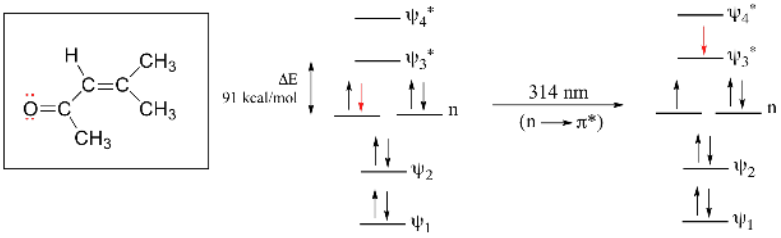


In molecules with extended pi systems, the HOMO-LUMO energy gap becomes so small that absorption occurs in the visible rather then the UV region of the electromagnetic spectrum. Beta-carotene, with its system of 11 conjugated double bonds, absorbs light with wavelengths in the blue region of the visible spectrum while allowing other visible wavelengths – mainly those in the red-yellow region - to be transmitted. This is why carrots are orange.



β-carotene

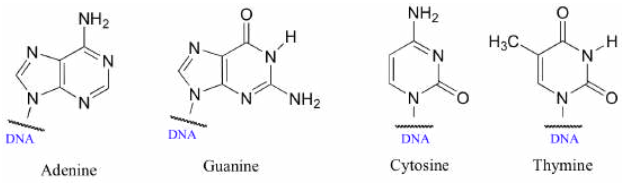
The conjugated pi system in 4-methyl-3-penten-2-one gives rise to a strong UV absorbance at 236 nm due to a π - π* transition. However, this molecule also absorbs at 314 nm. This second absorbance is due to the transition of a non-bonding (lone pair) electron on the oxygen up to a π* antibonding MO:



This is referred to as an $n - \pi^*$ transition. The nonbonding (*n*) MO's are higher in energy than the highest bonding p orbitals, so the energy gap for an $n - \pi^*$ transition is smaller that that of a $\pi - \pi^*$ transition – and thus the $n - \pi^*$ peak is at a longer wavelength. In general, $n - \pi^*$ transitions are weaker (less light absorbed) than those due to $\pi - \pi^*$ transitions.

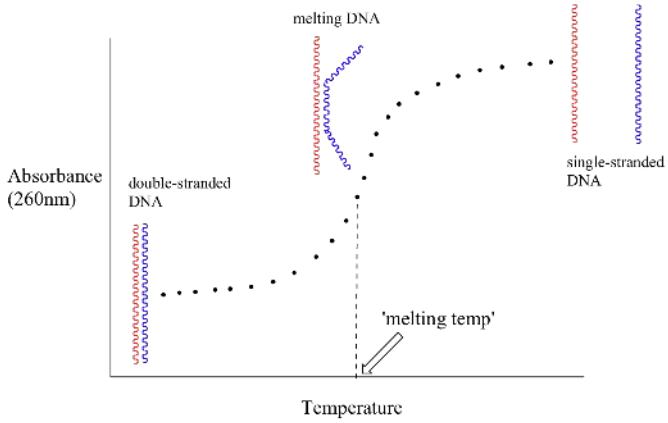
Use of UV/Vis Spectroscopy in Biological Systems

The bases of DNA and RNA are good chromophores:



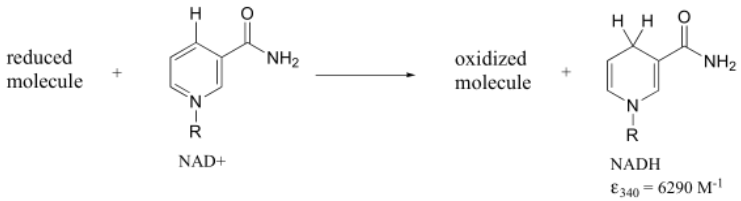
Biochemists and molecular biologists often determine the concentration of a DNA sample by assuming an average value of ε = 0.020 ng^{-1}×mL for double-stranded DNA at its λ_{max} of 260 nm (notice that concentration in this application is expressed in mass/volume rather than molarity: ng/mL is often a convenient unit for DNA concentration when doing molecular biology).

Because the extinction coefficient of double stranded DNA is slightly lower than that of single stranded DNA, we can use UV spectroscopy to monitor a process known as DNA melting. If a short stretch of double stranded DNA is gradually heated up, it will begin to 'melt', or break apart, as the temperature increases (recall that two strands of DNA are held together by a specific pattern of hydrogen bonds formed by 'base-pairing').

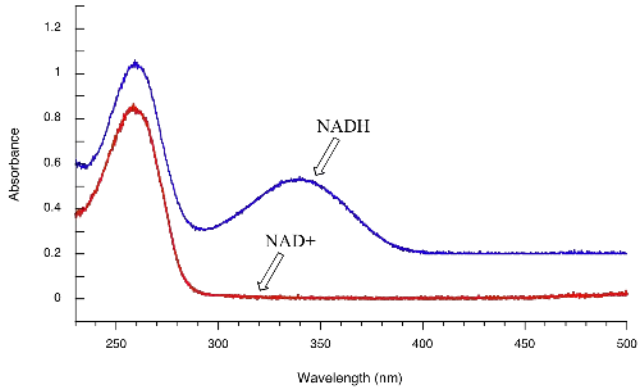


As melting proceeds, the absorbance value for the sample increases, eventually reaching a high plateau as all of the double-stranded DNA breaks apart, or 'melts'. The mid-point of this process, called the 'melting temperature', provides a good indication of how tightly the two strands of DNA are able to bind to each other.

Later we will see how the Beer - Lambert Law and UV spectroscopy provides us with a convenient way to follow the progress of many different enzymatic redox (oxidation-reduction) reactions. In biochemistry, oxidation of an organic molecule often occurs concurrently with reduction of nicotinamide adenine dinucleotide (NAD^+, the compound whose spectrum we saw earlier in this section) to NADH:

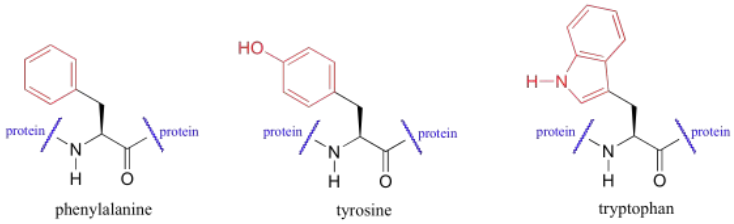


Both NAD^+ and NADH absorb at 260 nm. However NADH, unlike NAD^+, has a second absorbance band with λ_{max} = 340 nm and ε = 6290 $L_*mol^{-1}{}_*cm^{-1}$. The figure below shows the spectra of both compounds superimposed, with the NADH spectrum offset slightly on the y-axis:



By monitoring the absorbance of a reaction mixture at 340 nm, we can 'watch' NADH being formed as the reaction proceeds, and calculate the rate of the reaction.

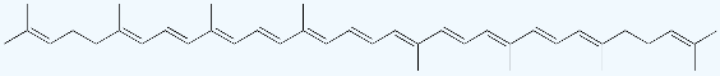
UV spectroscopy is also very useful in the study of proteins. Proteins absorb light in the UV range due to the presence of the aromatic amino acids tryptophan, phenylalanine, and tyrosine, all of which are chromophores.



Biochemists frequently use UV spectroscopy to study conformational changes in proteins - how they change shape in response to different conditions. When a protein undergoes a conformational shift (partial unfolding, for example), the resulting change in the environment around an aromatic amino acid chromophore can cause its UV spectrum to be altered.

? Exercise 9.2.1.1

1. 50 microliters of an aqueous sample of double stranded DNA is dissolved in 950 microliters of water. This diluted solution has a maximal absorbance of 0.326 at 260 nm. What is the concentration of the original (more concentrated) DNA sample, expressed in micrograms per microliter?
2. What is the energy range for 300 nm to 500 nm in the ultraviolet spectrum? How does this compare to energy values from NMR and IR spectroscopy?
3. Identify all isolated and conjugated pi bonds in lycopene, the red-colored compound in tomatoes. How many pi electrons are contained in the conjugated pi system?



lycopene

Answer

1) Using ε = A/c, we plug in our values for ε and A and find that c = 3.27 x 10^{-5}M, or 32.7 mM.

2)

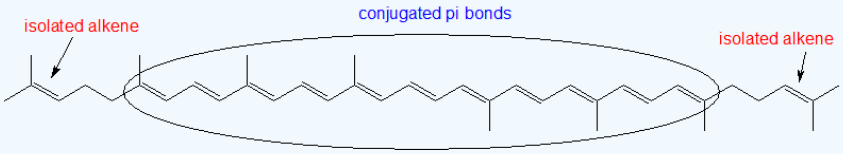
$E = hc/\lambda$

$E = (6.62 \times 10^{-34}\ \text{Js})(3.00 \times 10^{8}\ \text{m/s})/(3.00 \times 10^{-7}\ \text{m})$

$E = 6.62 \times 10^{-19}\ \text{J}$

The range of 3.972×10^{-19} to 6.62×10^{-19} joules. This energy range is greater in energy than the in NMR and IR.

3)



Objective

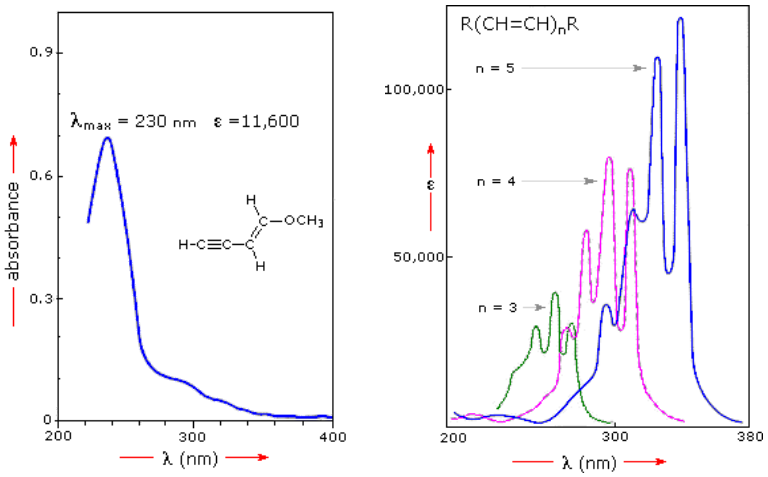
After completing this section, you should be able to use data from ultraviolet spectra to assist in the elucidation of unknown molecular structures.

Study Notes

It is important that you recognize that the ultraviolet absorption maximum of a conjugated molecule is dependent upon the extent of conjugation in the molecule.

The Importance of Conjugation

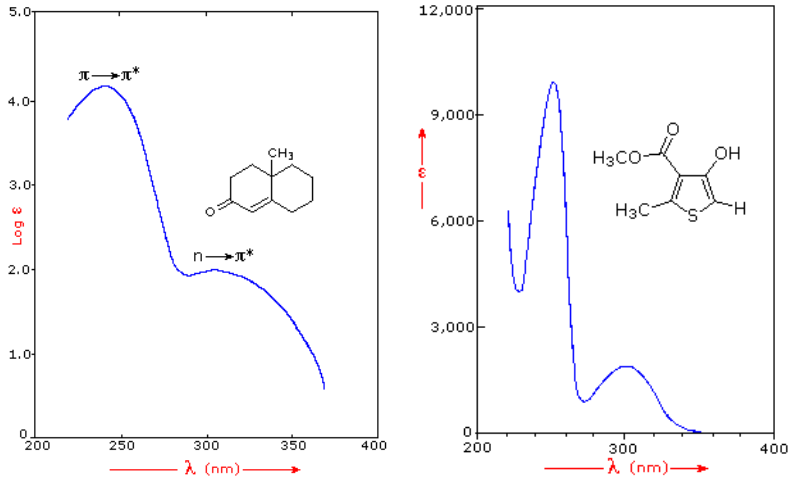
A comparison of the UV/Vis absorption spectrum of 1-butene, λ_{max} = 176 nm, with that of 1,3-butadiene, λ_{max} = 292 nm, clearly demonstrates that the effect of increasing conjugation is to shift toward longer wavelength (lower frequency, lower energy) absorptions. Further evidence of this effect is shown below. The spectrum on the left illustrates that conjugation of double and triple bonds also shifts the absorption maximum to longer wavelengths. From the polyene spectra displayed in the right it is clear that each additional double bond in the conjugated pi-electron system increases the absorption maximum about 30 nm. Also, the molar absorptivity (ε) roughly doubles with each new conjugated double bond. Spectroscopists use the terms defined in the table below when describing shifts in absorption. Thus, extending conjugation generally results in bathochromic and hyperchromic shifts in absorption.



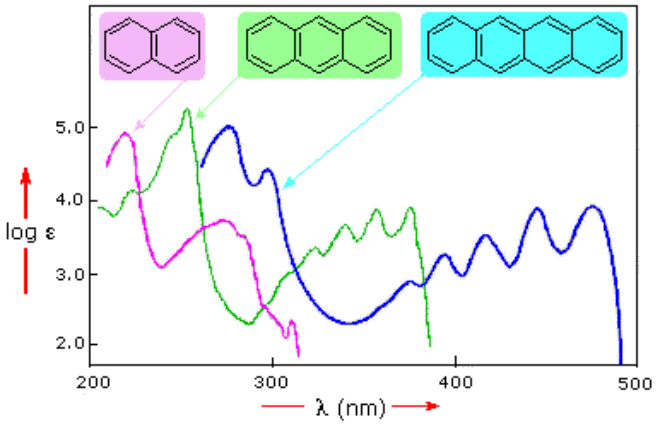
Terminology for Absorption Shifts

Nature of Shift	Descriptive Term
To Longer Wavelength	Bathochromic
To Shorter Wavelength	Hypsochromic
To Greater Absorbance	Hyperchromic
To Lower Absorbance	Hypochromic

Many other kinds of conjugated pi-electron systems act as chromophores and absorb light in the 200 to 800 nm region. These include unsaturated aldehydes and ketones and aromatic ring compounds. A few examples are displayed below. The spectrum of the unsaturated ketone (on the left) illustrates the advantage of a logarithmic display of molar absorptivity. The $\pi \rightarrow \pi^*$ absorption located at 242 nm is very strong, with an ε = 18,000. The weak $n \rightarrow \pi^*$ absorption near 300 nm has an ε = 100.

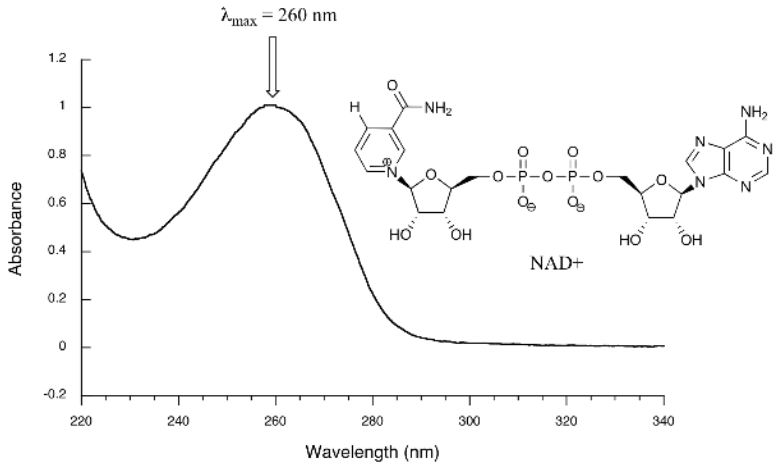


Benzene exhibits very strong light absorption near 180 nm (ε > 65,000) , weaker absorption at 200 nm (ε = 8,000) and a group of much weaker bands at 254 nm (ε = 240). Only the last group of absorptions are completely displayed because of the 200 nm cut-off characteristic of most spectrophotometers. The added conjugation in naphthalene, anthracene and tetracene causes bathochromic shifts of these absorption bands, as displayed in the chart below. All the absorptions do not shift by the same amount, so for anthracene (green shaded box) and tetracene (blue shaded box) the weak absorption is obscured by stronger bands that have experienced a greater red shift. As might be expected from their spectra, naphthalene and anthracene are colorless (with their absorptions in the UV range), but tetracene is orange (since its absorptions move into the visible range).

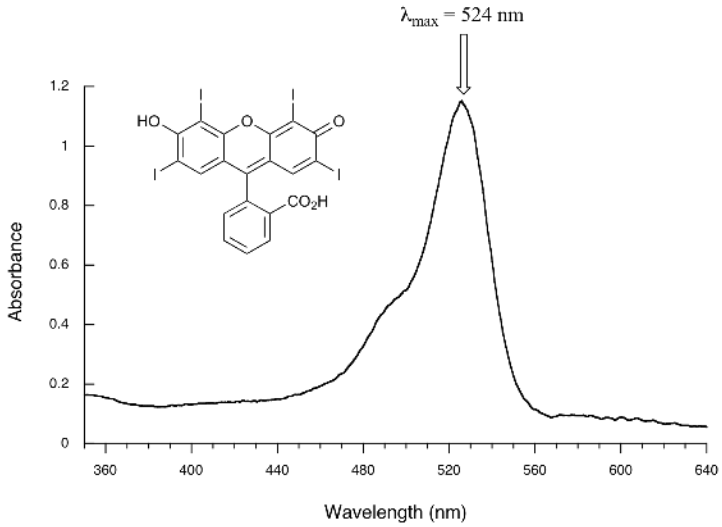


Looking at UV-Vis Spectra

Below is the absorbance spectrum of an important biological molecule called nicotinamide adenine dinucleotide, abbreviated NAD^+. This compound absorbs light in the UV range due to the presence of conjugated pi-bonding systems.



Below is the absorbance spectrum of the common food coloring Red #3. The extended system of conjugated pi bonds causes the molecule to absorb light in the visible range. Because the λ_{max} of 524 nm falls within the green region of the spectrum, the compound appears red to our eyes (recalling the color wheel from Section 14.7).



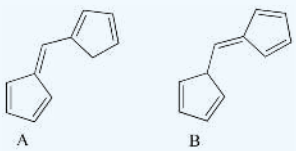
Example 14.8.2

How large is the π - π* transition in 4-methyl-3-penten-2-one?

Solution

Example 14.8.3

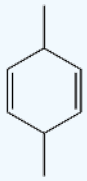
Which of the following molecules would you expect absorb at a longer wavelength in the UV region of the electromagnetic spectrum? Explain your answer.

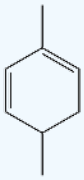


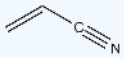
Solution

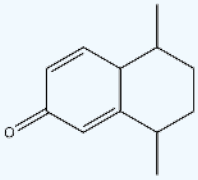
Exercise 9.2.1.1

Which of the following would show UV absorptions in the 200-300 nm range?

A) 

B) 

C) 

D) 

Answer

B and D would be in that range.

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9.2.2: Electronic Spectra - Ultraviolet and Visible Spectroscopy - Transition Metal Compounds and Complexes

UV-Vis Spectroscopy

UV-Vis spectroscopy is an analytical chemistry technique used to determine the presence of various compounds, such as transition metals/transition metal ions, highly conjugated organic molecules, and more. However, due to the nature of this course, only transition metal complexes will be discussed. UV-Vis spectroscopy works by exciting a metal's d-electron from the ground state configuration to an excited state using light. In short, when energy in the form of light is directed at a transition metal complex, a d-electron will gain energy and a UV-Vis spectrophotometer measures the abundance of transition metal atoms with excited electrons at a specific wavelength of light, from the visible light region to the UV region.

When using a UV-Vis Spectrophotometer, the solution to be analyzed is prepared by placing the sample in a cuvette then placing the cuvette inside the spectrophotometer. The machine then shines light waves from the visible and ultraviolet wavelengths and measures how much light of each wavelength the sample absorbs and then emits.

Absorbance of the sample can be calculated via Beer's Law: A=εlc where A is the absorbance, ε is the molar absorptivity of the sample, l is the length of the cuvette used, and c is the concentration of the sample.[1] When the spectrophotometer produces the absorption graph, the molar absorptivity can then be calculated.

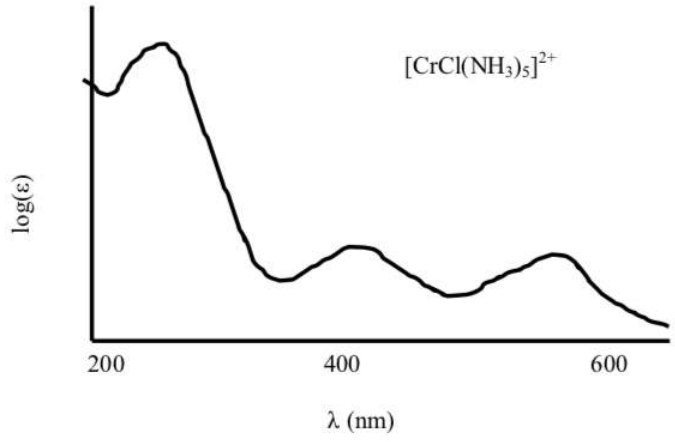


Figure 9.2.2.1. UV-Vis Spectrum of a Chromium(III) complex

To illustrate what this looks like, you will find a sample absorbance spectrum to the right.[2] 404-5</ref> </ref> As can be seen, the y-axis represents absorbance and the x-axis represents the wavelengths of light being scanned. This specific transition metal complex, $[CrCl(NH_3)_5]^{2+}$, has the highest absorbance in the UV region of light, right around 250-275 nm, and two slight absorbance peaks near 400 nm and 575 nm respectively. The two latter peaks are much less pronounced than the former peak due to the electron's transition being partially forbidden—a concept that will be discussed later in this chapter. If a transition is forbidden, not many transition metal electrons will undergo the excitation.

Theory Behind UV-Vis Spectroscopy

Splitting of the D-Orbitals

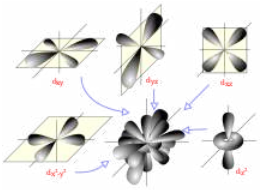


Figure 9.2.2.2. D orbitals

As is widely known, the d-orbitals contain five types of sub-orbitals: d_{xy}, d_{yz}, d_{xz}, d_x^2-$_y^2$, and d_z^2 which are all shown to the right[3]. When in the absence of a magnetic field—such as when there are no electrons present—all the sub-orbitals combine together to form a degenerate spherical orbital. This singular orbital promptly differentiates back into its sub-orbitals when electrons are introduced or the transition metal is bonded to a set of ligands.

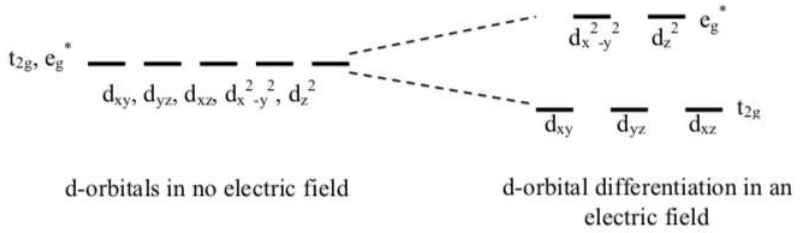
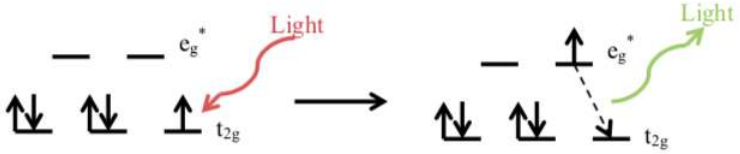


Figure 9.2.2.3. This differentiation gives rise to the origin of color in metallic complexes.

The Origination of Color in Transition Metal Complexes

When looking at color in transition metal complexes, it is necessary to pay attention to the differentiated d-orbitals. Color in this sense originates from the excitation of d-orbital electrons from one energy level to the next. For instance, an electron in the t_{2g} bonding orbital can be excited by light to the e_g^* bonding orbital and upon its descent back to the ground state, energy is released in the form of light:



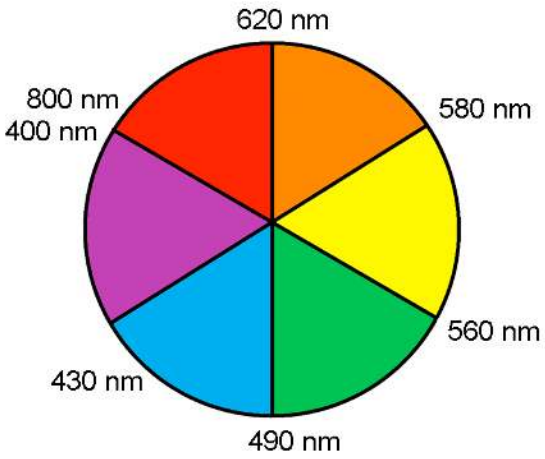


Figure 9.2.2.4. Colorwheel wavelengths

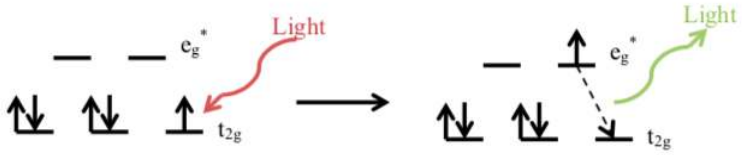
The specific wavelength of light required to excite an electron to the e_g^* orbital directly correlates to the color given off when the electron moves back down to the ground state. The figure on the right helps visualize the properties of transition metal color. Whichever color is absorbed, the complimentary color (directly opposite from the color in the figure) is emitted. For instance, if a metal complex emits green light we can figure out that the complex absorbed red light with a wavelength between 630 nm-750 nm in length.

Rules of Color Intensity and Forbidden Transitions

The intensity of the emitted color is based on two rules:[4]

1. Spin multiplicity: the spin multiplicity of a complex cannot change when an electron is excited. Multiplicity can be calculated via the equation 2S+1 where S is calculated by (1/2)(number of unpaired d-electrons).
2. If there is a center of symmetry in the molecule (i.e. center of inversion) then a g to g or u to u electron excitation is not allowed.

If a complex breaks one of these rules, we say it is a forbidden transition. If one rule is broken, it is singly forbidden. If two rules are broken, we call it double forbidden and so on. Even though the convention is to call it forbidden, this does not mean it will not happen; rather, the more rules the complex breaks, the more faded its color will be because the more unlikely the chances the transition will happen. Let's again look at the previous example:



If we apply the intensity rules to it:

1. Multiplicity before transition=2(0.5[1 unpaired electron])+1=3, Multiplicity after transition=2(0.5[1 unpaired electron])+1=3. Both multiplicities are the same, so this transition is allowed under rule 1.
2. If we assume this molecule is octahedral in symmetry, this means it has an inversion center and thus the transition of e_g^* to t_{2g} is forbidden under rule 2 due to both orbitals being gerade (g).
3. We are only exciting one electron and thus it is allowed under rule 3.

Based on these rules, we can see that this transition is only singly forbidden, and thus it will appear only slightly faded and light rather than a deep, rich green.

Ligand Field Theory: How Ligands Affect Color

As it turns out, the atoms bonded to a transition metal affect the wavelength that the complex needs to absorb in order to give off light; we refer to this as Ligand Field Theory. While certain transition metals like to absorb different wavelengths than other transition metals, the ligand(s) plays the most important role in determining the wavelength of light needed for electron excitation. [5]

The terms low spin and high spin are used to describe the difference in energy levels of the t_{2g} and e_g^* orbitals, which correlates to the wavelength of light needed to excite an electron from t_{2g} to e_g^*. When a complex is characterized as low spin, the ligands attached to the metal raise the energy of the e_g^* orbital so much that the ground state configuration of the complex fills the first six electrons in the t_{2g} orbital before the e_g^* orbital is filled. As a result, high energy wavelengths of light—violet, blue, and green—are needed to successful excite an electron to the e_g^* bonding orbital. This means that the transition metal complex will emit yellow, orange, and red light, respectively. Conversely, high spin complexes have ligands which lower the energy level of the e_g^* orbital so that low energy light—red, orange, and yellow—or even high energy light can successfully excite an electron. Thus, a high spin complex can emit any color. High spin complexes can be thought of as all-inclusive while low spin complexes are half-exclusive in terms of the wavelengths needed to excite an electron.

To determine whether a complex is high spin or low spin:

1. Look at the transition metal. First row metals will want to be high spin unless the ligand(s) forces it to be low spin. Second row metals want to be low spin unless the ligand(s) forces it high spin. Third row metals will low spin.
2. Look at the ligand(s) as they will be the ultimate determining factor. If the ligand matches the transition metal in terms of high spin/low spin, then the complex's spin will be whatever is "agreed" upon. If they differ, follow the ligand's spin type. If the ligand is classified neither as high nor low spin, follow the transition metal's spin type. Ligand spin types are enumerated below.
3. If there are multiple ligands with differing spin types, go with whichever spin type is most abundant in the complex.

The ligands below are ranked from low spin (greatest energy difference between t_{2g} and e_g^*) to high spin (lowest energy difference):[6]

$$\underbrace{CO > CN^- > PR_3 >}_{\text{Low spin}} \underbrace{\text{ethylene diamine (en)} > NH_3 > H_2O}_{\text{Neither high nor low spin}} \underbrace{> F^- > Cl^- > Br^- > I^-}_{\text{High Spin}}$$

To illustrate this concept, let's take the following complexes:

$[Ni(NH_3)_6]^{2+}$, $[Ni(CN)_4]^{2-}$

The nickel complexes all have the same oxidation state on the metal (2+), and thus the same d-electron count. In the first complex, nickel wants to be high spin, while ammonia prefers neither high nor low spin. Therefore the complex will be high spin and emit blue light, which is an absorbance of orange—weak energy—light. For the second complex, nickel again wants to be high spin, but cyanide prefers low spin. As a result, the complex becomes low spin and will emit yellow light, which is an absorbance of violet—strong energy—light.

References

1. Inorganic Chemistry, Miessler, Fischer, and Tarr, 2013, Pages 404 and 405
2. ↑ Chemistry LibreTexts, Electronic Spectroscopy: Interpretation, https://chem.libretexts.org/Core/Physical_and_Theoretical_Chemistry/Spectroscopy/Electronic_Spectroscopy/Electronic_Spectroscopy%3A_Interpretation
3. ↑ Principles of Inorganic Chemistry, Brian William Pfennig, 2015, Page 88
4. ↑ Inorganic Chemistry, Miessler, Fischer, and Tarr, 2013, Page 414
5. ↑ Principles of Inorganic Chemistry, Brian William Pfennig, 2015, Page 526
6. ↑ Principles of Inorganic Chemistry, Brian William Pfennig, 2015, Page 523

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9.2.3: Electronic Spectra - Ultraviolet and Visible Spectroscopy - Metal to Ligand and Ligand to Metal Charge Transfer Bands

In the field of inorganic chemistry, color is commonly associated with d–d transitions. If this is the case, why is it that some transition metal complexes show intense color in solution, but possess no d electrons? In transition metal complexes a change in electron distribution between the metal and a ligand gives rise to charge transfer (CT) bands when performing Ultraviolet-visible spectroscopy experiments. For complete understanding, a brief introduction to electron transfer reactions and **Marcus-Hush theory** is necessary.

Outer Sphere Charge Transfer Reactions

Electron transfer reactions(charge transfer) fall into two categories: **Inner-sphere mechanisms** and **Outer-sphere mechanisms**. Inner-sphere mechanisms involve electron transfer occurring via a covalently bound bridging ligand (Figure **9.2.3.1**).

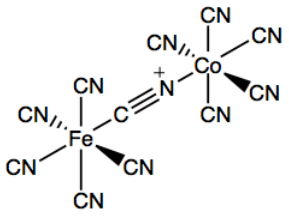


Figure **9.2.3.1**: Intermediate formed in the reaction between $[Fe(CN)_6]^{3-}$ and $[Co(CN)_5]^{3-}$

Outer-sphere mechanisms involve electron transfer occurring without a covalent linkage between reactants, e.g.,

$$[ML_6]^{2+} + [ML_6]^{3+} \longrightarrow [ML_6]^{3+} + [ML_6]^{2+} \tag{9.2.3.1}$$

Here, we focus on outer sphere mechanisms.

In a self-exchange reaction the reactant and product side of a reaction are the same. No chemical reaction takes place and only an electron transfer is witnessed. This reductant-oxidant pair involved in the charge transfer is called the precursor complex. The Franck-Condon approximation states that a molecular electronic transition occurs much faster than a molecular vibration.

Consider the reaction described in Equation ???. This process has a Franck-Condon restriction: Electron transfer can only take place when the **M–L** bond distances in the **ML(II)** and **ML(III)** states are the same. This means that vibrationally excited states with equal bonds lengths must be formed in order to allow electron transfer to occur. This would mean that the $[ML_6]^{2+}$ bonds must be compressed and $[ML_6]^{3+}$ bonds must be elongated for the reaction to occur.

Self exchange rate constants vary, because the activation energy required to reach the vibrational states varies according to the system. The greater the changes in bond length required to reach the precursor complex, the slower the rate of charge transfer.[1]

A Brief Introduction to Marcus-Hush Theory

Marcus-Hush theory relates kinetic and thermodynamic data for two self-exchange reactions with data for the cross-reaction between the two self-exchange partners. This theory determines whether an outer sphere mechanism has taken place. This theory is illustrated in the following reactions

Self exchange 1: $[ML_6]^{2+} + [ML_6]^{3+} \rightarrow [ML_6]^{3+} + [ML_6]^{2+}$ $\Delta G^O = 0$

Self exchange 2: $[ML_6]^{2+} + [ML_6]^{3+} \rightarrow [ML_6]^{3+} + [ML_6]^{2+}$ $\Delta G^O = 0$

Cross Reaction: $[ML_6]^{2+} + [ML_6]^{3+} \rightarrow [ML_6]^{3+} + [ML_6]^{2+}$

The Gibbs free energy of activation $\Delta G^{\ddagger}$ is represented by the following equation:

$$\Delta G^{\ddagger} = \Delta_w G^{\ddagger} + \Delta_o G^{\ddagger} + \Delta_s G^{\ddagger} + RT\ln(k'T/hZ)$$

- T = temperature in K
- R = molar gas constant

- k' = Boltzmann constant
- h = Planck's constant
- Z = effective frequency collision in solution ~ 10^{11} dm^3 mol^{-1} s^{-1}
- $\Delta_w G^{\ddagger}$ = the energy associated with bringing the reactants together, includes the work done to counter any repulsion
- $\Delta_0 G^{\ddagger}$ = energy associated with bond distance changes
- $\Delta_s \Delta G^{\ddagger}$= energy associated with the rearrangements taking place in the solvent spheres
- ln (k'T / hZ) = accounts for the energy lost in the formation of the encounter complex

The rate constant for the self-exchange is calculated using the following reaction

$$k = \kappa Z e^{-\Delta G^{\ddagger}/RT}$$

where κ is the transmission coefficient ~1.

The Marcus-Hush equation is given by the following expression

$$k_{12} = (k_{11} k_{22} K_{12} f_{12})^{1/2} \qquad (9.2.3.2)$$

where:

$$\log f_{12} = \frac{(\log K_{12})^2}{4 \log(\frac{k_{11} k_{22}}{Z^2})}$$

- Z is the collision frequency
- k_{11} and $\Delta G^{\ddagger}_{11}$ correspond to self exchange 1
- k_{22} and $\Delta G^{\ddagger}_{22}$ correspond to self exchange 2
- k_{12} and $\Delta G^{\ddagger}_{12}$ correspond to the cross-reaction
- K_{12} = cross reaction equilibrium constant
- ΔG^{O}_{12}= standard Gibbs free energy of the reaction

The following equation is an approximate from of the Marcus-Hush equation (Equation 9.2.3.2):

$$\log k_{12} \approx 0.5 \log k_{11} + 0.5 \log \log$$

since $f \approx 1$ and $\log f \approx 0$.

How is the Marcus-Hush equation used to determine if an outer sphere mechanism is taking place?

- values of k_{11}, k_{22}, K_{12}, and k_{12} are obtained experimentally
- k_{11} and k_{22} are theoretically values
- K_{12} is obtained from E_{cell}

If an outer sphere mechanism is taking place the calculated values of k_{12} will match or agree with the experimental values. If these values do not agree, this would indicate that another mechanism is taking place.[1]

The Laporte Selection Rule and Weak d–d Transitions

d- d transitions are forbidden by the Laporte selection rule.

- Laporte Selection Rule: \(\Delta l = \pm 1\)
- Laporte allowed transitions: a change in parity occurs i.e. s → p and p → d.
- Laporte forbidden transitions: the parity remains unchanged i.e. p → p and d → d.

d-d transitions result in weak absorption bands and most d-block metal complexes display low intensity colors in solution (exceptions d^0 and d^{10} complexes). The low intensity colors indicate that there is a low probability of a d-d transition occurring.

Ultraviolet-visible (UV/Vis) spectroscopy is the study of the transitions involved in the rearrangements of valence electrons. In the field of inorganic chemistry, UV/Vis is usually associated with d – d transitions and colored transition metal complexes. The color of the transition metal complex solution is dependent on: the metal, the metal oxidation state, and the number of metal d-electrons. For example iron(II) complexes are green and iron(III) complexes are orange/brown.[2]

Charge Transfer Bands

If color is dependent on d-d transitions, why is it that some transition metal complexes are intensely colored in solution but possess no d electrons?



Figure **9.2.3.2**: Fullerene oxides are intensely colored in solution, but possess no d electrons. Solutions from left to right: C_{60}, $C_{60}O$, $C_{60}O$, and $C_{60}O_2$. Fullerenes, nanometer-sized closed cage molecules, are comprised entirely of carbons arranged in hexagons and pentagons. Fullerene oxides, with the formula $C_{60}O_n$, have epoxide groups directly attached to the fullerene cage.

In transition metal complexes a change in electron distribution between the metal and a ligand give rise to charge transfer (CT) bands.[1] CT absorptions in the UV/Vis region are intense (ε values of 50,000 L mole^{-1} cm^{-1} or greater) and selection rule allowed. The intensity of the color is due to the fact that there is a high probability of these transitions taking place. Selection rule forbidden d-d transitions result in weak absorptions. For example octahedral complexes give ε values of 20 L mol^{-1} cm^{-1} or less.[2] A charge transfer transition can be regarded as an internal oxidation-reduction process. [2]

Ligand to Metal and Metal to Ligand Charge Transfer Bands

Ligands possess σ, σ^*, π, π^*, and nonbonding (n) molecular orbitals. If the ligand molecular orbitals are full, charge transfer may occur from the ligand molecular orbitals to the empty or partially filled metal d-orbitals. The absorptions that arise from this process are called ligand-to-metal charge-transfer (LMCT) bands (Figure 3).[2] LMCT transitions result in intense bands. Forbidden d-d transitions may also take place giving rise to weak absorptions. Ligand to metal charge transfer results in the reduction of the metal.

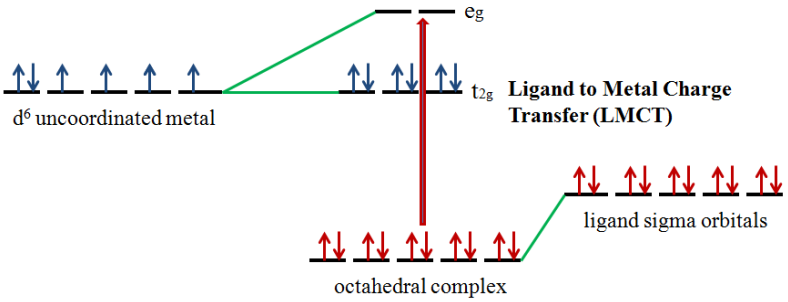


Figure **9.2.3.3**: Ligand to Metal Charge Transfer (LMCT) involving an octahedral d^6 complex.

If the metal is in a low oxidation state (electron rich) and the ligand possesses low-lying empty orbitals (e.g., CO or CN^-) then a metal-to-ligand charge transfer (MLCT) transition may occur. MLCT transitions are common for coordination compounds having π-acceptor ligands. Upon the absorption of light, electrons in the metal orbitals are excited to the ligand π^* orbitals.[2] Figure 4 illustrates the metal to ligand charge transfer in a d^5 octahedral complex. MLCT transitions result in intense bands. Forbidden d–d transitions may also occur. This transition results in the oxidation of the metal.

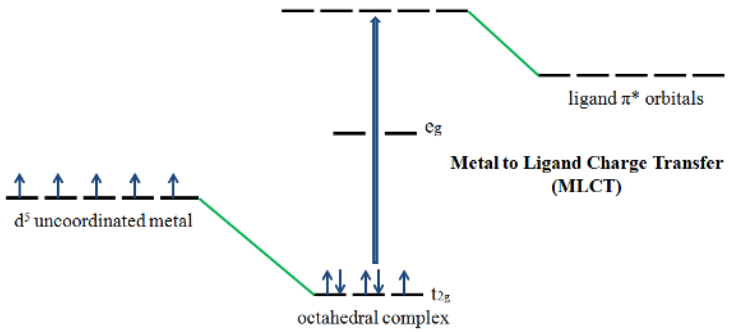


Figure **9.2.3.4**. Metal to Ligand Charge Transfer (MLCT) involving an octahedral d^5 complex.

Effect of Solvent Polarity on CT Spectra

This effect only occurs if the species being studied is an ion pair

The position of the CT band is reported as a transition energy and depends on the solvating ability of the solvent. A shift to lower wavelength (higher frequency) is observed when the solvent has high solvating ability.

Polar solvent molecules align their dipole moments maximally or perpendicularly with the ground state or excited state dipoles. If the ground state or excited state is polar an interaction will occur that will lower the energy of the ground state or excited state by solvation. The effect of solvent polarity on CT spectra is illustrated in the following example.

Example 9.2.3.1

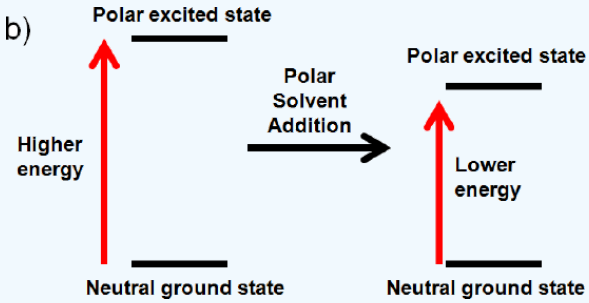
You are preparing a sample for a UV/Vis experiment and you decide to use a polar solvent. Is a shift in wavelength observed when:

a) Both the ground state and the excited state are neutral

When both the ground state and the excited state are neutral a shift in wavelength is not observed. No change occurs. Like dissolves like and a polar solvent won't be able to align its dipole with a neutral ground and excited state.

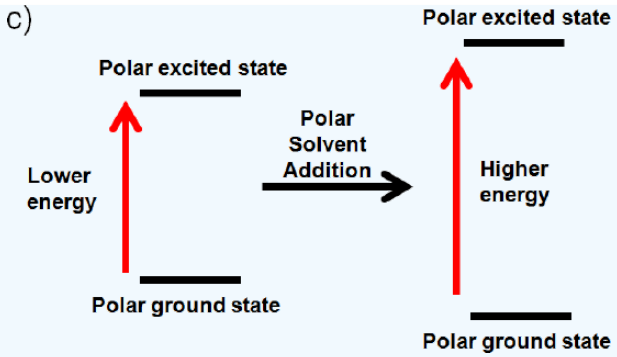
b) The excited state is polar, but the ground state is neutral

If the excited state is polar, but the ground state is neutral the solvent will only interact with the excited state. It will align its dipole with the excited state and lower its energy by solvation. This interaction will lower the energy of the polar excited state. (increase wavelength, decrease frequency, decrease energy)



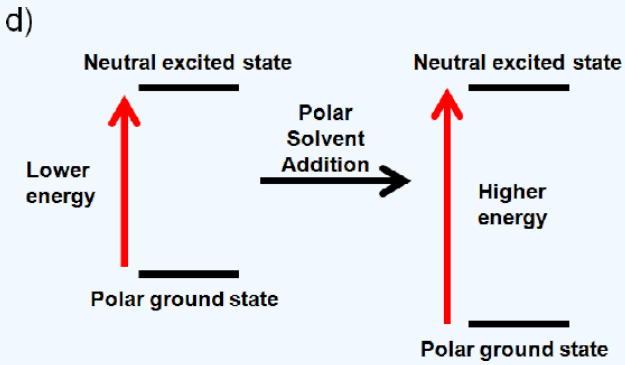
c) The ground state and excited state is polar

If the ground state is polar the polar solvent will align its dipole moment with the ground state. Maximum interaction will occur and the energy of the ground state will be lowered. (increased wavelength, lower frequency, and lower energy) The dipole moment of the excited state would be perpendicular to the dipole moment of the ground state, since the polar solvent dipole moment is aligned with the ground state. This interaction will raise the energy of the polar excited state. (decrease wavelength, increase frequency, increase energy)



d) The ground state is polar and the excited state is neutral

If the ground state is polar the polar solvent will align its dipole moment with the ground state. Maximum interaction will occur and the energy of the ground state will be lowered. (increased wavelength, lower frequency, and lower energy). If the excited state is neutral no change in energy will occur. Like dissolves like and a polar solvent won't be able to align its dipole with a neutral excited state. Overall you would expect an increase in energy (Illustrated below), because the ground state is lower in energy (decrease wavelength, increase frequency, increase energy).[4]



How to Identify Charge Transfer Bands

CT absorptions are selection rule allowed and result in intense (ε values of 50,000 L mole^{-1} cm^{-1} or greater) bands in the UV/Vis region.[2] Selection rule forbidden d-d transitions result in weak absorptions. For example octahedral complexes give ε values of 20 L mol^{-1} cm^{-1} or less.[2] CT bands are easily identified because they:

- Are very intense, i.e. have a large extinction coefficient
- Are normally broad
- Display very strong absorptions that go above the absorption scale (dilute solutions must be used)

✔ Example 9.2.3.2: Ligand to Metal Charge Transfer

$KMnO_4$ dissolved in water exhibits intense CT Bands. The one LMCT band in the visible is observed around 530 nm.

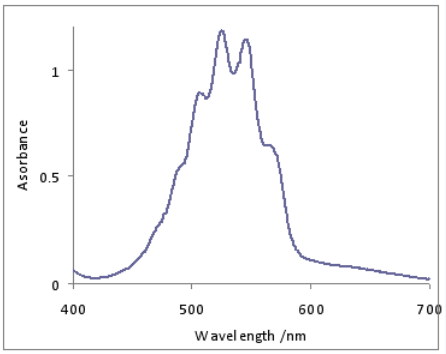


Figure 9.2.3.5: Absorption spectrum of an aqueous solution of potassium permanganate, showing a vibronic progression. (CC BY-SA 3; Petergans via Wikipedia)

The band at 528 nm gives rise to the deep purple color of the solution. An electron from a “oxygen lone pair” character orbital is transferred to a low lying Mn orbital.[1]

Example 9.2.3.3: Metal to Ligand Charge Transfer

Tris(bipyridine)ruthenium(II) dichloride ($[Ru(bpy)_3]Cl_2$) is a coordination compound that exhbits a CT band is observed (Figure 9.2.3.6)

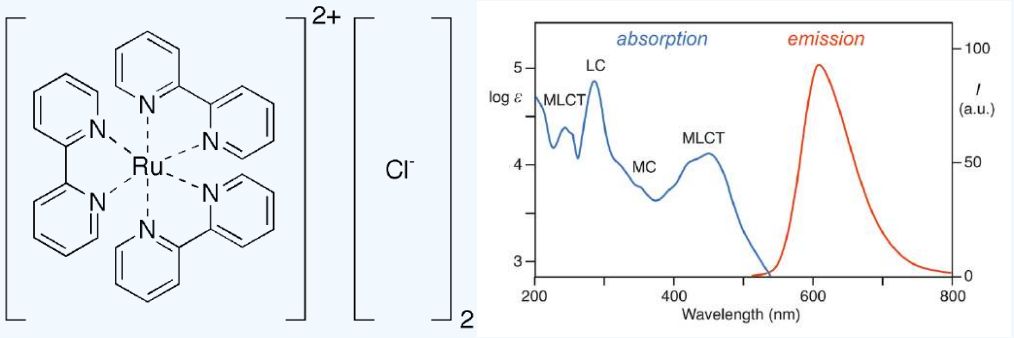


Figure 9.2.3.6: (left) Structure of $[Ru(bpy)_3]Cl_2$, (right) CT band observed in its V/Vis spectrum. (CC BY-SA 4.0; Albris via Wikipedia)

A d electron from the ruthenium atom is excited to a bipyridine anti-bonding orbital. The very broad absorption band is due to the excitation of the electron to various vibrationally excited states of the π* electronic state.[6]

Practice Problems

1. You perform a UV/Vis on a sample. The sample being studied has the ability to undergo a charge transfer transition. A charge transfer transitions is observed in the spectra. Why would this be an issue if you want to detect d-d transitions? How can you solve this problem?
2. What if both types of charge transfer are possible? For example a complex has both σ-donor and π-accepting orbitals? Why would this be an issue?
3. If the ligand has chromophore functional groups an intraligand band may be observed. Why would this cause a problem if you want to observe charge transfer bands? How would you identify the intraligand bands? State a scenario in which you wouldn’t be able to identify the intraligand bands.

Answers to Practice Problems

1. This is an issue when investigating weak d-d transitions, because if the molecule undergoes a charge transfer transitions it results in an intense CT band. This makes the d – d transitions close to impossible to detect if they occur in the same region as the charge transfer band. This problem is solved by performing the UV/Vis experiment on a more concentrated solution, resulting in minor peaks becoming more prominent.

2. Octahedral complexes such as $Cr(CO)_6$, have both σ-donor and π-accepting orbitals. This means that they are able to undergo both types of charge transfer transitions. This makes it difficult to distinguish between LMCT and MLCT.
3. This would cause a problem because CT bands may overlap intraligand bands. Intraligand bands can be identified by comparing the complex spectrum to the spectrum of the free ligand. This may be difficult, since upon coordination to the metal, the ligand orbital energies may change, compared to the orbital energies of the free ligand. It would be very difficult to identify an intraligand band if the ligand doesn't exist as a free ligand. If it doesn't exist as a free ligand you wouldn't be able to take a UV/Vis, and thus wouldn't be able to use this spectrum in comparison to the complex spectrum.

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9.2.4: Electronic Spectra - Ultraviolet and Visible Spectroscopy - Lanthanides and Actinides

The ions of most lanthanide and actinide ions absorb light in the UV and visible regions. The transitions involve only a redistribution of electrons within the 4f orbitals (**f --> f' transitions**) are ***orbitally-forbidden*** by the quantum mechanical selection rules. Consequently, the compounds of lanthanide ions are generally pale in color.

Unlike transition metal complexes, the crystal/ligand field effects for the lanthanide 4f orbitals are virtually **insignificant**. This is a result of the extensive shielding of the 4f electrons by the 5s and 5p orbitals. Consequently the f --> f' absorption bands are **very sharp** (useful **fingerprinting** and **quantitation** of Ln^{III}) and the optical spectra are **virtually independent of environment.**

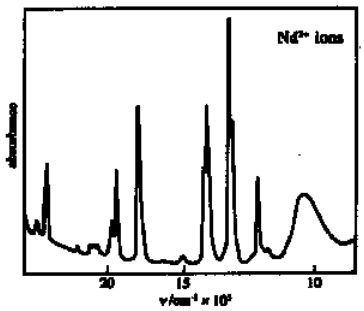


Figure**9.2.4.1**: The spectrum of a neodymium(III) complex over the range of 25,000 cm-1 to 9100 cm-1 (400 nm to 1100 nm). The sharp "atom-like" spectral features for Ln^{3+} ion complexes are the same be thay be in the gas, solid or solution phase.

The insensitivity of the f --> f' transitions leads to limited use in study of lanthanide materials. **Ce^{III}** and **Tb^{III}** complexes have the **highest intensity bands in the UV** due to **$4f^n$ --> $4f^{n-1}5d^1$** transitions. The f -->d are not orbitally forbidden (n-1 = 0 (empty sub-shell) for Ce^{III} = 7 (half-filled sub-shell) for Tb^{III}).

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9.3: Qualitative Applications of Ultraviolet Visible Absorption Spectroscopy

In contrast to NMR and IR, spectroscopy in the UV and visible regions is of very limited in terms of the information content contained in a a molecule's spectrum. The small number of peaks, the broadness of the peaks makes the unambiguous identification of a compound from its UV-Vis spectrum very unlikely.

Methods of Plotting Spectra

UV/vis spectra are plotted in a number of different ways. The most common quantities plotted as the ordinate are absorbance, % transmittance, log absorbance, or molar absorptivity. The most common quantities plotted as the abscissa are wavelength, wavenumber (cm^{-1}), or frequency.

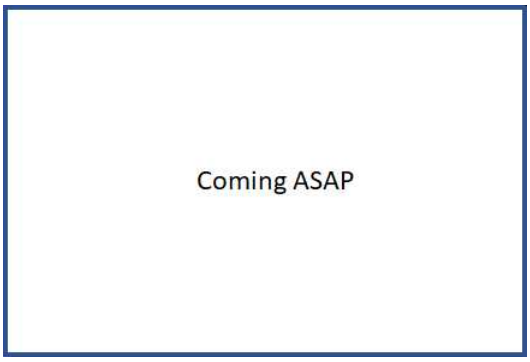


Figure **9.3.1**: Two plots of the absorbance spectrum of a 1.00 x 10^{-5} M solution of 1-naphthol in terms of (a) absorbance and (b) %transmittance.

Generally, plots in terms of absorbance give the largest difference in peak heights as a function of analyte concentration, the exception being for solution that do not absorb very much and where the curve to curve differences are larger with % transmittance.

Solvents

In addition to solubility, consideration must be given to the transparency of the solvent and the effect of the solvent on the spectrum of the analyte.

Any colorless solvent will be transparent in the visible region. For the UV, a low wavelength cutoff for common solvents can be easily found. This low wavelength cutoff is the wavelength below which the solvent absorbance increases to obscure the analyte's absorbance. A short table is given below

Solvent	Low wavelength cutoff (nm)
water	190
ethanol	210
n-hexane	195
cyclohexane	210
benzene	280
diethyl ether	210
acetone	310
acetonitrile	195
carbon tetrachloride	265
methylene chloride	235
chloroform	245

Spectral fine structure, often observed in the gas phase spectra, is either broadened or obscured by the solvent. In addition, the absorbance peak positions in a polar solvent are often shifted to longer wavelengths, red-shifted, relative to their position sin a non polar solvent. The shift in peak position can be as large as 40 nm and results from the stabilization or destabilization of the ground state and excited state energy levels by the solvent. Both the loss of fine structure and shift in peak positions is evident in the three spectra of flavone shown in Figure 9.3.2 obtained in methanol, acetonitrile, and cyclohexane. Focusing on the bands at around 290 nm the two bands (peak and shoulder) clearly observed in cyclohexane, the least polar solvent, is largely gone in methanol, the most polar solvent. Also a red shift on the order of 10 nm is evident for methanol relative to cyclhexane.

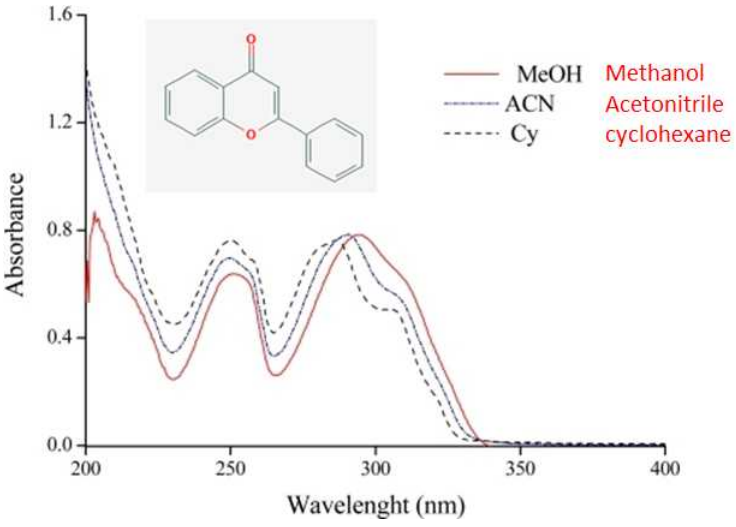


Figure **9.3.2**: The UV spectrum of flavone in three different solvents. The spectra are from Spectroscopic Study of Solvent Effects on the Electronic Absorption Spectra of Flavone and 7-Hydroxyflavone in Neat and Binary Solvent Mixtures, Sancho, Amadoz, Blanco and Castro, *Int. J. Mol. Sci.* 2011, *12*(12), 8895-8912

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9.4: Quantitative Analysis by Absorption Measurements

Adsorption spectroscopy is one of the most useful and widely used tools in modern analytical chemistry for the quantitative analysis of analytes in solution. Important characteristics of spectrophotometric methods include:

1) wide applicability to many organic and inorganic species that absorb

2) sensitivities to 10^{-5} M

3) moderate to high selectivity by choice of wavelength

4) good accuracy

5) precision on the order of 1 - 3 % RSD

Numerous selective reagents are available to extend spectrophotometry to non absorbing species. An important examples is the Griess method for nitrite. As shown in Figure 9.4.1, the Griess method involves two acid catalyzed reactions to produce a strongly absorbing colored azo dye.

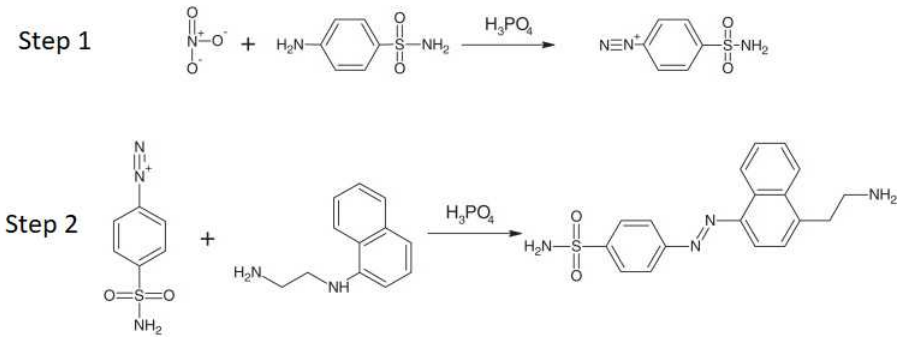


Figure 9.4.1: The reaction scheme for the conversion of the colorless nitite ion to a colored product by the Griess method.

Wavelength Selection

The best choice of wavelength for Beer's law analysis is one which is selective for the chosen analyte, has a large ϵ and where the absorption curve is relatively flat. As shown in Figure 9.4.1, a peak with a large ϵ will yield a better sensitivity than a peak with a smaller (\{\epsilon}\). Choosing a wavelength where the absorption curve is relatively flat will minimize polychromatic error and will minimize uncertainties form a failure to precisely set the instrument to the exact wavelength for each measurement.

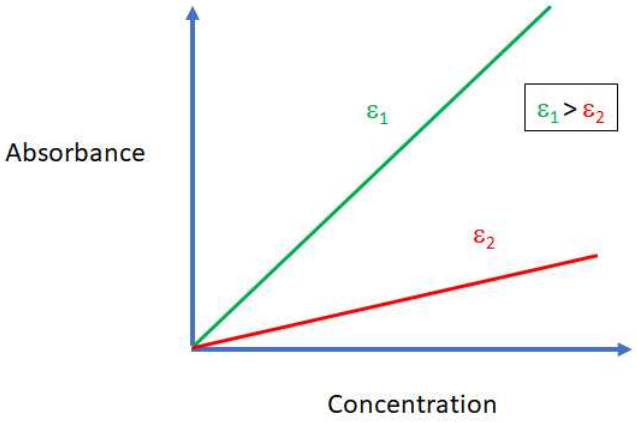


Figure 9.4.2: The cartoon above illustrates the better sensitivity for a Beer's law analysis when ϵ_2 is larger than ϵ_1

Variables that Influence Absorbance

Common variables that influence the absorption spectrum of an analyte include pH, solvent, temperature, electrolyte concentration and the presence of interfering substances. The effect of these variables should be known and controlled if needed (i.e. buffered

solutions). These effects are especially important for analytes that participate in association or dissociation reactions.

Cells

Accurate spectrophotometric analysies required good quality, matched cuvettes that are clean and transmit at the chosen wavelength. Care should be taken to avoid scratching and leaving fingerprints on the surfaces of the cuvettes. Prior to measurements, it is recommended that the outer surfaces of a cuvette be cleaned with lens paper wet with spectro-grade methanol.

Determination of the Relationship between Absorbance and Concentration

For any spectrophotometric analysis it is necessary to prepared a series of external standards that bracket the concentration range of the unknown sample and construct a calibration curve. It is unwise to use a single standard solution to determine the molar absorptivity and while literature values for the molar absorptivity are useful they should not be used to determine analytical results.

The composition of the external standards should approximate as best as possible the samples to be analyzed. Consideration should be given to the other species expected in the samples as well as the pH, ionic strength. Failure to do so leads to matrix matching errors.

If the non analyte components of the sample, the matrix, are not known but are expected to be a problem, the standard addition method is recommended (see section 5.3).

The Analysis of Mixtures

The total absorbance of a solution at a given wavelength is equal to the sum of the absorbances of the individual components present in the solution. Consider the cartoon spectra shown in Figure 9.4.3. for two absorbing species R and G. In Figure 9.4.3 the black curve is the sum of the red and green curves.

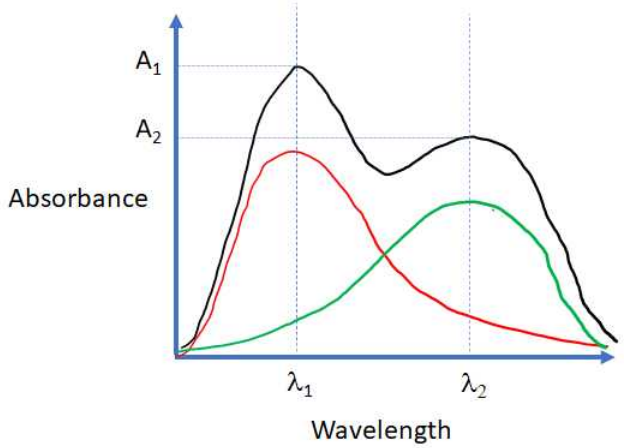


Figure**9.4.3**: The illustration below shows the additivity of absorbances. In the cartoon, the black curve is the sum of the REd curve and hte Green curve.

The absorbance A_1 is the sum of the absorbance of R and the absorbance of G at λ_1 and the absorbance A_2 is is the sum of the absorbance of R and the absorbance of G at λ_2. This is described by the two equations shown below.

$$A_1 = \epsilon_R^1 b C_R + \epsilon_G^1 b C_G \text{ (at } \lambda_1)$$
$$A_2 = \epsilon_R^2 b C_R + \epsilon_G^2 b C_G \text{ (at } \lambda_2)$$

Beers law calibration curves standard solutions for R and G collected at λ_2 and λ_2 can be used to determine the four ϵb terms. At this point the system of equations is minimally solvable for two unknowns, C_R and C_G.

Modern instruments employing the techniques of linear algebra will use absorbance values at many more wavelengths for both the standard solution and the mixture to over-determine the system and provided calculated uncertainties for the unknown concentrations

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9.5: Photometric and Spectrophotometric Titrations

A photometric titration curve is a plot of absorbance, corrected for volume changes, as a function of the volume of titrant. If the conditions are properly chosen the curve will consisits of two linear regions of different slopes. One region occurs at the start of the titration and the other is well after the equivalence point. The end point is the volume of titrant corresponding to the point where the two linear regions intersect. A few examples of photometric titration curves are shown in Figure 9.5.1 where *A* indicates the analyte, *P* indicates the product and *T* indicates the titration for the reaction A + T --> P.

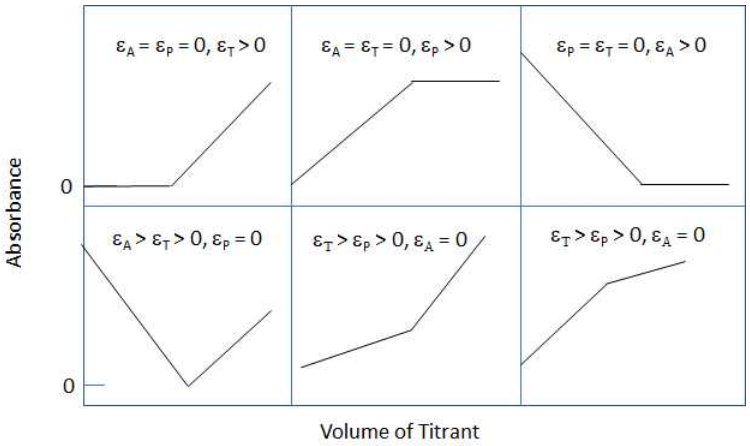


Figure 9.5.1: The illustration above shows some typical photmetric titration cuves where A indicates the analyte, T indicates the titrant, P indicates the product and ϵ indicates the molar absorptivity.

In order to obtain a satisfactory endpoint the absorbing system(s) must obey Beer's law and the absorbance values need be correct for the volume changes due to the addition of the volume of titrant; A' = A (V + v)/V where V is the original volume and v is the total volume of titrant added.

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9.6: Spectrophotometric Kinetic Methods

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9.6.1: Kinetic Techniques versus Equilibrium Techniques

In an equilibrium method the analytical signal is determined by an equilibrium reaction that involves the analyte or by a steady-state process that maintains the analyte's concentration. When we determine the concentration of iron in water by measuring the absorbance of the orange-red $\mathbf{Fe(phen)_3^{2+}}$ complex, the signal depends upon the concentration of $\mathbf{Fe(phen)_3^{2+}}$, which, in turn, is determined by the complex's formation constant. In the flame atomic absorption determination of Cu and Zn in tissue samples, the concentration of each metal in the flame remains constant because each step in the process of atomizing the sample is in a steady-state. In a ***kinetic method*** the analytical signal is determined by the rate of a reaction that involves the analyte or by a nonsteady-state process. As a result, the analyte's concentration changes during the time in which we monitor the signal.

In many cases we can choose to complete an analysis using either an equilibrium method or a kinetic method by changing when we measure the analytical signal. For example, one method for determining the concentration of nitrite, $\mathbf{NO_2^-}$, in groundwater utilizes the two-step diazotization re-action shown in Figure 13.1.1 [Method 4500-NO_2^- B in *Standard Methods for the Analysis of Waters and Wastewaters*, American Public Health Association: Washington, DC, 20th Ed., 1998]. The final product, which is a reddish-purple azo dye, absorbs visible light at a wavelength of 543 nm. Because neither reaction in Figure 13.1.1 is rapid, the absorbance —which is directly proportional to the concentration of nitrite—is measured 10 min after we add the last reagent, a lapse of time that ensures that the concentration of the azo dyes reaches the steady-state value required of an equilibrium method.

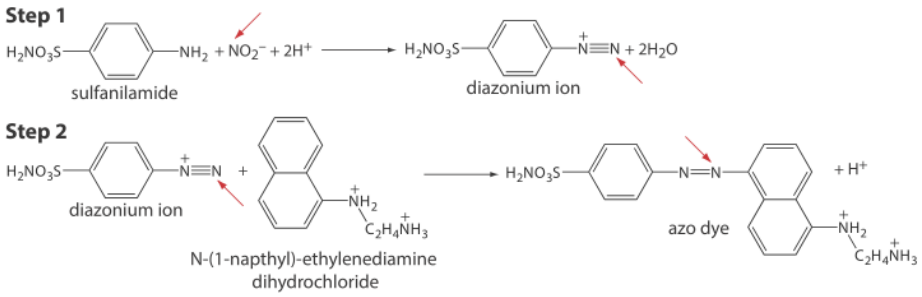


Figure 13.1.1 . Analytical scheme for the analysis of NO-2 in groundwater. The red arrows highlights the nitrogen in $\mathbf{NO_2^-}$ that becomes part of the azo dye.

We can use the same set of reactions as the basis for a kinetic method if we measure the solution's absorbance during this 10-min development period, obtaining information about the reaction's rate. If the measured rate is a function of the concentration of $\mathbf{NO_2^-}$, then we can use the rate to determine its concentration in the sample [Karayannis, M. I.; Piperaki, E. A.; Maniadaki, M. M. *Anal. Lett.* **1986**, *19*, 13–23].

There are many potential advantages to a kinetic method of analysis, perhaps the most important of which is the ability to use chemical reactions and systems that are slow to reach equilibrium. In this chapter we examine three techniques that rely on measurements made while the analytical system is under kinetic control: chemical kinetic techniques, in which we measure the rate of a chemical reaction; radiochemical techniques, in which we measure the decay of a radioactive element; and flow injection analysis, in which we inject the analyte into a continuously flowing carrier stream, where its mixes with and reacts with reagents in the stream under conditions controlled by the kinetic processes of convection and diffusion.

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9.6.2: Chemical Kinetics

The earliest analytical methods based on chemical kinetics—which first appear in the late nineteenth century—took advantage of the catalytic activity of enzymes. In a typical method of that era, an enzyme was added to a solution that contained a suitable substrate and their reaction was monitored for a fixed time. The enzyme's activity was determined by the change in the substrate's concentration. Enzymes also were used for the quantitative analysis of hydrogen peroxide and carbohydrates. The development of chemical kinetic methods continued in the first half of the twentieth century with the introduction of nonenzymatic catalysts and noncatalytic reactions.

Despite the diversity of chemical kinetic methods, by 1960 they no longer were in common use. The principal limitation to their broader acceptance was a susceptibility to significant errors from uncontrolled or poorly controlled variables—temperature and pH are two such examples—and the presence of interferents that activate or inhibit catalytic reactions. By the 1980s, improvements in instrumentation and data analysis methods compensated for these limitations, ensuring the further development of chemical kinetic methods of analysis [Pardue, H. L. *Anal. Chim. Acta* **1989**, *216*, 69–107].

Theory and Practice

Every chemical reaction occurs at a finite rate, which makes it a potential candidate for a chemical kinetic method of analysis. To be effective, however, the chemical reaction must meet three necessary conditions: (1) the reaction must not occur too quickly or too slowly; (2) we must know the reaction's rate law; and (3) we must be able to monitor the change in concentration for at least one species. Let's take a closer look at each of these requirements.

> The material in this section assumes some familiarity with chemical kinetics, which is part of most courses in general chemistry. For a review of reaction rates, rate laws, and integrated rate laws, see the material in Appendix 17.

Reaction Rate

The ***rate*** of the chemical reaction—how quickly the concentrations of reactants and products change during the reaction—must be fast enough that we can complete the analysis in a reasonable time, but also slow enough that the reaction does not reach equilibrium while the reagents are mixing. As a practical limit, it is not easy to study a reaction that reaches equilibrium within several seconds without the aid of special equipment for rapidly mixing the reactants.

> We will consider two examples of instrumentation for studying reactions with fast kinetics later in this chapter.

Rate Law

The second requirement is that we must know the reaction's ***rate law***—the mathematical equation that describes how the concentrations of reagents affect the rate—for the period in which we are making measurements. For example, the rate law for a reaction that is first order in the concentration of an analyte, *A*, is

$$\text{rate} = -\frac{d[A]}{dt} = k[A] \tag{9.6.2.1}$$

where *k* is the reaction's ***rate constant***.

> Because the concentration of *A* decreases during the reactions, *d*[*A*] is negative. The minus sign in equation 9.6.2.1 makes the rate positive. If we choose to follow a product, *P*, then *d*[*P*] is positive because the product's concentration increases throughout the reaction. In this case we omit the minus sign.

An ***integrated rate law*** often is a more useful form of the rate law because it is a function of the analyte's initial concentration. For example, the integrated rate law for equation 9.6.2.1 is

$$\ln [A]_t = \ln [A]_0 - kt \tag{9.6.2.2}$$

or

$$[A]_t = [A]_0 e^{-kt} \tag{9.6.2.3}$$

where $[A]_0$ is the analyte's initial concentration and $[A]_t$ is the analyte's concentration at time t.

Unfortunately, most reactions of analytical interest do not follow a simple rate law. Consider, for example, the following reaction between an analyte, A, and a reagent, R, to form a single product, P

$$A + R \rightleftharpoons P$$

where k_f is the rate constant for the forward reaction, and k_r is the rate constant for the reverse reaction. If the forward and the reverse reactions occur as single steps, then the rate law is

$$\text{rate} = -\frac{d[A]}{dt} = k_f[A][R] - k_r[P] \tag{9.6.2.4}$$

The first term, $k_f[A][R]$ accounts for the loss of A as it reacts with R to make P, and the second term, $k_r[P]$ accounts for the formation of A as P converts back to A and to R.

Although we know the reaction's rate law, there is no simple integrated form that we can use to determine the analyte's initial concentration. We can simplify equation 9.6.2.4 by restricting our measurements to the beginning of the reaction when the concentration of product is negligible.

Under these conditions we can ignore the second term in equation 9.6.2.4, which simplifies to

$$\text{rate} = -\frac{d[A]}{dt} = k_f[A][R] \tag{9.6.2.5}$$

The integrated rate law for equation 9.6.2.5, however, is still too complicated to be analytically useful. We can further simplify the kinetics by making further adjustments to the reaction conditions [Mottola, H. A. *Anal. Chim. Acta* **1993**, *280*, 279–287]. For example, we can ensure pseudo-first-order kinetics by using a large excess of R so that its concentration remains essentially constant during the time we monitor the reaction. Under these conditions equation 9.6.2.5 simplifies to

$$\text{rate} = -\frac{d[A]}{dt} = k_f[A][R]_0 = k'[A] \tag{9.6.2.6}$$

where $k' = k_f[R]_0$. The integrated rate law for equation 9.6.2.6 then is

$$\ln [A]_t = \ln [A]_0 - k't \tag{9.6.2.7}$$

or

$$[A]_t = [A]_0 e^{-k't} \tag{9.6.2.8}$$

It may even be possible to adjust the conditions so that we use the reaction under pseudo-zero-order conditions.

$$\text{rate} = -\frac{d[A]}{dt} = k_f[A]_0[R]_0 = k''t \tag{9.6.2.9}$$

$$[A]_t = [A]_0 - k''t \tag{9.6.2.10}$$

where $k'' = k_f [A]_0[R]_0$.

To say that the reaction is pseudo-first-order in A means the reaction behaves as if it is first order in A and zero order in R even though the underlying kinetics are more complicated. We call k' a pseudo-first-order rate constant. To say that a reaction is pseudo-zero-order means the reaction behaves as if it is zero order in A and zero order in R even though the underlying kinetics are more complicated. We call k'' the pseudo-zero-order rate constant.

Monitoring the Reaction

The final requirement is that we must be able to monitor the reaction's progress by following the change in concentration for at least one of its species. Which species we choose to monitor is not important: it can be the analyte, a reagent that reacts with the analyte, or a product. For example, we can determine the concentration of phosphate by first reacting it with Mo(VI) to form 12-molybdophosphoric acid (12-MPA).

$$H_3PO_4(aq) + 6Mo(VI)(aq) \longrightarrow 12 - MPA(aq) + 9H^+(aq) \quad (9.6.2.11)$$

Next, we reduce 12-MPA to heteropolyphosphomolybdenum blue, PMB. The rate of formation of PMB is measured spectrophotometrically, and is proportional to the concentration of 12-MPA. The concentration of 12-MPA, in turn, is proportional to the concentration of phosphate [see, for example, (a) Crouch, S. R.; Malmstadt, H. V. *Anal. Chem.* **1967**, *39*, 1084–1089; (b) Crouch, S. R.; Malmstadt, H. V. *Anal. Chem.* **1967**, *39*, 1090–1093; (c) Malmstadt, H. V.; Cordos, E. A.; Delaney, C. J. *Anal. Chem.* **1972**, *44(12)*, 26A–41A]. We also can follow reaction 13.11 spectrophotometrically by monitoring the formation of the yellow-colored 12-MPA [Javier, A. C.; Crouch, S. R.; Malmstadt, H. V. *Anal. Chem.* **1969**, *41*, 239–243].

Reaction 9.6.2.11 is, of course, unbalanced; the additional hydrogens on the reaction's right side come from the six Mo(VI) that appear on the reaction's left side where Mo(VI) is thought to be present as the molybdate dimer $HMo_2O_6^+$.

There are several advantages to using the reaction's ***initial rate*** (t = 0). First, because the reaction's rate decreases over time, the initial rate provides the greatest sensitivity. Second, because the initial rate is measured under nearly pseudo-zero-order conditions, in which the change in concentration with time effectively is linear, it is easier to determine the slope. Finally, as the reaction of interest progresses competing reactions may develop, which complicating the kinetics: using the initial rate eliminates these complications. One disadvantage of the initial rate method is that there may be insufficient time to completely mix the reactants. This problem is avoided by using an intermediate rate measured at a later time (t > 0).

As a general rule (see Mottola, H. A. "Kinetic Determinations of Reactants Utilizing Uncatalyzed Reactions," *Anal. Chim. Acta* **1993**, *280*, 279–287), the time for measuring a reaction's initial rate should result in the consumption of no more than 2% of the reactants. The smaller this percentage, the more linear the change in concentration as a function of time.

Representative Method 13.2.1: Determination of Creatinine in Urine

Description of Method

Creatine is an organic acid in muscle tissue that supplies energy for muscle contractions. One of its metabolic products is creatinine, which is excreted in urine. Because the concentration of creatinine in urine and serum is an important indication of renal function, a rapid method for its analysis is clinically important. In this method the rate of reaction between creatinine and picrate in an alkaline medium is used to determine the concentration of creatinine in urine. Under the conditions of the analysis the reaction is first order in picrate, creatinine, and hydroxide.

$$\text{rate} = k[\text{ picrate }][\text{ creatinine }][OH^-]$$

The reaction is monitored using a picrate ion selective electrode.

Procedure

Prepare a set of external standards that contain 0.5–3.0 g/L creatinine using a stock solution of 10.00 g/L creatinine in 5 mM H_2SO_4, diluting each standard to volume using 5 mM H_2SO_4. Prepare a solution of 1.00×10^{-2} M sodium picrate. Pipet 25.00 mL of 0.20 M NaOH, adjusted to an ionic strength of 1.00 M using Na_2SO_4, into a thermostated reaction cell at 25ºC. Add 0.500 mL of the 1.00×10^{-2} M picrate solution to the reaction cell. Suspend a picrate ion selective in the solution and monitor the potential until it stabilizes. When the potential is stable, add 2.00 mL of a creatinine external standard and record the potential as a function of time. Repeat this procedure using the remaining external standards. Construct a calibration curve of $\Delta E/\Delta t$ versus the initial concentration of creatinine. Use the same procedure to analyze samples, using 2.00 mL of urine in place of the external standard. Determine the concentration of creatinine in the sample using the calibration curve.

Questions

1. The analysis is carried out under conditions that are pseudo-first order in picrate. Show that under these conditions the change in potential as a function of time is linear.

The potential, E, of the picrate ion selective electrode is given by the Nernst equation

$$E = K - \frac{RT}{F} \ln [\text{ picrate }]$$

where K is a constant that accounts for the reference electrodes, the junction potentials, and the ion selective electrode's asymmetry potential, R is the gas constant, T is the temperature, and F is Faraday's constant. We know from equation 9.6.2.7 that for a pseudo-first-order reaction, the concentration of picrate at time t is

$$\ln [\text{picrate}]_t = \ln [\text{picrate}]_0 - k't$$

where k' is the pseudo-first-order rate constant. Substituting this integrated rate law into the ion selective electrode's Nernst equation leaves us with the following result.

$$E_t = K - \frac{RT}{F} (\ln [\text{picrate}]_0 - k't)$$

$$E_t = K - \frac{RT}{F} \ln [\text{picrate}]_0 + \frac{RT}{F} k't$$

Because K and $(RT/F)\ln[\text{picrate}]_0$ are constants, a plot of E_t versus t is a straight line with a slope of $\frac{RT}{F}k'$.

2. Under the conditions of the analysis, the rate of the reaction is pseudo-first-order in picrate and pseudo-zero-order in creatinine and OH^-. Explain why it is possible to prepare a calibration curve of $\Delta E/\Delta t$ versus the concentration of creatinine.

The slope of a plot of E_t versus t is $\Delta E/\Delta t = RTk'/F$ = RTk'/F (see the previous question). Because the reaction is carried out under conditions where it is pseudo-zero-order in creatinine and OH^-, the rate law is

$$\text{rate} = k[\text{picrate}][\text{creatinine}]_0[OH^-]_0 = k'[\text{picrate}]$$

The pseudo-first-order rate constant, k', is

$$k' = k[\text{ creatinine }]_0 [OH^-]_0 = c[\text{creatinine}]_0$$

where c is a constant equivalent to $k[OH^-]_0$. The slope of a plot of E_t versus t, therefore, is linear function of creatinine's initial concentration

$$\frac{\Delta E}{\Delta t} = \frac{RTk'}{F} = \frac{RTc}{F} [\text{creatinine}]_0$$

and a plot of $\Delta E/\Delta t$ versus the concentration of creatinine can serve as a calibration curve.

3. Why is it necessary to thermostat the reaction cell?

The rate of a reaction is temperature-dependent. The reaction cell is thermostated to maintain a constant temperature to prevent a determinate error from a systematic change in temperature, and to minimize indeterminate errors from random fluctuations in temperature.

4. Why is it necessary to prepare the NaOH solution so that it has an ionic strength of 1.00 M?

The potential of the picrate ion selective electrode actually responds to the activity of the picrate anion in solution. By adjusting the NaOH solution to a high ionic strength we maintain a constant ionic strength in all standards and samples. Because the relationship between activity and concentration is a function of ionic strength, the use of a constant ionic strength allows us to write the Nernst equation in terms of picrate's concentration instead of its activity.

Making Kinetic Measurements

When using Representative Method 13.2.1 to determine the concentration of creatinine in urine, we follow the reactions kinetics using an ion selective electrode. In principle, we can use any of the analytical techniques in Chapters 8–12 to follow a reaction's kinetics provided that the reaction does not proceed to an appreciable extent during the time it takes to make a measurement. As you might expect, this requirement places a serious limitation on kinetic methods of analysis. If the reaction's kinetics are slow relative to the analysis time, then we can make a measurement without the analyte undergoing a significant change in concentration. If the reaction's rate is too fast—which often is the case—then we introduce a significant error if our analysis time is too long.

One solution to this problem is to stop, or quench the reaction by adjusting experimental conditions. For example, many reactions show a strong dependence on pH and are quenched by adding a strong acid or a strong base. Figure **9.6.2.6** shows a typical example for the enzymatic analysis of *p*-nitrophenylphosphate, which uses the enzyme wheat germ acid phosphatase to hydrolyze the analyte to *p*-nitrophenol. The reaction has a maximum rate at a pH of 5. Increasing the pH by adding NaOH quenches the reaction and converts the colorless *p*-nitrophenol to the yellow-colored *p*-nitrophenolate, which absorbs at 405 nm.

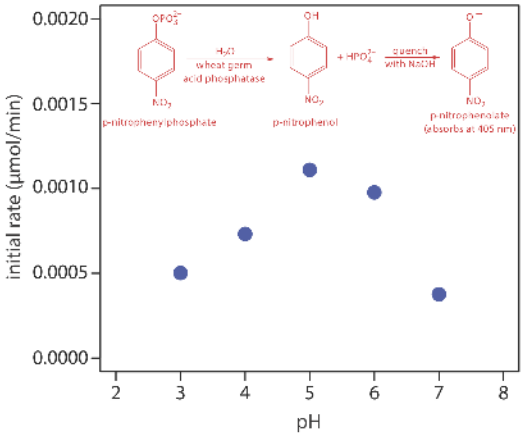


Figure 9.6.2.6. Initial rate for the enzymatic hydrolysis of *p*-nitrophenylphosphate using wheat germ acid phosphatase. Increasing the pH quenches the reaction and coverts colorless *p*-nitrophenol to the yellow-colored *p*-nitrophenolate, which absorbs at 405 nm.

An additional problem when the reaction's kinetics are fast is ensuring that we rapidly and reproducibly mix the sample and the reagents. For a fast reaction, we need to make our measurements within a few seconds—or even a few milliseconds—of combining the sample and reagents. This presents us with a problem and an advantage. The problem is that rapidly and reproducibly mixing the sample and the reagent requires a dedicated instrument, which adds an additional expense to the analysis. The advantage is that a rapid, automated analysis allows for a high throughput of samples. Instruments for the automated kinetic analysis of phosphate using reaction 9.6.2.11, for example, have sampling rates of approximately 3000 determinations per hour.

A variety of instruments have been developed to automate the kinetic analysis of fast reactions. One example, which is shown in Figure **9.6.2.7**, is the ***stopped-flow analyzer***. The sample and the reagents are loaded into separate syringes and precisely measured volumes are dispensed into a mixing chamber by the action of a syringe drive. The continued action of the syringe drive pushes the mixture through an observation cell and into a stopping syringe. The back pressure generated when the stopping syringe hits the stopping block completes the mixing, after which the reaction's progress is monitored spectrophotometrically. With a stopped-flow analyzer it is possible to complete the mixing of sample and reagent, and initiate the kinetic measurements in approximately 0.5 ms. By attaching an autosampler to the sample syringe it is possible to analyze up to several hundred samples per hour.

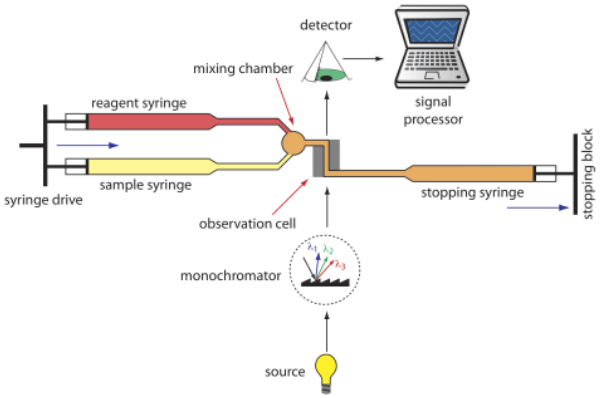


Figure 9.6.2.7. Schematic diagram of a stopped-flow analyzer. The blue arrows show the direction in which the syringes are moving.

Another instrument for kinetic measurements is the ***centrifugal analyzer***, a partial cross section of which is shown in Figure **9.6.2.8**. The sample and the reagents are placed in separate wells, which are oriented radially around a circular transfer disk. As the centrifuge spins, the centrifugal force pulls the sample and the reagents into the cuvette where mixing occurs. A single optical

source and detector, located below and above the transfer disk's outer edge, measures the absorbance each time the cuvette passes through the optical beam. When using a transfer disk with 30 cuvettes and rotating at 600 rpm, we can collect 10 data points per second for each sample.

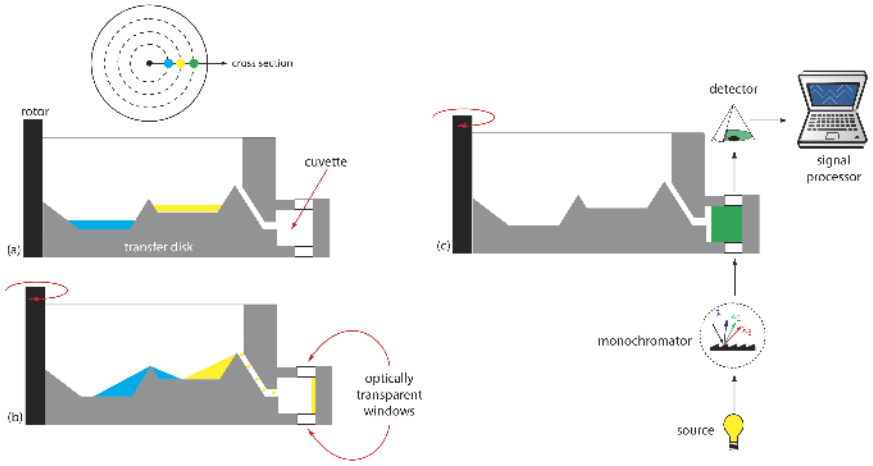


Figure 9.6.2.8. Cross sections through a centrifugal analyzer showing (a) the wells that hold the sample and the reagents, (b) the mixing of the sample and the reagents, and (c) the configuration of the spectrophotometric detector.

The ability to collect lots of data and to collect it quickly requires appropriate hardware and software. Not surprisingly, automated kinetic analyzers developed in parallel with advances in analog and digital circuitry—the hardware—and computer software for smoothing, integrating, and differentiating the analytical signal. For an early discussion of the importance of hardware and software, see Malmstadt, H. V.; Delaney, C. J.; Cordos, E. A. "Instruments for Rate Determinations," *Anal. Chem.* **1972**, *44(12)*, 79A–89A.

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9.7: Spectrophotometric Studies of Complex Ions

Absorption spectroscopy is one of the most powerful methods for determining the formulas of complex ions in solution and in determining their formation constants. The only requirement is that either or both the reactant or product absorbs light or that either the reactant of product can be caused to participate in a competing equilibrium that does not produce an absorbing species.

The three most common methods employed for studies of complex ions are (1) the method of continuous variations [also called the Job's method], (2) the mole-ratio methods, and (3) the slope-ratio method.

Method of Continuous Variations

Posted on July 29, 2013 by David Harvey on the Analytical Sciences Digital Library (asdlib.org)

The method of continuous variations, also called Job's method, is used to determine the stoichiometry of a metal-ligand complex. In this method we prepare a series of solutions such that the total moles of metal and ligand, n_{total}, in each solution is the same. If $(n_M)_i$ and $(n_L)i$ are, respectively, the moles of metal and ligand in solution i, then

$$n_{total} = (n_M)_i + (n_L)i$$

The relative amount of ligand and metal in each solution is expressed as the mole fraction of ligand, $(X_L)i$, and the mole fraction of metal, $(X_M)i$,

$$(X_L)i = (n_L)i/n_{total}$$

$$(X_M)i = 1 - (n_L)i/n_{total} = (n_M)i/n_{total}$$

The concentration of the metal–ligand complex in any solution is determined by the limiting reagent, with the greatest concentration occurring when the metal and the ligand are mixed stoichiometrically. If we monitor the complexation reaction at a wavelength where the metal–ligand complex absorbs only, a graph of absorbance versus the mole fraction of ligand will have two linear branches—one when the ligand is the limiting reagent and a second when the metal is the limiting reagent. The intersection of these two branches represents a stoichiometric mixing of the metal and the ligand. We can use the mole fraction of ligand at the intersection to determine the value of y for the metal–ligand complex MLy.

$$y = (n_L/n_M) = (X_L/X_M) = (X_L/1 - X_M)$$

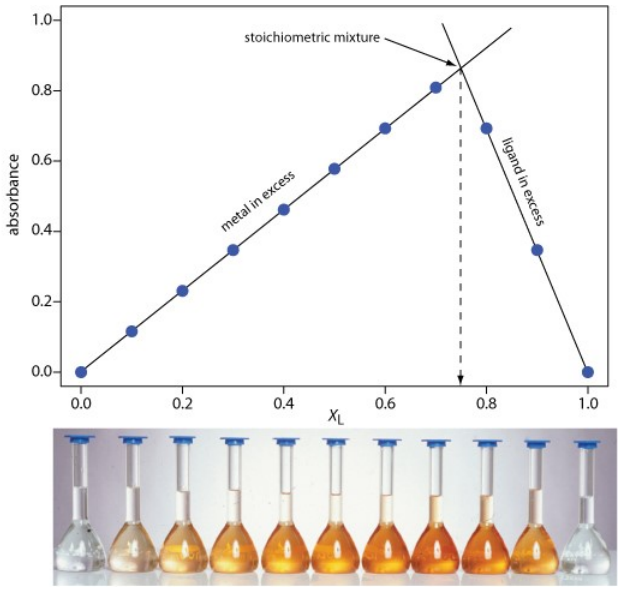


Figure 9.7.1: The illustration below shows a continuous variations plot for the metal–ligand complex between Fe^{2+} and *o*-phenanthroline. As shown here, the metal and ligand form the 1:3 complex Fe(*o*-phenanthroline)$_3^{2+}$.

Mole-Ratio Method for Determining Metal-Ligand Stoichiometry

Posted on July 29, 2013 by David Harvey on the Analytical Sciences Digital Library (asdlib.org)

An alternative to the method of continuous variations for determining the stoichiometry of metal-ligand complexes is the mole-ratio method in which the amount of one reactant, usually the moles of metal, is held constant, while the amount of the other reactant is varied. Absorbance is monitored at a wavelength where the metal–ligand complex absorbs.

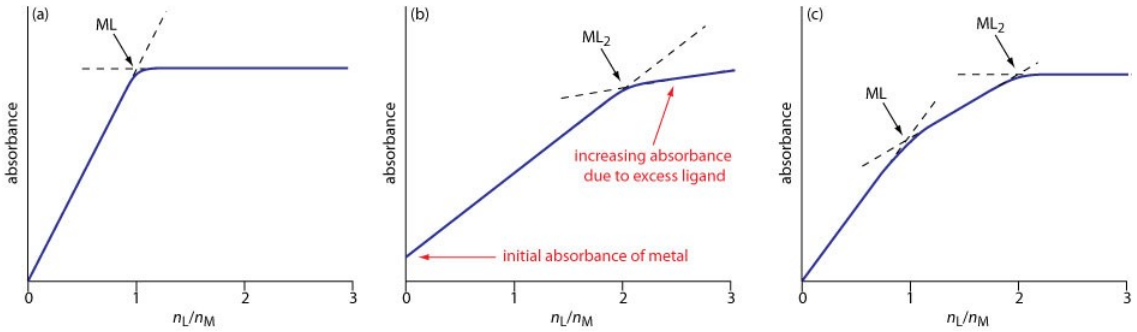


Figure **9.7.2**: The illustrations below show typical results: (a) the mole-ratio plot for the formation of a 1:1 complex in which the absorbance is monitored at a wavelength where only the complex absorbs; (b) the mole-ratio plot for a 1:2 complex in which all three species—the metal, the ligand, and the complex—absorb at the selected wavelength; and (c) the mole-ratio plot for the step-wise formation of ML and ML_2.

The Slope – Ratio Method

This method is useful for weak complexes and is applicable only to systems where a single complex is formed. The method assumes that the complex formation reaction can be forced to completion by a large excess of either the reactant metal ion or ligand and that Beer's law is followed.

For the reaction $mM + lL \rightleftarrows M_mL_l$

the following equation can be written, when L is present in very large excess.

$[M_mL_l] \approx F_M/m$

If Beer' law is obeyed

$Am = \epsilon b[M_mL_l] = \epsilon bF_M/m$

And a plot of A_m with respect to F_M will be linear. When M is very large with respect to L

$[M_mL_l] \approx F_L/l$

and

$A_l = \epsilon b[MmLl] = \epsilon bF_L/l$

A plot of A_l with respect to F_L will be linear. The slopes of straight line, (A_m/F_M) and (A_l/F_L) and the ratio of the slopes yields the combining ration of ligand to metal; l/m.

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CHAPTER OVERVIEW

10: Molecular Luminescence Spectrometry

Luminescence is emission of light by a substance not resulting from heat; it is thus a form of cold-body radiation. It can be caused by chemical reactions, electrical energy, subatomic motions, or stress on a crystal, which all are ultimately caused by Spontaneous emission. This distinguishes luminescence from incandescence, which is light emitted by a substance as a result of heating.

10.1: Fluorescence and Phosphorescence

10.2: Fluorescence and Phosphorescence Instrumentation

10.3: Applications of Photoluminescence Methods

10.3.1: Intrinsic and Extrinsic Fluorophores

10.3.2: The Stokes Shift

10.3.3: The Detection Advantage

10.3.4: The Fluorescence Lifetime and Quenching

10.3.5: Fluorescence Polarazation Analysis

10.3.6: Fluorescence Microscopy

Thumbanil: Chemiluminescence after a reaction of hydrogen peroxide and luminol. This is an image from video https://youtu.be/8_82cNtZSQE. Image used with permission (CC BY-SA 4.0; Tavo Romann).

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10.1: Fluorescence and Phosphorescence

Fluorescence and phosphorescence are types of molecular luminescence methods. A molecule of analyte absorbs a photon and excites a species. The emission spectrum can provide qualitative and quantitative analysis. The term fluorescence and phosphorescence are usually referred as photoluminescence because both are alike in excitation brought by absorption of a photon. Fluorescence differs from phosphorescence in that the electronic energy transition that is responsible for fluorescence does not change in electron spin, which results in short-live electrons ($<10^{-5}$ s) in the excited state of fluorescence. In phosphorescence, there is a change in electron spin, which results in a longer lifetime of the excited state (second to minutes). Fluorescence and phosphorescence occurs at longer wavelength than the excitation radiation.

Introduction

Fluorescence can occur in gaseous, liquid, and solid chemical systems. The simple kind of fluorescence is by dilute atomic vapors. A fluorescence example would be if a 3s electron of a vaporized sodium atom is excited to the 3p state by absorption of a radiation at wavelength 589.6 and 589.0 nm. After 10^{-8} s, the electron returns to ground state and on its return it emits radiation of the two wavelengths in all directions. This type of fluorescence in which the absorbed radiation is remitted without a change in frequency is known as resonance fluorescence. Resonance fluorescence can also occur in molecular species. Molecular fluorescence band centers at wavelengths longer than resonance lines. The shift toward longer wavelength is referred to as the Stokes Shift.

Singlet and Triplet Excited State

Understanding the difference between fluorescence and phosphorescence requires the knowledge of electron spin and the differences between singlet and triplet states. The Pauli Exclusion principle states that two electrons in an atom cannot have the same four quantum numbers (n, l, m_l, m_s) and only two electrons can occupy each orbital where they must have opposite spin states. These opposite spin states are called spin pairing. Because of this spin pairing, most molecules do not exhibit a magnetic field and are diamagnetic. In diamagnetic molecules, electrons are not attracted or repelled by the static electric field. Free radicals are paramagnetic because they contain unpaired electrons have magnetic moments that are attracted to the magnetic field.

Singlet state is defined when all the electron spins are paired in the molecular electronic state and the electronic energy levels do not split when the molecule is exposed into a magnetic field. A doublet state occurs when there is an unpaired electron that gives two possible orientations when exposed in a magnetic field and imparts different energy to the system. A singlet or a triplet can form when one electron is excited to a higher energy level. In an excited singlet state, the electron is promoted in the same spin orientation as it was in the ground state (paired). In a triplet excited stated, the electron that is promoted has the same spin orientation (parallel) to the other unpaired electron. The difference between the spins of ground singlet, excited singlet, and excited triplet is shown in Figure **10.1.1**. Singlet, doublet and triplet is derived using the equation for multiplicity, 2S+1, where S is the total spin angular momentum (sum of all the electron spins). Individual spins are denoted as spin up (s = +1/2) or spin down (s = -1/2). If we were to calculated the S for the excited singlet state, the equation would be 2(+1/2 + -1/2)+1 = 2(0)+1 = 1, therefore making the center orbital in the figure a singlet state. If the spin multiplicity for the excited triplet state was calculated, we obtain 2(+1/2 + +1/2)+1 = 2(1)+1 =3, which gives a triplet state as expected.

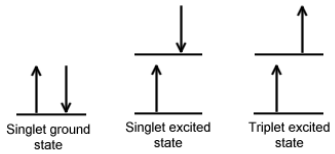


Figure **10.1.1**: Spin in the ground and excited states.

The difference between a molecule in the ground and excited state is that the electrons is diamagnetic in the ground state and paramagnetic in the triplet state.This difference in spin state makes the transition from singlet to triplet (or triplet to singlet) more improbable than the singlet-to-singlet transitions. This singlet to triplet (or reverse) transition involves a change in *electronic* state. For this reason, the lifetime of the triplet state is longer the singlet state by approximately 10^4 seconds fold difference.The radiation that induced the transition from ground to excited triplet state has a low probability of occurring, thus their absorption bands are less intense than singlet-singlet state absorption. The excited triplet state can be populated from the excited singlet state of certain

molecules which results in phosphorescence. These spin multiplicities in ground and excited states can be used to explain transition in photoluminescence molecules by the Jablonski diagram.

Jablonski Diagrams

The Jablonski diagram that drawn below is a partial energy diagram that represents the energy of photoluminescent molecule in its different energy states. The lowest and darkest horizontal line represents the ground-state electronic energy of the molecule which is the singlet state labeled as S_o. At room temperature, majority of the molecules in a solution are in this state.

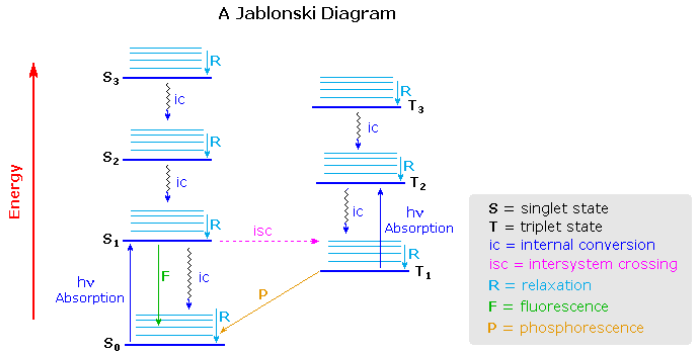


Figure **10.1.2**: Partial Jablonski Diagram for Absorption, Fluorescence, and Phosphorescence. from Bill Reusch.

The upper lines represent the energy state of the three excited electronic states: $S_1$and S_2 represent the electronic singlet state (left) and T_1 represents the first electronic triplet state (right). The upper darkest line represents the ground vibrational state of the three excited electronic state.The energy of the triplet state is lower than the energy of the corresponding singlet state.

There are numerous vibrational levels that can be associated with each electronic state as denoted by the thinner lines. Absorption transitions (blues lines in Figure **10.1.2**) can occur from the ground singlet electronic state (S_o) to various vibrational levels in the singlet excited vibrational states. It is unlikely that a transition from the ground singlet electronic state to the triplet electronic state because the electron spin is parallel to the spin in its ground state (Figure **10.1.1**). This transition leads to a change in multiplicity and thus has a low probability of occurring which is a **forbidden transition.** Molecules also go through vibration relaxation to lose any excess vibrational energy that remains when excited to the electronic states (S_1 and S_2) as demonstrated in wavy lines in Figure **10.1.2**. The knowledge of forbidden transition is used to explain and compare the peaks of absorption and emission.

Absorption and Emission Rates

The table below compares the absorption and emission rates of fluorescence and phosphorescence.The rate of photon absorption is very rapid. Fluorescence emission occurs at a slower rate.Since the triplet to singlet (or reverse) is a forbidden transition, meaning it is less likely to occur than the singlet-to-singlet transition, the rate of triplet to singlet is typically slower. Therefore, phosphorescence emission requires more time than fluorescence.

Table **10.1.1**: Rates of Absorption and Emission comparison.

Process	Transition	Timescale (sec)
Light Absorption (Excitation)	$S_0 \rightarrow S_n$	ca. 10^{-15} (instantaneous)
Internal Conversion	$S_n \rightarrow S_1$	10^{-14} to 10^{-11}
Vibrational Relaxation	$S_n^* \rightarrow S_n$	10^{-12} to 10^{-10}
Intersystem Crossing	$S_1 \rightarrow T_1$	10^{-11} to 10^{-6}
Fluorescence	$S_1 \rightarrow S_0$	10^{-9} to 10^{-6}
Phosphorescence	$T_1 \rightarrow S_0$	10^{-3} to 100
Non-Radiative Decay	$S_1 \rightarrow S_0$ $T_1 \rightarrow S_0$	10^{-7} to 10^{-5} 10^{-3} to 100

Deactivation Processes

A molecule that is excited can return to the ground state by several combinations of mechanical steps that will be described below and shown in Figure **10.1.2**.The deactivation process of fluorescence and phosphorescence involve an emission of a photon radiation as shown by the straight arrow in Figure **10.1.2**. The wiggly arrows in Figure **10.1.2** are deactivation processes without the use of radiation. The favored deactivation process is the route that is most rapid and spends less time in the excited state.If the rate constant for fluorescence is more favorable in the radiationless path, the fluorescence will be less intense or absent.

- **Vibrational Relaxation:** A molecule maybe to promoted to several vibrational levels during the electronic excitation process.Collision of molecules with the excited species and solvent leads to rapid energy transfer and a slight increase in temperature of the solvent. Vibrational relaxation is so rapid that the lifetime of a vibrational excited molecule ($<10^{-12}$) is less than the lifetime of the electronically excited state. For this reason, fluorescence from a solution always involves the transition of the lowest vibrational level of the excited state. Since the space of the emission lines are so close together, the transition of the vibrational relaxation can terminate in any vibrational level of the ground state.
- **Internal Conversion:** Internal conversion is an intermolecular process of molecule that passes to a lower electronic state without the emission of radiation.It is a crossover of two states with the same multiplicity meaning singlet-to-singlet or triplet-to-triplet states.The internal conversion is more efficient when two electronic energy levels are close enough that two vibrational energy levels can overlap as shown in between S_1 and S_2. Internal conversion can also occur between S_0 and S_1 from a loss of energy by fluorescence from a higher excited state, but it is less probable. The mechanism of internal conversion from S_1 to S_0 is poorly understood. For some molecules, the vibrational levels of the ground state overlaps with the first excited electronic state, which leads to fast deactivation.These usually occur with aliphatic compounds (compound that do not contain ring structure), which would account for the compound is seldom fluorescing. Deactivation by energy transfer of these molecules occurs so rapidly that the molecule does not have time to fluoresce.

- **External Conversion**: Deactivation of the excited electronic state may also involve the interaction and energy transfer between the excited state and the solvent or solute in a process called **external conversion.** Low temperature and high viscosity leads to enhanced fluorescence because they reduce the number of collision between molecules, thus slowing down the deactivation process.
- **Intersystem Crossing:** Intersystem crossing is a process where there is a crossover between electronic states of different multiplicity as demonstrated in the singlet state to a triplet state (S_1 to T_1) on Figure **10.1.1**. The probability of intersystem crossing is enhanced if the vibration levels of the two states overlap. Intersystem crossing is most commonly observed with molecules that contain heavy atom such as iodine or bromine. The spin and orbital interaction increase and the spin become more favorable.Paramagnetic species also enhances intersystem crossing, which consequently decreases fluorescence.
- **Phosphorescence**: Deactivation of the electronic excited state is also involved in phosphorescence. After the molecule transitions through intersystem crossing to the triplet state, further deactivation occurs through internal or external fluorescence or phosphorescence. A triplet-to-singlet transition is more probable than a singlet-to-singlet internal crossing. In phosphorescence, the excited state lifetime is inversely proportional to the probability that the molecule will transition back to the ground state. Since the lifetime of the molecule in the triplet state is large (10^{-4} to 10 second or more), transition is less probable which suggest that it will persist for some time even after irradiation has stopped. Since the external and internal conversion compete so effectively with phosphorescence, the molecule has to be observed at lower temperature in highly viscous media to protect the triplet state.

Variables that affect Fluorescence

After discussing all the possible deactivation processes, variable that affect the emissions to occur. Molecular structure and its chemical environment influence whether a substance will fluoresce and the intensities of these emissions. The **quantum yield** or **quantum efficiency** is used to measure the probability that a molecule will fluoresce or phosphoresce. For fluorescence and phosphorescence is the ratio of the number of molecules that luminescent to the total number of excited molecules. For highly fluoresce molecules, the quantum efficiency approaches to one.Molecules that do not fluoresce have quantum efficiencies that approach to zero.

Fluorescence quantum yield (ϕ) for a compound is determined by the relative rate constants (k) of various deactivation processes by which the lowest excited singlet state is deactivated to the ground state. The deactivation processes including fluorescence (kf), intersystem crossing (k_i), internal conversion (kic), predissociation (kpd), dissociation (kd), and external conversion (kec) allows one to qualitatively interpret the structural and environmental factors that influence the intensity of the fluorescence. They are related by the quantum yield equation given below:

$$\frac{k_f}{k_f + k_i + k_{ec} + k_{ic} + k_{pd} + k_d} \tag{10.1.1}$$

Using this equation as an example to explain fluorescence, a high fluorescence rate (k_f) value and low values of the all the other relative rate constant terms ($k_f + k_i + k_{ec} + k_{ic} + k_{pd} + k_d$) will give a large ϕ, which suggest that fluorescence is enhanced. The magnitudes of kf , kd, and kpd depend on the chemical structure, while the rest of the constants ki, kec, and kic are strongly influenced by the environment.

Fluorescence rarely results from absorption of ultraviolet radiation of wavelength shorter than 250 nm because radiation at this wavelength has sufficient energy to deactivate the electron in the excited state by predissociation or dissociation. The bond of some organic molecules would rupture at 140 kcal/mol, which corresponds to 200-nm of radiation. For this reason, $\sigma \rightarrow \sigma^*$ transition in fluorescence are rarely observed. Instead, emissions from the less energetic transition will occur which are either $\pi^* \rightarrow \pi$ or $\pi^* \rightarrow n$ transition.

Molecules that are excited electronically will return to the lowest excited state by rapid vibrational relaxation and internal conversion, which produces no radiation emission. Fluorescence arises from a transition from the lowest vibrational level of the first excited electronic state to one of the vibrational levels in the electronic ground state. In most fluorescent compounds, radiation is produced by a $\pi^* \rightarrow \pi$ or $\pi^* \rightarrow n$ transition depending on which requires the least energy for the transition to occur.

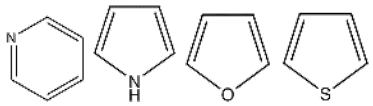
Fluorescence is most commonly found in compounds in which the lowest energy transition is $\pi \rightarrow \pi^*$ (excited singlet state) than $n \rightarrow \pi^*$ which suggest that the quantum efficiency is greater for $\pi \rightarrow \pi^*$ transitions. The reason for this is that the molar absorptivity,

which measures the probability that a transition will occur, of the $\pi \rightarrow \pi^*$ transition is 100 to 1000 fold greater than $n \rightarrow \pi^*$ process. The lifetime of $\pi \rightarrow \pi^*$ (10^{-7} to 10^{-9} s) is shorter than the lifetime of $n \rightarrow \pi^*$ (10^{-5} to 10^{-7}).

Phosphorescent quantum efficiency is the opposite of fluorescence in that it occurs in the $n \rightarrow \pi^*$ excited state which tends to be short lived and less suceptable to deactivation than the $\pi \rightarrow \pi^*$ triplet state. Intersystem crossing is also more probable for $\pi \rightarrow \pi^*$ excited state than for the $n \rightarrow \pi^*$state because the energy difference between the singlet and triplet state is large and spin-orbit coupling is less likely to occur.

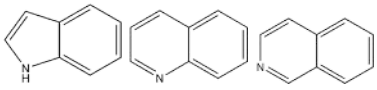
Fluorescence and Structure

The most intense fluorescence is found in compounds containing aromatic group with low-energy $\pi \rightarrow \pi^*$ transitions. A few aliphatic, alicyclic carbonyl, and highly conjugated double-bond structures also exhibit fluorescence as well. Most unsubstituted aromatic hydrocarbons fluoresce in solution too. The quantum efficiency increases as the number of rings and the degree of condensation increases. Simple heterocycles such as the structures listed below do not exhibit fluorescence.



Pyridine Pyrrole Furan Thiophene

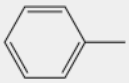
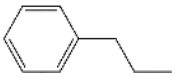
With nitrogen heterocyclics, the lowest energy transitions is involved in $n \rightarrow \pi^*$ system that rapidly converts to the triplet state and prevents fluorescence. Although simple heterocyclics do not fluoresce, fused-ring structures do. For instance, a fusion of a benzene ring to a hetercyclic structure results in an increase in molar absorptivity of the absorption band. The lifetime of the excited state in fused structure and fluorescence is observed. Examples of fluorescent compounds is shown below.



quinoline

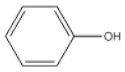
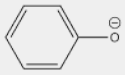
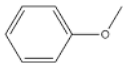
Benzene ring substitution causes a shift in the absorption maxima of the wavelength and changes in fluorescence emission. The table below is used to demonstrate and visually show that as benzene is substituted with increasing methyl addition, the relative intensity of fluorescence increases.

Table 2. Relative intensity of fluorescence comparison with alkane substituted benzenes.

Compound	Structure	Wavelength of Fluorescence (nm)	Relative intensity of Fluorescence
Benzene		270-310	10
Toluene		270-320	17
Propyl Benzene		270-320	17

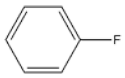
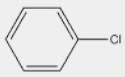
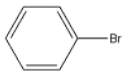
The relative intensity of fluorescence increases as oxygenated species increases in substitution. The values for such increase is demonstrated in the table below.

Table **10.1.3**: Relative intensity of fluorescence comparison with benzene with oxygenated substituted benzene

Compound	Structure	Wavelength of Fluorescence (nm)	Relative intensity of Fluorescence
Phenol		285-365	18
Phenolate ion		310-400	10
Anisole		285-345	20

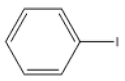
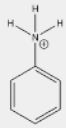
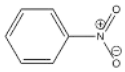
Influence of a halogen substitution decreases fluorescence as the molar mass of the halogen increases. This is an example of the "heavy atom effect" which suggest that the probability of intersystem crossing increases as the size of the molecule increases. As demonstrated in the table below, as the molar mass of the substituted compound increases, the relative intensity of the fluorescence decreases.

Table 10.1.4: Relative intensity fluorescence comparison with halogen substituted compounds

Compound	Structure	Wavelength of Fluorescence (nm)	Relative intensity of Fluorescence
Fluorobenzene		270-320	10
Chlorobenzene		275-345	7
Bromobenzene		290-380	5

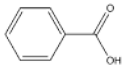
In heavy atom substitution such as nitro derivatives or heavy halogen substitution such as iodobenzene, the compounds are subject to predissociation. These compounds have bonds that easily rupture that can then absorb excitation energy and go through internal conversion. Therefore, the relative intensity of fluorescence and fluorescent wavelength is not observed and this is demonstrated in the table below.

Table 10.1.5: Relative fluorescent intensities of iodobenzene and nitro derivative compounds

Compound	Structure	Wavelength of Fluorescence (nm)	Relative intensity of Fluorescence
Iodobenzene		None	0
Anilinium ion		None	0
Nitrobenzene		None	0

Carboxylic acid or carbonyl group on aromatic ring generally inhibits fluorescence since the energy of the $n \rightarrow \pi^*$ transition is less than $\pi \rightarrow \pi^*$ transition. Therefore, the fluorescence yield from $n \rightarrow \pi^*$ transition is low.

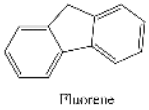
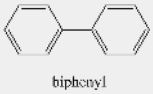
Table **10.1.6**: *Relative fluorescent intensity of benzoic acid*

Compound	Structure	Wavelength of Fluorescence (nm)	Relative intensity of Fluorescence
Benzoic Acid		310-390	3

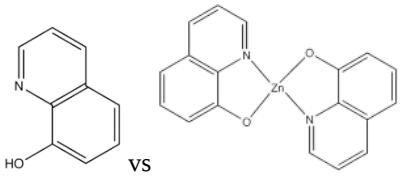
Effect of Structural Rigidity on Fluorescence

Fluorescence is particularly favored in molecules with rigid structures. The table below compares the quantum efficiencies of fluorine and biphenyl which are both similar in structure that there is a bond between the two benzene group. The difference is that fluorene is more rigid from the addition methylene bridging group. By looking at the table below, rigid fluorene has a higher quantum efficiency than unrigid biphenyl which indicates that fluorescence is favored in rigid molecules.

Table **10.1.7**: Quantum Efficiencies in Rigid vs. Nonrigid structures

Compound	Structure	Quantum Efficiency
Fluorene		1.0
Biphenyl		0.2

This concept of rigidity was used to explain the increase in fluorescence of organic chelating agent when the compound is complexed with a metal ion. The fluorescence intensity of 8-hydroxyquinoline is much less than its zinc complex.



8-hydroxyquinoline 8-hydroxyquinoline with Zinc complexed

The explanation for lower quantum efficiency or lack of rigidity in caused by the enhanced internal conversion rate (k_{ic}) which increases the probability that there will be radiationless deactivation. Nonrigid molecules can also undergo low-frequency vibration which accounts for small energy loss.

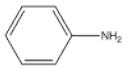
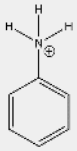
Temperature and Solvent Effects

Quantum efficiency of Fluorescence decreases with increasing temperature. As the temperature increases, the frequency of the collision increases which increases the probability of deactivation by external conversion. Solvents with lower viscosity have higher possibility of deactivation by external conversion. Fluorescence of a molecule decreases when its solvent contains heavy atoms such as carbon tetrabromide and ethyl iodide, or when heavy atoms are substituted into the fluorescing compound. Orbital spin interaction result from an increase in the rate of triplet formation, which decreases the possibility of fluorescence. Heavy atoms are usually incorporated into solvent to enhance phosphorescence.

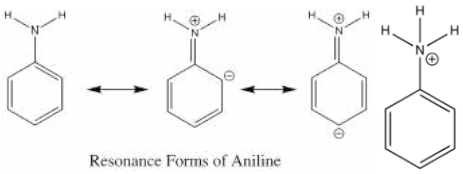
Effect of pH on Fluorescence

The fluorescence of aromatic compound with basic or acid substituent rings are usually pH dependent. The wavelength and emission intensity is different for protonated and unprotonated forms of the compound as illustrated in the table below:

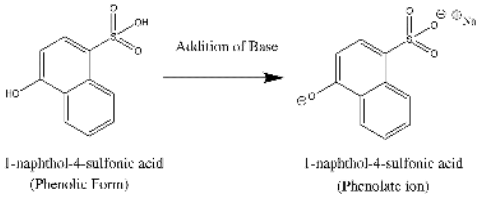
Table **10.1.8**: Quantum efficiency comparison due to protonation

Compound	Structure	Wavelength of Fluorescence (nm)	Relative intensity of Fluorescence
aniline		310-405	20
Anilinium ion		None	0

The emission changes of this compound arises from different number of resonance structures associated with the acidic and basic forms of the molecule.The additional resonance forms provides a more stable first excited state, thus leading to fluorescence in the ultraviolet region.The resonance structures of basic aniline and acidic anilinium ion is shown below:



basic Aniline Fluorescence of certain compounds have been used a detection of end points in acid-base titrations.An example of this type of fluorescence seen in compound as a function of pH is the phenolic form of 1-naphthol-4-sulfonic acid.This compound is not detectable with the eye because it occurs in the ultraviolet region, but with an addition of a base, it becomes converted to a phenolate ion, the emission band shifts to the visible wavelength where it can be visually seen. Acid dissociation constant for excited molecules differs for the same species in the ground state.These changes in acid or base dissociation constant differ in four or five orders of magnitude.



Dissolved oxygen reduces the intensity of fluorescence in solution, which results from a photochemically induced oxidation of fluorescing species.Quenching takes place from the paramagnetic properties of molecular oxygen that promotes intersystem crossing and conversion of excited molecules to triplet state.Paramagnetic properties tend to quench fluorescence.

Effects of Concentration on Fluorescence Intensity

The power of fluorescence emission F is proportional to the radiant power is proportional to the radiant power of the excitation beam that is absorbed by the system. The equation below best describes this relationship.

$$F=\phi_f K''(P_0-P)=K'(P_0-P) \quad (1)$$

Since $\phi_f K''$ is constant in the system, it is represented at K'. The table below defines the variables in this equation.

Table 9: Definitions of all the variables defined in the Fluorescence Emission (F) in Equation 1.

Variable	Definition
F	Power of fluorescence emission
P0	Power of incident beam on solution
P	Power after transversing length b in medium
K”	Constant dependent on geometry and other factors
ϕ_f	Quantum efficiency

Fluorescence emission (F) can be related to concentration (c) using Beer’s Law stating:

$$F = \epsilon bc \tag{10.1.2}$$

where ϵ is the molar absorptivity of the molecule that is fluorescing. Rewriting Equation 2 gives:

$$P = P_0 10^{-\epsilon bc} \tag{10.1.3}$$

Substituting Equation 10.1.3 into Equation 10.1.2 and factoring out P_0 gives us this equation:

$$F = K' P_0 \left(1 - 10^{-\varepsilon bc}\right) \tag{10.1.4}$$

The MacLaurin series could be used to solved the exponential term.

$$F = K' P_0 \left[2.303\varepsilon bc - \frac{(2.303\varepsilon bc)^2}{2!} + \frac{(2.303\varepsilon bc)^3}{3!} + \frac{(2.303\varepsilon bc)^4}{4!} + \ldots \frac{(2.303\varepsilon bc)^n}{n!}\right]_1 \tag{10.1.5}$$

Given that $(2.303\epsilon bc = \text{Absorbance} < 0.05$, all the subsequent terms after the first can be dropped since the maximum error is 0.13%. Using only the first term, Equation 10.1.5 can be rewritten as:

$$F = K' P_0 2.303\varepsilon bc \tag{10.1.6}$$

Equation 10.1.6 can be expanded to the equation below and simplified to compare the fluorescence emission F with concentration. If the equation below were to be plotted with F versus c, a linear relation would be observed.

$$F = \phi_f K'' P_0 2.303\varepsilon bc \tag{10.1.7}$$

If c becomes so great that the absorbance > 0.05, the higher terms in Equation 10.1.5 start to become more important and the linearity is lost. F then lies below the extrapolation of the straight-line plot. This excessive absorption is the **primary absorption.** Another cause of this negative downfall of linearity is the **secondary absorption** when the wavelength of emission overlaps the absorption band. This occurs when the emission transverse the solution and gets reabsorbed by other molecules by analyte or other species in the solution, which leads to a decrease in fluorescence.

Quenching Methods

Dynamic Quenching is a nonradiative energy transfer between the excited and the quenching agent species (Q).The requirements for a successful dynamic quenching are that the two collision species the concentration must be high so that there is a higher possibility of collision between the two species.Temperature and quenching agent viscosity play a role on the rate of dynamic quenching.Dynamic quenching reduces fluorescence quantum yield and the fluorescence lifetime.

Dissolved oxygen in a solution increases the intensity of the fluorescence by photochemically inducing oxidation of the fluorescing species.Quenching results from the paramagnetic properties of molecular oxygen that promotes intersystem crossing and converts the excited molecules to triplet state.Paramagnetic species and dissolved oxygen tend to quench fluorescence and quench the triplet state.

Static quenching occurs when the quencher and ground state fluorophore forms a dark complex.Fluorescence is usually observed from unbound fluorophore.Static quenching can be differentiated from dynamic quenching in that the lifetime is not affected in

static quenching.In long range (Förster) quenching, energy transfer occurs without collision between molecules, but dipole-dipole coupling occurs between excited fluorophore and quencher.

Emission and Excitation Spectra

One of the ways to visually distinguish the difference between each photoluminescence is to compare the relative intensities of emission/excitation at each wavelength. An example of the three types of photoluminescence (absorption, fluorescence and phosphorescence) is shown for phenanthrene in the spectrum below.In the spectrum, the luminescent intensity is measure in a wavelength is fixed while the excitation wavelength is varied. The spectrum in red represents the excitation spectrum, which is identical to the absorption spectrum because in order for fluorescence emission to occur, radiation needs to be absorbed to create an excited state.The spectrum in blue represent fluorescence and green spectrum represents the phosphorescence.

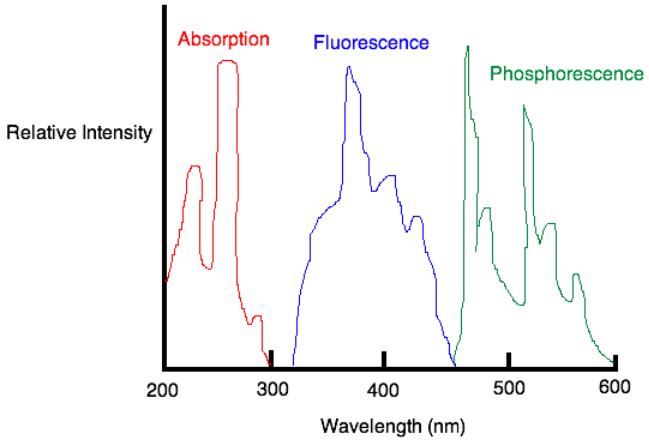


Figure 3: Wavelength Intensities of Absorption, Fluorescence, and Phosphorescence

As a real example illustrating the "mirror" relationship between the absorbance and emission spectra is shown in Figure 4. Figure 4 depicts a small portion of the absorbance spectrum for fluorescein in blue as well as the and fluorescence spectra in red. Fluorescein is a common dye used in analytical chemistry as well as ophthalmology.

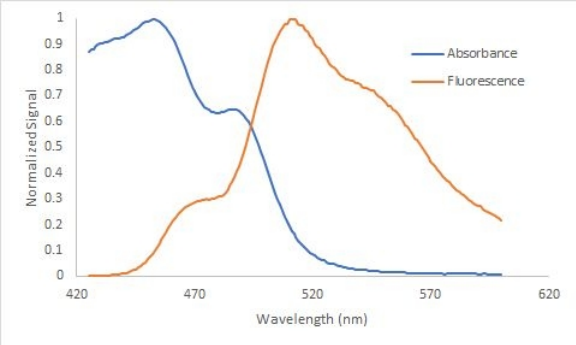


Figure 4. The absorbance and fluorescence spectrum for fluorescein illustrating the "mirroring" between the two spectra because of the similarity in energy level spacing within the ground state and lowest excited state. The two spectra also illustrate the Stokes shift, the emission of light at a longer wavelength than the excitation wavelength, λ_{max} for emission ~ 520 nm and λ_{max} for excitation ~ 450 nm. Both spectra are normalized and the concentration for the absorbance spectrum is 3 x 10^{-6} M and the the fluorescence spectrum 1 x 10^{-7} M.

Fluorescence and Phosphorescence occur at wavelengths that are longer than their absorption wavelengths.Phosphorescence bands are found at a longer wavelength than fluorescence band because the excited triplet state is lower in energy than the singlet state.The difference in wavelength could also be used to measure the energy difference between the singlet and triplet state of the molecule. The wavelength (λ) of a molecule is inversely related to the energy (E) by the equation below:

$$E = \frac{hc}{\lambda} \tag{10.1.8}$$

As the wavelength increases, the energy of the molecule decrease and vice versa.

References

1. D. A. Skoog, et al. "Principles of Instrumental Analysis" 6th Edition, Thomson Brooks/Cole. 2007
2. D. C. Harris and M.D. Bertolucci "Symmetry and Spectroscopy, An Introduction to Vibrational and Electronic Spectroscopy" Dover Publications, Inc., New York. 1989.

Problems

1. Draw and label the Jablonski Diagram.
2. How do spin states differ in ground singlet state versus excite singlet state and triplet excited state?
3. Describe the rates of deactivation process.
4. What is quantum yield and how is it used to compare the fluorescence of different types of molecule?
5. What roles do solvent play in fluorescence?

Contributors

- Diana Wong (UCD)

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10.2: Fluorescence and Phosphorescence Instrumentation

The components of instruments designed for fluorescence spectroscopy use many of the same components found in instruments for absorbaance spectroscopy in the UV -Vis.

Figure 10.2.1 shows a typical configuration for a filter based fluorometer or a monochromotor based spectrofluorometer.

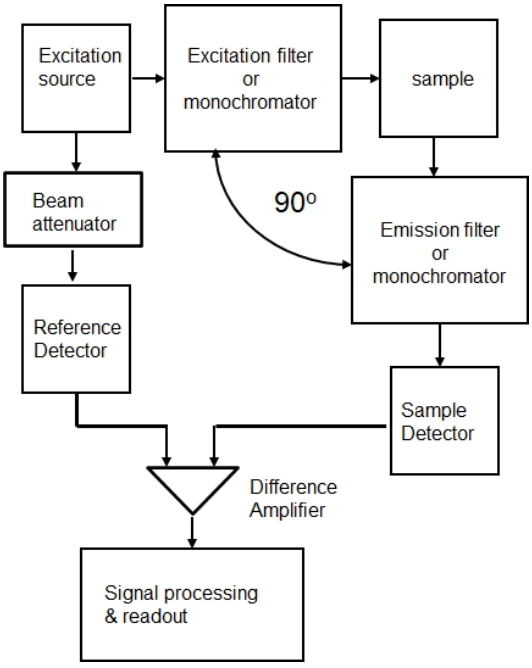
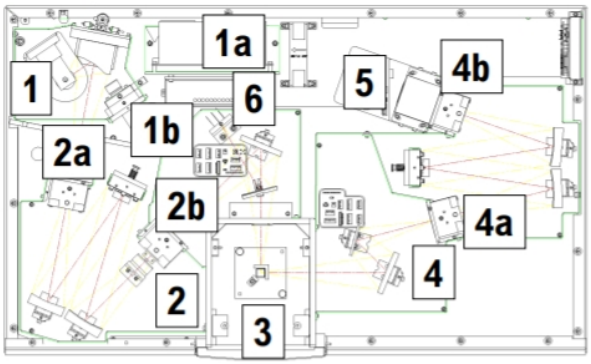


Figure **10.2.1**: Components of a fluorometer or spectroflorometer.

A fluorometer is a filter based, fixed wavelength, instrument suitable for established quantitative fluorescence methods. A spectrofluorometer is equipped with two scanning monochromoators permitting variation in excitation wavelength, the emission wavelength or both (constant energy difference mode). The selectivity offered by spectrofluorometers is important to investigators, especially physical chemists, concerned with the spectral and structural characterization of molecules. When operated in the emission mode, with the excitation wavelength fixed and the emission wavelengths scanned, the intensity of the light emitted offers a map of the vibrational energy levels (and rotational levels for gas phase samples) of the ground electronic state. When operated in the excitation mode, with the emission wavelength fixed and the excitation wavelengths scanned and with suitable corrections for variations in the excitation intensity, the intensity of the light emitted offers a map of the vibrational energy levels (and again rotational energy levels for gas phase samples) of the excited state and a spectrum that closely resembles an absorption spectrum.

The optical layout of a Horiba Scientific FluorMax 4 spectropfluorometer is shown below in Figure 10.2.2



1 Xenon arc-lamp and lamp housing
1a Xenon-lamp power supply
1b Xenon flash lamp (FluoroMax®-4P only)
2 Excitation monochromator
2a & 2b Slits
3 Sample compartment
4 Emission monochromator
4a & 4b Slits
5 Signal detector (photomultiplier tube and housing)
6 Reference detector (photodiode and current-acquisition module)
Host computer (not on diagram)

Figure **10.2.2**: The optical layout of a FluoroMax 4 spectrofluorometer. This image was taken from the FluorMax 4 installation manual (https://www.horiba.com/fileadmin/upl...Manual_USB.pdf)

Specific components

Sources

In most applications optical sources with larger output intensities greater than a tungsten halogen lamp or a deuterium lamp are required (See Equation
10.1.7).

The most common source for simple fluorometers is the low pressure mercury lamp which produce line emission at 254 , 302, 313, 546, 578, 691, and 773 nm. Individual lines from a mercury lamp can be isolated with an interference. More recently LED's with wavelengths reaching into the UV have become useful. LEDs with narrow band emission (+/- 15 nm) are available at 365, 385, 395 or 405 as well as numerous wavelengths into the visible and near IR.

The most common source spectrofluorometers is the high pressure xenon arc lamp. Available in sizes ranging from 75 to 450 Watt, the high pressure xenon arc lamp produces near blackbody radiation covering a range from 300 nm to 1300 nm.

Wavelength Selectors

Interference filters are the most commonly used filter to select the excitation wavelength and both interference and short wavelenght cut-off (or long pass) filters are commonly used to select the emission light. Most sprectrofluorometers are equipped with two grating based monochromoators.

Transducers

Typical fluorescence signals are of low light intensities and consequently the most common transducers used are large gain photomultiplier tubes (PMTs). Often the PMTs are operated in photon-counting mode to gain an improvement in signal-to-noise. Cooling of the transducer is sometimes used to improve the signal-to-noise. Diode array and CCD transducers are sometimes used in spectrofluorometers, especially in small hand held instruments and in detectors for liquid chromatography and capillary electrophoresis.

Cells

Both cylindrical and rectangular cells fabricated from glass, fused silica or plastic are employed for fluorescence measurement. Rectangular cells need to have four polished sides. Care should be taken in the grade of optical materials and in the design of the cell compartment to reduce the amount of scattered light. Even more than in absorbance measurements, it is important to avoid fingerprints on cells because skin oils often fluorescence

Instrument Standardization and Calibration

Because of variations in source intensity, transducer sensitivity, and other instrument variables, it is impossible to obtain with a given fluorometer or spectrofluorometer exactly the same reading for a solution or set of solutions from day to day. For this reason it is common practice to standardize an instrument and set it to a reproducible sensitivity level. Standardization is often carried out with a standard solution of a stable fluorophore. The most common standard reagent is quinine sulfate having a concentration of about 10-5 M. Quinine sulfate can be excited at 350 nm and it emits strongly at 450 nm.

Quinine sulfate in 0.1 M acid solution is also a commonly used standard for quantum yield measurements. In these measurements, the integrated fluorescence is recorded for a series of quinine sulfate solutions (excitation 350 nm, emission 400 - 650 nm) with absorbances less than 0.03 a.u.. A similar set of measurements is made for a fluorophore with unknown quantum yield. The integrated fluorescence is plotted versus the solution absorbance for both quinine sulfate and the unknown and the slope of the best fit lines are calculated. The quantum yield of the unknown is then the product of the established quantum yield for quinine sulfate (0.54) and the ratio of the slopes (unknown / quinine sulfate).

Calibration of the wavelength scales for the monochromators in a spectrofluorometer is done using cell containing clean water. The calibration of the excitation wavelength scale is based on the scattered light coming from the xenon arc lamp. In the excitation mode, with the emission monochromator set to 350 nm (1 nm slit width) the scattered light is recorded by scanning the excitation wavelength from 200 to 600 nm (1 nm slit width). The strongest sharp spectral feature should be observed at 467 nm or adjusted to be so. The calibration of the emission monochromator is based on the Raman spectrum of water. In the emission mode, with the excitation monochromator set to 350 nm (5 nm slit width), the Raman spectrum of water is recorded by scanning the emission monochromator from 365 to 450 nm (5 nm slit width). The single peak observed should be centered at 397 nm or adjusted to be so.

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10.3: Applications of Photoluminescence Methods

In the following sub-sections the topics listed below will be presented to show the wide impact of fluorescence spectroscopy in chemistry and biochemistry.

1. Intrinsic and extrinsic fluorophores
2. The Stokes shift
3. The detection advantage
4. The fluorescence lifetime and quenching
5. Fluorescence polarization analysis
6. Fluorescence microscopy

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10.3.1: Intrinsic and Extrinsic Fluorophores

Intrinsic and Extrinsic Fluorophores

An intrinsic fluorophore is a ion, molecule or macromolecule that fluoresces strongly in it native form while an extrinsic fluorophore is a species that has been made to fluoresce strongly through reaction with a fluorometric reagent.

Among organic molecules only a small fraction are intrinsic fluorophores. These molecules tend to possess rigid structures, that hinder release of the excited state energy to the bath, and extensive delocalized ***pi*** bonding networks. Fluorescein and quinine are good examples of fluorophores with large quantum yields.

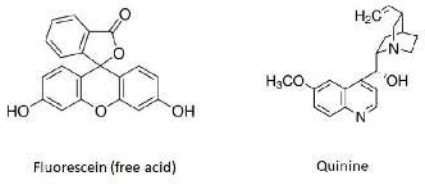


Figure **10.3.1.1**: The structures of fluorescein and quinine.

Both the quantum yield and the emission wavelength can change for fluorophores that can participate in acid base chemistry depending on whether they are protonated or not.

Most metal ions, be they main group ions, transition metal ions do not fluoresce and only a few lanthanide ions fluoresce weakly. In fact metal ions that are paramagnetic are more likely phosphoresce following intersystem crossing or to act to quench the fluorescence of an excited state fluorophore. Also for transition metal ions, they are characterized by many closely spaced energy levels which increases the likelihood of deactivation by internal conversion.

Extrinsic fluorophores can be formed via chelation reactions with many metal ions. A metal chelate is a combination of a metal ion with an organic molecule to which the metal ion is attached at one point by a primary bond and to another part of the molecule by a secondary bond. Common linkages are from between a metal ion with a 2,2'-dihydroxy azo dye or 8-quinolinol and are shown below in Figure**10.3.1.2**: for (1) Al and (2) Be.

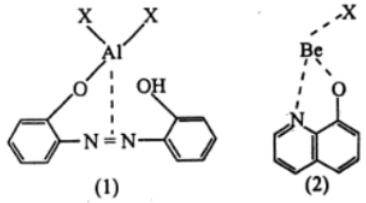


Figure **10.3.1.2**: Two examples of metal chelate structures

This combination usually results in the metal ion being a member of a 5 or 6 membered ring, increasing the rigidity of the organic molecule. This combination is not the cause or origin of the fluorescence because the molecule is likely to be weakly fluorescent. However, in many cases the emission is shifted to a longer wavelength and the fluorescence intensity is greatly increased.

For the lanthanides ion, the fluorescence of the metal chelate complexes is most intense when the metal ion has the oxidation state of of 3+. Not all lanthanide metals can be used and the most common are: Sm(III), Eu(III), Tb(III), and Dy(III)

In the realm of biochemistry and bioanalytical chemistry, tryptophan is the only intrinsic fluorophore among the natural amino acids and it's excitation and emission wavelengths are in the UV (maximum absorption of 280 nm and an emission peak that is solvatochromic, ranging from 300 to 350 nm depending in the polarity of the local environment). DNA and RNA are not intrinsic fluorophores.

Proteins, DNA and RNA can be made fluorescent thorough well established labeling chemistry with fluormetric reagents that are either reactive towards amines and carboxylic acids or in the case of DNA, intercalate between the base pairs. Two example of fluorometric reagents that react with amines are show in Figure**10.3.1.3**: below.

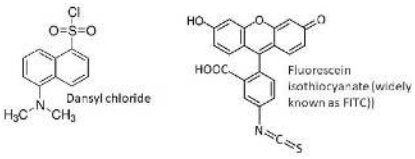


Figure **10.3.1.3**: Two fluorometric reagents used to put a fluorescent label on a biological macromolecule through a reaction with an amine group.

One of the most important developments in bioanalytical chemistry has been the development and application of the green fluorescent protein first isolated from jelly fish in the 1960's and 1970's . The original close of the green fluorescent protein were not particularly useful due to dual peaked excitation spectra, pH sensitivity, chloride sensitivity, poor fluorescence quantum yield, poor photostability and poor folding at 37 °C. However, due to the potential for widespread usage and the evolving needs of researchers, many different mutants of GFP have been engineered. The first major improvement was a single point mutation (S65T) reported in 1995 in *Nature* by Roger Tsien. This mutation dramatically improved the spectral characteristics of GFP, resulting in increased fluorescence, photostability, and a shift of the major excitation peak to 488 nm, with the peak emission kept at 509 nm. As shown in Figure **10.3.1.4**: below, many other mutations are available, including different color mutants; in particular, blue fluorescent protein, cyan fluorescent protein, and yellow fluorescent protein derivatives.

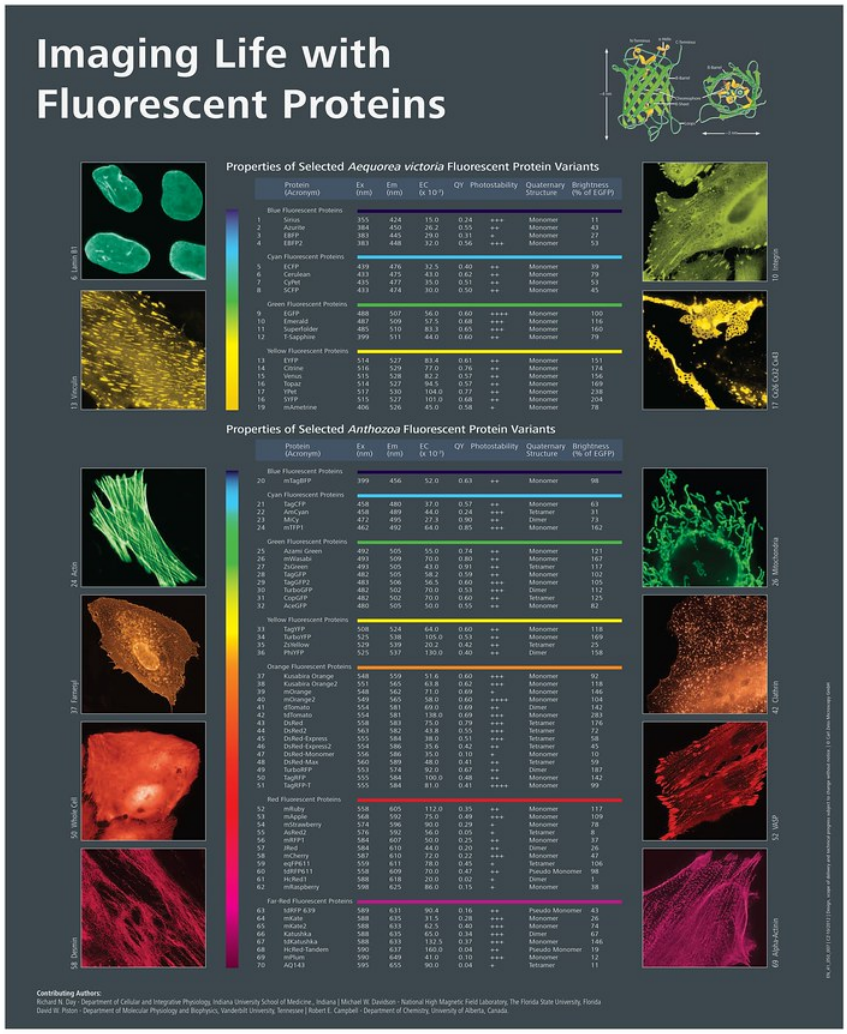


Figure **10.3.1.4**: "Imaging Life with Fluorescent Proteins" by ZEISS Microscopy is licensed under CC BY-SA 2.0

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10.3.2: The Stokes Shift

The Stokes shift is the term used to describe the difference in the wavelength at which a molecule emits light is relative to the wavelength at which the molecule was excited. As shown in the Jablonski diagram below in Figure **10.3.2.1**, the energy associated with the blue arrows indicating fluorescence is never longer (more energy) than the green arrows indicating absorption.

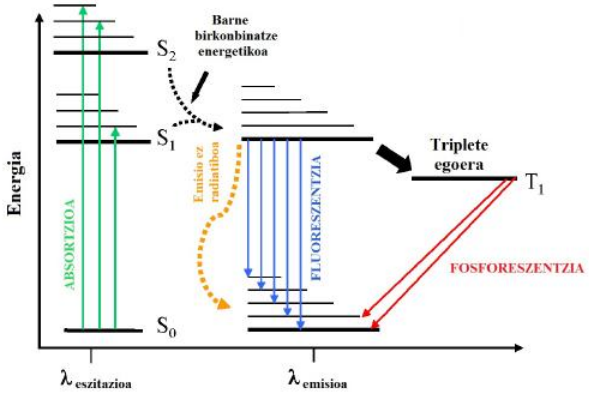


Figure **10.3.2.1**: "File:Diagramme de Jablonski-EU.jpg" by Iratipedia is licensed under CC BY-SA 4.0

The absorption of a photon is a very fast process taking place on the femtosecond time scale or faster. Consequently, not only are the nuclei in the species of interest frozen in position during the time (the Franck-Condon Principle) are any molecules in the bath surrounding the species of interest. Thus any interaction of the bath molecules with the species of interest is largely limited to the ground electronic state. These interactions lead to the relatively small solvent dependent shifts observed in absorption spectroscopy in the UV and Vis (2 - 40 nm).

Fluorescence in condensed media takes place on the nanosecond time scale, a time scale during which solvent molecules in the bath can reorientate or relax around the molecule in the excited state. Figure **10.3.2.2**: is a simple energy level diagram for illustrating the absorption, solvent reorientation, and emission processes. For most species, the excited state is much more polar than the ground state and μ_e is much larger than μ_g. As shown in Figure**10.3.2.2**: the polar solvent molecules can quickly reorientate their dipoles to stabilize the energy of the excited state to a much greater extent than for the ground state. Consequently, the energy difference between the excited and ground state can be much less following solvent reorientation and the Stokes shift much greater.

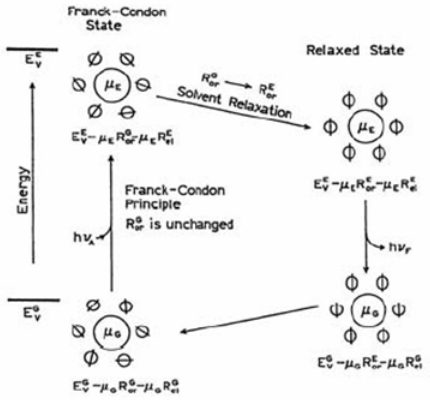
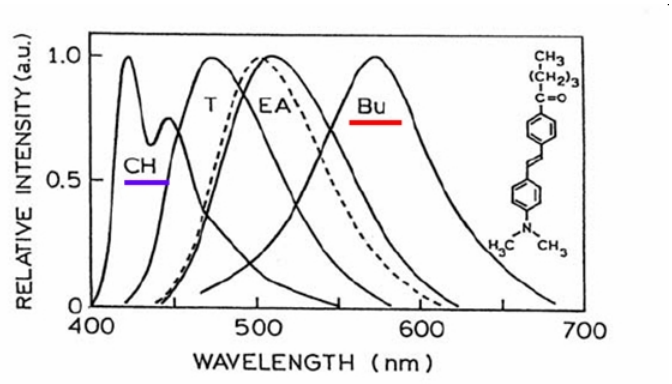


Figure **10.3.2.2**: A simple energy level diagram for the absorption and emission processes in the presence of a strongly interacting solvent. Lakowicz principles of fluorescence spectroscopy

Thus the Stokes shift for fluorescence is a much more sensitive reported for the local environment of the fluorophore. In Figure **10.3.2.3** the fluorescence of a lipophilic styrene fluorophore of the kind used in studies of biomembranes is shown for a series

of solvents ranging from very nonpolar (cyclohexane) to very polar (butanol). Over this range of solvents, the peak in the emission intensity ranges from 420 nm to almost 600 nm and the peak in emission intensity when the fluorophore is interacting with a model DPPC membrane vesicle reveals the fluorophore is contained in volume of polar molecules, as found in the inside of the membrane based vesicle.



Corrected fluorescence emission spectra of DOS in cyclohexane (CH), toluene (T), ethylacetate (EA) and butanol (Bu). The dashed line shows the emission of DOS from DPPC vesicles.

Figure **10.3.2.3**: Lakowicz principles of fluorescence spectroscopy

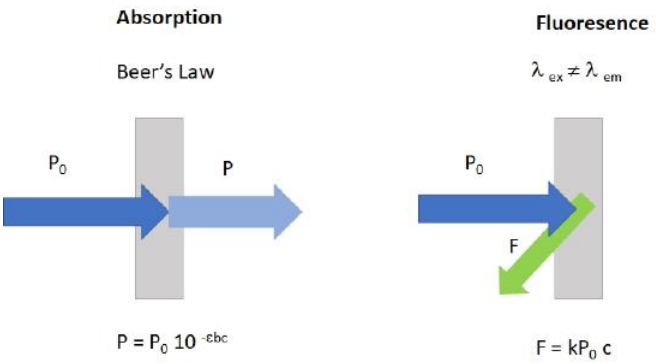
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10.3.3: The Detection Advantage

Fluorescence methods are among the most sensitive analytical methods available to chemists. In fact with a specialized instrument setup, one can detect the fluorescence from a single molecule. The harder trick in this experiment is isolating the single fluorophore in the focal area of the instrument.

In general and for experiments routinely available instrumentation, fluorescence methods have sensitivities that are one to three orders of magnitude better that absorbance measurements. The enhanced sensitivity for fluorescence arises from the difference between the wavelength of emission relative to wavelength of excitation and the fact that the concentration related parameter for fluorescence, the fluorescence intensity F, can be measured independent of the power of the optical source, P_0. In contrast, an absorbance measurement requires evaluation of both P_0 and P, because absorbance, which according to Beer's Law is proportional to concentration, is dependent on the ratio of the two quantities.

Consider Figure **10.3.3.1** illustrating the difference between the absorbance and fluorescence experiments.



As we saw in Chapter 8, as the concentration of the absorbing species decreases the difference between P_0 and P gets smaller and the uncertainty with measuring this difference increases. This lead to a lower limit of detection for a strongly absorbing species with a molar absorptivity of 50,000 liter/(mole cm) to approximately 10^{-6} M.

In the case of fluorescence there is no light to detect at the emission wavelength (not even scattered light at that wavelength) unless the fluorophore is present in the sample. One can either collect the fluorescence signal, F, for a longer time or increase P_0 to detect measurable signal. Consequently the lower limit of detection in fluorescence is on the of 10^{-10} M.

It is also true that the minimum volume of required sample is much less for fluorescence than for absorbance. However, the upper limit in terms of concentration for absorbance is much larger and the accuracy and precision for quantitative fluorescence methods is poorer by a factor of about 5X.

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10.3.4: The Fluorescence Lifetime and Quenching

The fluorescence Lifetime is the average time it takes for a molecule after absorption to return to its ground state. While the fluorescence process for a individual fluorophore is a stochastic process Absorption and emission processes are almost always studied on populations of molecules and the average properties of a molecule of the population are deduced from the macroscopic properties of the process.

In order to describe the behavior of the excited state population we define the following:

• n is the number of molecules in the ground state (•) at time t.

• n * is the number of excited molecules (•) at time t.

• Γ is the rate constant of emission. The dimensions of Γ are sec^{-1} (transitions per molecule per unit time).

• f(t) is an arbitrary function of the time, describing the time course of the excitation .

In Figure**10.3.4.1**: a laser beam is shown passing through a solution containing fluorophores. At any given time some fluorophores will be excited (•), while the rest will be in its ground state (•).

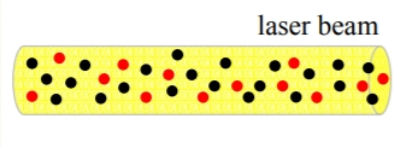


Figure**10.3.4.1**: was taken from Fluorescence Workshop UMN Physics June 8-10, 2006 Fluorescence Lifetime + Polarization (2) presented by Joachim Mueller (https://fitzkee.chemistry.msstate.ed...90/Fluoro5.pdf)

The excited state population of fluorophores is described by a rate equation:

$$\frac{dn^*}{dt} = -n^*\Gamma + nf(t)$$

where n + n* = n_0 (n_0 describes the total number of molecules and i s a constant)

As shown in Figure**10.3.4.2**: , the excitation intensity in a steady-state experiment is constant. In other words the function f(t) is constant. The solution of the rate equation for a constant f(t) is given by a constant excited state population:

$$n^* = \frac{n_0 I_{ex}}{\Gamma + I_{ex}}$$

where f(t) = I_{ex} describes the excitation intensity. Note that the fluorescence intensity is directly proportional to the excited state population: $I_F \propto n^*$

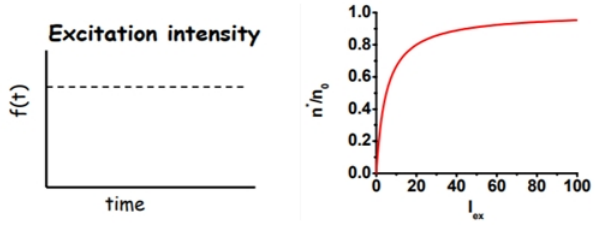


Figure**10.3.4.2** was taken from Fluorescence Workshop UMN Physics June 8-10, 2006 Fluorescence Lifetime + Polarization (2) presented by Joachim Mueller (https://fitzkee.chemistry.msstate.ed...90/Fluoro5.pdf)

The excited state population is initially directly proportional to the excitation intensity I_{ex} (linear regime), but saturates at higher excitation intensities (because one can not drive more molecules in the excited state than are available).

In general, steady state experiments are conducted under conditions where we are far from saturation, where $n^* \propto I_{ex}$

Lets consider a different experiment, shown in Figure**10.3.4.3**, involving a short pulse of excitation light, much shorter than the lifetime of the fluorophore, say less than 10^{-12} s in duration) applied to the sample at t = 0.

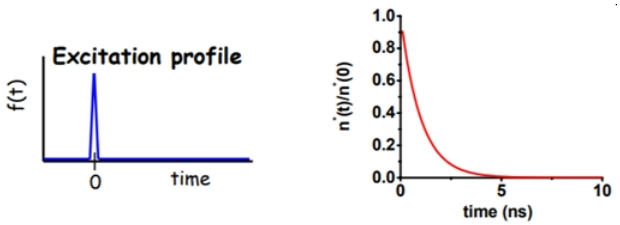


Figure**10.3.4.3** was taken from Fluorescence Workshop UMN Physics June 8-10, 2006 Fluorescence Lifetime + Polarization (2) presented by Joachim Mueller (https://fitzkee.chemistry.msstate.ed...90/Fluoro5.pdf)

The solution of the rate equation is given by an exponential decay of the excited state population:

$$n^*(t) = n^*(0)e^{-rt}$$

If a population of fluorophores are excited, the lifetime is the time it takes for the number of excited molecules to decay to 1/e or 36.8% of the original population according to:

$$\frac{n^*(t)}{n^*(0)} = e^{-t/\tau}$$

and we can define the lifetime, τ as equal to Γ^{-1}.

Experimentally we can only observe the radiative (fluorescent) decay. Nonradiative processes are alternative paths for the molecule to return to its ground state. These additional paths lead to a faster decay (of the excited state population. Thus we observe a faster decay of the fluorescence intensity.

The lifetime and quantum yield for a given fluorophore is often dramatically affected by its environment. In the gas phase fluorescence lifetimes are the long because there is little interaction with bath molecules leading to non radiative deactivation. In condensed media the lifetime is a reporter of the local environment of the fluorophore. For example, NADH, has a lifetime in water of ~ 0.4 ns but when bound to dehydrogenases the lifetime can be a long as 9 ns.

Commercial instruments for fluorescence lifetime measurements are readily available and based either the impulse response (as described above) or the harmonic response method which relies on a modulated excitation beam and the relative phase lag of the emitted light. In principle both methods have the same information content. These methods are also referred to as either the “time domain” method or the “frequency domain” method and will be left to the curious student to discover and learn about on their own.

Fluorescence Quenching and Fluorescence Resonance Energy Transfer

As said in the section on the Stokes shift, fluorescence is a very sensitive method for studying the local environment around the fluorophore. Two methods are very commonly employed to learn about the local environment on a molecular scale are quenching and Fluorescence Resonance Energy Transfer (FRET and not yet discussed in this LibreText). Quenching can also be employed for quantitative work and the interactions between anions and quenchers is a useful way to measure the concentration of the anions.

Quenching occurs via two distinct pathways. Collisional quenching occurs when the excited state fluorophore is deactivated upon contact with some quencher molecule in solution. Static quenching occurs when a fluorophore forms a non-fluorescent complex with a quencher and is no longer excitable.

Let's consider the quantum yield for a fluorophore in the absence of a quenching species

$$\phi_{\text{with out quench}} = \frac{k_f}{k_f + k_{isc} + k_{ic}}$$

and in the presence of a quencher, Q

$$\phi_{\text{with quench}} = \frac{k_f}{k_f + k_{isc} + k_{ic} + k_{\text{quench}}[Q]}$$

the ratio of these two equations yields

$$\frac{\phi_{\text{withoutquench}}}{\phi_{\text{withquench}}} = 1 + \frac{k_{\text{quench}}[Q]}{k_f + k_{isc} + k_{ic}} = 1 + k_{\text{quench}}[Q] = \mathrm{F_0/F}$$

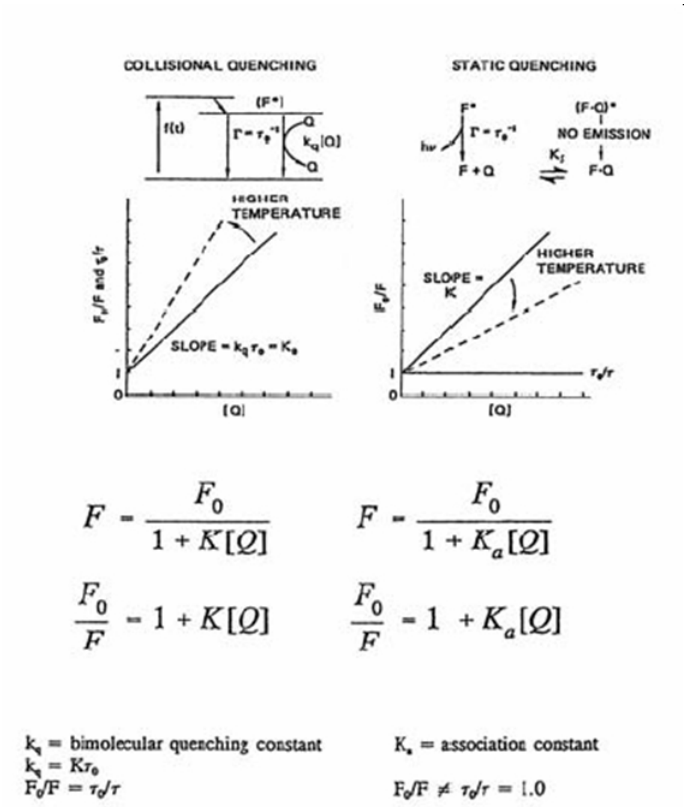
The expression

$$\frac{\mathrm{F_0}}{\mathrm{F}} = \frac{\tau_0}{\tau} = 1 + K[\mathrm{Q}]$$

is known as the Stern-Volmer equation and the equivalence to the ratio of the fluorescence lifetimes without, τ_0, and with, τ, the quencher only holds for the collisional quencher case.

An experiment done last year in the physical chemistry laboratory was focused on the time scales for the quenching of anthracene's fluorescence by carbon tetrachloride. In the laboratory portion of this course we will examine the quenching of a sulfonated quinine dye, SPQ by chloride to develop a method for the measurement of the concentration of chloride in solution.

One can often determine whether or not one is dealing with a collisional quencher or a static quenching by examining the extent of quenching as a function of quencher concentration at at least two temperatures. Consider Figure**10.3.4.4** comparing the two forms of quenching.



$$F = \frac{F_0}{1 + K[Q]} \qquad F = \frac{F_0}{1 + K_a[Q]}$$

$$\frac{F_0}{F} = 1 + K[Q] \qquad \frac{F_0}{F} = 1 + K_a[Q]$$

k_q = bimolecular quenching constant
$k_q = K\tau_0$
$F_0/F = \tau_0/\tau$

K_a = association constant
$F_0/F \neq \tau_0/\tau = 1.0$

Figure**10.3.4.4**: image source currently unknown

If quenching is occurring by a collisional deactivation of the excited state, according to the kinetic molecular theory, as the temperature of the system is increased the number of collisions between with fluorophore in it's excited state and the quencher will increase and the fluorescence signal will be smaller relative to the the intensity with the same concentration of quencher but at a lower temperature. Consequently the slope of the Stern-Volmer plot will be greater at the higher temperature relative to the slope at the lower temperature. The same will be true for a Stern-Volmer plot based on the ratio of the fluorescence lifetimes without, τ_0, and with, τ, the quencher.

If static quenching is causing the decrease in the fluorscence and increase in system temperature is more likely to rupture the bond associating the fluorophore and quencher resulting in an increase in the fluorescence intensity. For static quenching the slope of the Stern-Volmer plot will be less at the higher temperature relative to the slope at lower temperature. Since the observed liftime is only based on the free fluorphores the ratio of of the fluorescence lifetimes without, τ_0, and with, τ, the quencher will be unchanged.

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10.3.5: Fluorescence Polarazation Analysis

Fluorescence polarization measurements, or anisotropy measurements, provide information about the size and shape of molecules, the rigidity of molecular environments or the size of cavities.

In a steady state instrument one must add a linear polarization filter to select one polarization of the excitation light beam and a polarization analyzer to measure the fluorescence intensity perpendicular and parallel to the polarization of the excitation light. These are shown in Figure **10.3.5.1**. As suggested in this figure, the linear polarizer and polarization analyzer can often be a selected option for a research grade spectrofluorometer.

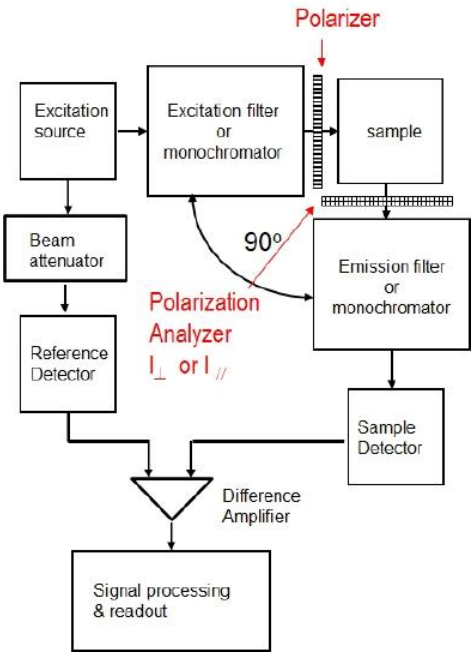


Figure **10.3.5.1**: A Block diagram of a steady state spectrofluorometer equipped with a excitation polarizer and an emission polarization analyzer.

As shown in the Figure **10.3.5.2**:parts (a) and (b), fluorophores preferentially absorb photons with electric field vectors that are aligned parallel to the transition moment of the fluorophore. Figure **10.3.5.2** (a) is the system before any fluorophores absorb light and Figure **10.3.5.1** (b) shows that only the fluorophores with an electric field vector that have absorbed a photon and are in the excited state.

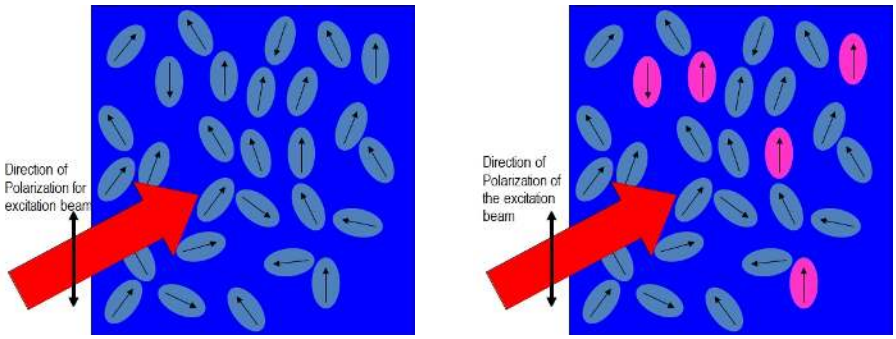


Figure**10.3.5.2**; parts (a) and (b): Illustrating the preferential excitation of fluorophores with transition moments parallel to the electric field vector of the linearly polarized excitation beam.

Emission also occurs with the light polarized along the transition moment of the excited state fluorophore. The relative angle between the polarization of the incident light and the polarization of the emission determines the anisotropy, r.

$$r = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + 2F_{\perp}} = \frac{r_0}{1 + (\tau/\omega)}$$

Where $F_{\parallel}$ is the intensity of the emission polarized parallel to the polarization of the excitation beam and $\mathbf{F}_{\perp}$ is the intensity of the emission polarized perpendicular to that of the excitation beam. In the simplest form of the experiment the emission is measured twice, once with the polarization analyzer orientated with it's direction parallel to the direction of the polarizer and a second time with its direction perpendicular to the direction of the polarizer. Based on the collection of the light being along only one direction, the anisotropy, r, can range from 0 to 0.4. A r value of 0 indicates the emitted light is randomly polarized or depolarized.

An alternative measure of the anisotropy of the emitted light is the polarization, P, of the system given by

$$P = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + F_{\perp}}$$

Small fluorophores rotate extensively in 50 – 100 psec and can rotate many times during the nanosecond fluorescence lifetime of the excited state. Macromolecules take much longer to rotate. A big change occurs for a small molecular fluorophore when it becomes bound to macromolecule, trapped in a viscous environment, or contained in a small restrictive cavity. Figure **10.3.5.31** is a illustration of the large difference in rotational correlation time when a small, quickly rotating, fluorophore (green rectangle) associates with the large, slowly rotating, macromolecule (purple blob).

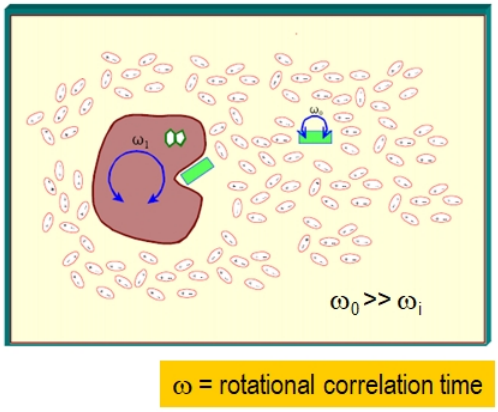


Figure **10.3.5.3**: image source currently unknown

Consequently, fluorescence polarization analysis can be a useful method to measure binding events between fluorophores and macromolecules. In the example shown below, the interaction between acridine orange AO, a DNA intercalator, and a DNA oligomer is shown. The anisotropy data, plotted over the range of the emission spectrum, shows the emission is much more polarized (r = 0.240) when the AO is bound to the DNA strand then when it is free in solution (r = 0.018) and largely depolarized.

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10.3.6: Fluorescence Microscopy

An important use of fluorescence spectroscopy techniques is in the area of microscopy, and this is especially true in the realm of biology and biochemistry. In this section a brief description of fluorescence microscopy will be presented with an emphasis on techniques to improve the resolution.

A fluorescence microscope is much the same as a conventional light microscope with added features to enhance its capabilities.

- The conventional microscope uses visible light (400-700 nanometers) to illuminate and produce a magnified image of a sample.
- A fluorescence microscope, on the other hand, uses a much higher intensity light source which excites a fluorescent species in a sample of interest. This fluorescent species in turn emits a lower energy light of a longer wavelength that produces the magnified image instead of the original light source.

Fluorescent microscopy is often used to image specific features of small specimens such as microbes. It is also used to visually enhance 3-D features at small scales. This can be accomplished by attaching fluorescent tags to anti-bodies that in turn attach to targeted features, or by staining in a less specific manner. When the reflected light and background fluorescence is filtered in this type of microscopy the targeted parts of a given sample can be imaged. This gives an investigator the ability to visualize desired organelles or unique surface features of a sample of interest.

A block diagram of a typical wide field fluorescence microscope and a picture of a readily available fluorescence microscope is shown in the Figure**10.3.6.1**.

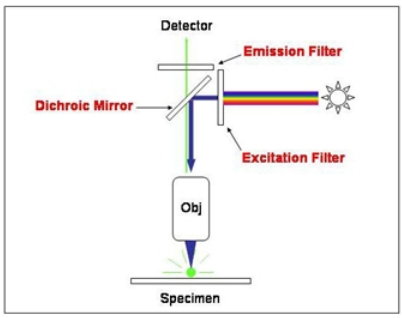




Figure**10.3.6.1**: A schematic diagram of a wide-field fluorescence microscope and a picture of a typical commercially available fluorescence microscope. these images were taken from Fluorescent Microscopy by George Rice, Montana State University (https://serc.carleton.edu/microbelif.../fluromic.html)

A wide field microscope contains a detector capable of recording the entire image in a single collection (a digital camera) without moving the sample. A dichroic mirror is a mirror that transmits one wavelength (color) of light but reflects another wavelength (color). Most fluorescence microscope come equipped with a small set of dichroic mirrors appropriate for a small set of fluorescence labels. Like all simple optical microscopes the spatial resolution is limited to approximately λ. However, the emitted light is collected from regions above and below the focal plane of the microscope objective leading to a lesser ability to discern sharp features in the sample.

The first major improvement in terms of resolution for a fluorescence microscope was the invention of the confocal fluorescence microscope. As shown in the Figure**10.3.6.2**, a pinhole has been introduced before the detector to spatially limit the volume from which the fluorescence is collected. The pinhole optical element along with the microscope objective give this instrument two focal points along the light path. Light is collected only from the focal point of the microscope objective and emitted light from above and below the focal point of the microscope objective is discriminated (blocked) by the focal point created by the pinhole. Images are collected by moving the sample in a point by point raster pattern and the image is reconstructed from the signal at each point. Confocal microscope are commercially available albeit at a much higher cost relative to a wide field fluorescence microscope.

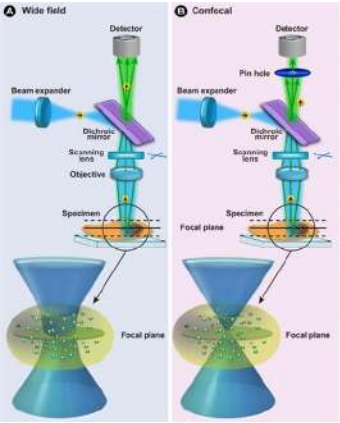


Figure**10.3.6.2**: An illustration of a confocal fluorescence microscopy along side an illustration of a wide field fluorescene microscopy. This image was adapted from one found in New approaches in renal microscopy - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/...fig2_299444264

Another major improvement in terms of resolution comes with the two-photon fluorescence microscope. A two photon fluorescence microscope employs a laser that emits light for which a single photon is insufficiently energetic enough to of excite the fluorophore. Because of the high spectral brightness of the laser, when the light focused by the microscope objective an intense electric field is created at the focal point and the energy associated with two photons is sufficient to excite the fluorophore. The difference between one photon excitation with short wavelength light and two photon excitation with long wavelength light is schematically shown in F Figure**10.3.6.3**.

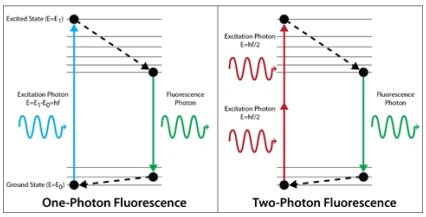


Figure**10.3.6.3**: An energy level diagram illustrating the difference between one photon excitation with short wavelenght light and two photon excitation with long wavelength light.

Because the electric field is sufficiently large only at the focal point the emission from the excited fluorophore only comes from this small volume region. Figure**10.3.6.4** is a picture showing the fluorescence in a one and two photon experiment in a cuvette. In Figure 10.3.6.4 each absorbed photon at 488 nm is capable of exciting the fluorophore and hence the emission is observed coming from a very large volume region. The 960 nm is insufficient of exciting the fluorophore with a single photon and only excites the fluorophore in the small volume region at the focal point .

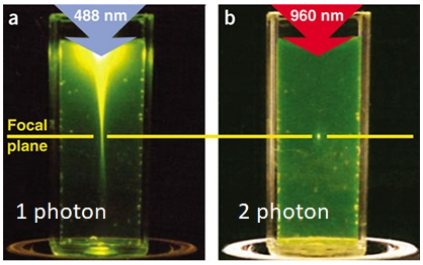


Figure**10.3.6.4**: In this photo the small volume region in which the excited fluorophores are produced with two photon excitation with 960 nm light is compared to the large volume region in which the excited fluorophores are produced with one photon excitation with 488 nm light. This image was taken from Zipfel, W., Williams, R. & Webb, W. Nonlinear magic: multiphoton microscopy in the biosciences. *Nat Biotechnol* **21,** 1369–1377 (2003). https://doi.org/10.1038/nbt899

A two photon fluorescence microscope is depicted in Figure**10.3.6.5** where red excitation beam shown is coming from a laser. Again in this type of fluorescence microscope the image is generate by translating the sample in a point by point raster pattern, collecting the signal at each point and reconstructing the image.

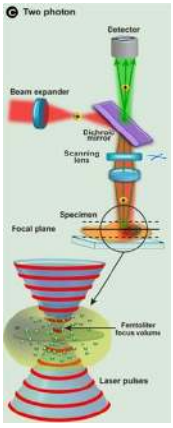


Figure**10.3.6.5**: An illustration of a two photon fluorescenc microscope. This image was adapted from one found in New approaches in renal microscopy - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/...fig2_299444264

Figure**10.3.6.6** shows a series of fluorescent images of a pollen sample illustrating the spatial resolution and ability to probe the sample as a function of depth for the three types of fluorescence microscopes described in this section. It should be readily observable that the confocal and two-photon microscopes provide far superior images to a standard wide field fluorescence microscope.

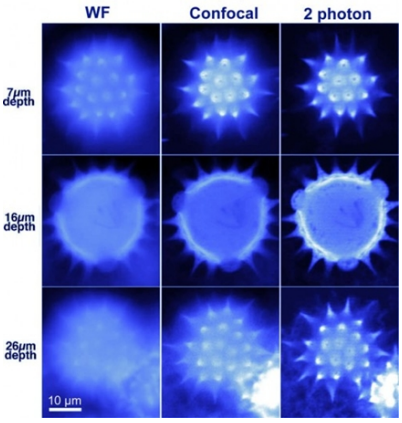


Figure**10.3.6.6**: Fluorescent pollen grain was imaged at three depths below its surface (7, 16, and 26 μm) by wide field, confocal, and 2 photon technique. Two-photon imaging improves image sharpness for deep optical sections by reducing scattering of excitation light and by eliminating fluorescence excitation outside the plane of focus. This image was taken from http://candle.am/microscopy/.

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CHAPTER OVERVIEW

11: Raman Spectroscopy

Raman spectroscopy is a chemical instrumentation technique that exploits molecular vibrations. The information tat can be gained using Raman spectroscopy is complementary to that from Infrared Spectroscopy but acquired using sources and detectors in the UV through Near Infrared spectral range.

11.1: Raman- Application
11.2: Introduction to Lasers
11.2.1: History
11.2.2: Basic Principles
11.2.2.1: Laser Radiation Properties
11.2.2.2: Laser Operation and Components
11.2.3: Types of Lasers
11.2.3.1: Gas Lasers
11.2.3.2: Solid State Lasers
11.2.3.3: Diode Lasers
11.2.3.4: Dye Lasers
11.2.4: References
11.3: Resonant vs. Nonresonant Raman Spectroscopy
11.4: Raman Spectroscopy - Review with a few questions
11.5: Problems/Questions

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11.1: Raman- Application

If one can extract all of the vibrational information corresponds a molecule, its molecular structure can then be determined. In the field of spectroscopy, two main techniques are applied in order to detect molecular vibrational motions: Infrared spectroscopy (IR) and Raman spectroscopy. Raman Spectroscopy has its unique properties which have been used very commonly and widely in Inorganic, Organic, Biological systems [1] and Material Science [2], [3], etc for both qualitative and quantitative applications

Introduction

Generally speaking, vibrational and rotational motions are unique for every molecule. The uniqueness to molecules are in analogous to fingerprint identification of people hence the term molecular fingerprint. Study the nature of molecular vibration and rotation is particularly important in structure identification and molecular dynamics. Two of the most important techniques in studying vibration/rotation information are IR spectroscopy and Raman spectroscopy. IR is an absorption spectroscopy which measures the transmitted light. Coupling with other techniques, such as Fourier Transform, IR has been highly successful in both organic and inorganic chemistry.

Unlike IR, Raman spectroscopy measures the scattered light (Figure 2). There are three types of scattered lights: Rayleigh scattering, Stokes scattering, and anti-stokes scattering. Rayleigh scattering is elastic scattering where there is no energy exchange between the incident light and the molecule. Stokes scattering happens when there is an energy absorption from the incident light, while anti-stokes scattering happens when the molecule emits energy to the incident light. Thus, Stokes scattering results in a red shift, while anti-stokes scattering results in a blue shift. (Figure 1) Stokes and Anti-Stokes scattering are called Raman scattering which can provide the vibration/rotation information

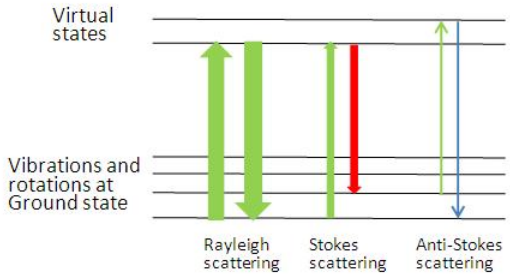


Figure 1: Scattered lights (The line thickness represents the intensity of the signal)

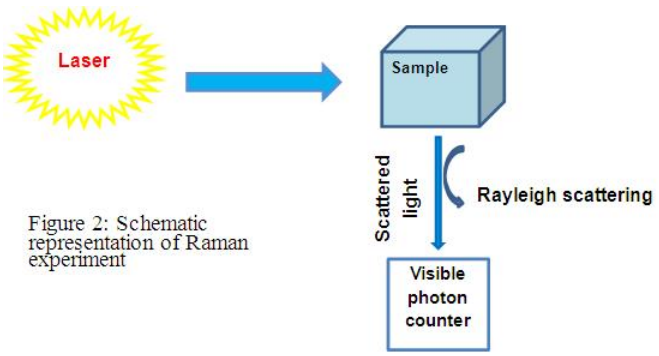


Figure 2: Schematic representation of Raman experiment

The intensity of Rayleigh scattering is around 10^7 times that of Stokes scattering. [4] According to the Boltzmann distribution, anti-Stokes is weaker than Stokes scattering. Thus, the main difficulty of Raman spectroscopy is to detect the Raman scattering by filtering out the strong Rayleigh scattering. In order to reduce the intensity of the Rayleigh scattering, multiple monochromators are

applied to selectively transmit the needed wave range. An alternative way is to use Rayleigh filters. There are many types of Rayleigh filters. One common way to filter the Rayleigh light is by interference.

Because of the weakness of Raman scattering, the resolving power of a Raman spectrometer is much higher than an IR spectrometer. A resolution of 10^5 is needed in Raman while 10^3 is sufficient in IR. [5] In order to achieve high resolving power, prisms, grating spectrometers or interferometers are applied in Raman instruments.

Numerous laser sources are available for both portable compact and bench top Raman instruments at wavelengths such as 266 nm, 355, nm, 441.6 nm, 514.5 nm, 532 nm, 632.8 nm, 785 nm and 1060 nm. Like other light scattering phenomena, Raman lines increases in intensity with the fourth power of the excitation frequency. For normal Raman spectroscopy, the excitation wavelength should be longer (less energy) that those absorbed for electronic transitions.

For quantitative work, spontaneous Raman scattering is directly proportional to the analyte concentration. And overall, the power of Raman lines can be expressed by:

$$P = cP_0\nu_s^4\alpha^2 \tag{11.1.1}$$

where c is the concentration, P_0 is the laser power, ν_s is the laser frequency and α is the scattering cross-section

Despite the limitations above, Raman spectroscopy has some advantages over IR spectroscopy as follows:

1. Raman Spectroscopy can be used in aqueous solutions (while water can absorb the infrared light strongly and affect the IR spectrum).
2. Because of the different selection rules, vibrations inactive in IR spectroscopy may be seen in Raman spectroscopy. This helps to complement IR spectroscopy.
3. There is no destruction to the sample in Raman Spectroscopy. In IR spectroscopy, samples need to disperse in transparent matrix. For example grind the sample in solid KBr. In RS, no such destruction of the sample is needed.
4. Glass vials can be used in RS *(this should only work in the visible region. If in the UV region for excitation, glass is not applicable because it can strongly absorb UV light)*
5. Raman Spectroscopy needs relative short time. So we can do Raman Spectroscopy detection very quickly.

After analysis of the advantages and disadvantages of Raman Spectroscopy technique, we can begin to consider the application of Raman Spectroscopy in inorganic, organic, biological systems and Material Science, etc.

Applications

Raman Spectroscopy application in inorganic systems

X-ray diffraction (XRD) has been developed into a standard method of determining structure of solids in inorganic systems. Compared to XRD, it is usually necessary to obtain other information (NMR, electron diffraction, or UV-Visible) besides vibrational information from IR/Raman in order to elucidate the structure. Nevertheless, vibrational spectroscopy still plays an important role in inorganic systems. For example, some small reactive molecules only exist in gas phase and XRD can only be applied for solid state. Also, XRD cannot distinguish between the following bonds: –CN vs. –NC, –OCN vs. –NCO,–CNO vs. –ONC, -SCN vs. –NCS. [7] Furthermore, IR and Raman are fast and simple analytical method, and are commonly used for the first approximation analysis of an unknown compound.

Raman spectroscopy has considerable advantages over IR in inorganic systems due to two reasons. First, since the laser beam used in RS and the Raman-scattered light are both in the visible region, glass (Pyrex) tubes can be used in RS. On the other hand, glass absorbs infrared radiation and cannot be used in IR. However, some glass tubes, which contain rare earth salts, will gives rises to fluorescence or spikes. Thus, using of glass tubes in RS still need to be careful. Secondly, since water is a very weak Raman scatter but has a very broad signal in IR, aqueous solution can be directly analyzed using RS.

Raman Spectroscopy and IR have different selection rules. RS detects the polarizability change of a molecule, while IR detects the dipole momentum change of a molecule. Principle about the RS and IR can be found at Chemwiki Infrared Theory and Raman Theory. Thus, some vibration modes that are active in Raman may not be active IR, vice versa. As a result, both of Raman and IR spectrum are provided in the stucture study. As an example, in the study of Xenon Tetrafluoride. There are 3 strong bands in IR and solid Raman shows 2 strong bands and 2 weaker bands. These information indicates that Xenon Tetrafluoride is a planar molecule

and has a symmetry of D4h. [8] Another example is the application of Raman Spectroscopy in homonuclear diatomic molecules. Homonuclear diatomic molecules are all IR inactive, fortunately, the vibration modes for all the homonuclear diatomic molecules are always Raman Spectroscopy active.

Raman Spectroscopy Application in Organic Systems

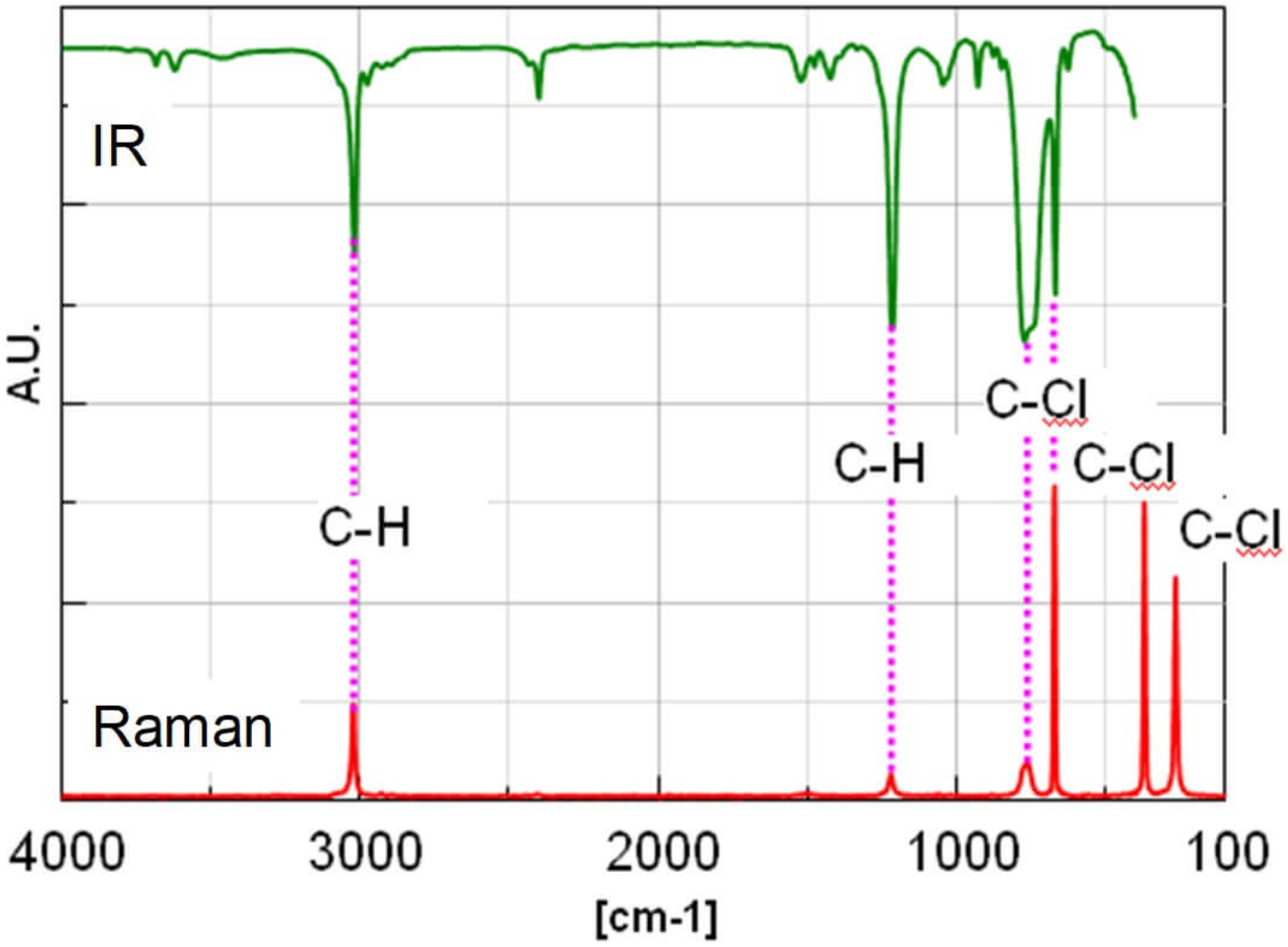
Unlike inorganic compounds, organic compounds have less elements mainly carbons, hydrogens and oxygens. And only a certain function groups are expected in organic specturm. Thus, Raman and IR spectroscopy are widely used in organic systems. Characteristic vibrations of many organic compounds both in Raman and IR are widely studied and summarized in many literature. [5] Qualitative analysis of organic compounds can be done base on the characteristic vibrations table.

Table 1: Characteristic frequencies of some organic function group in Raman and IR

Vibration	Region(cm^{-1})	Raman intensity	IR intensity
v(O-H)	3650~3000	weak	strong
v(N-H)	3500~3300	medium	medium
v(C=O)	1820~1680	strong~weak	very strong
v(C=C)	1900~1500	very strong~medium	0~weak

“RS is similar to IR in that they have regions that are useful for functional group detection and fingerprint regions that permit the identification of specific compounds.”[1] While from the different selection rules of Raman Spectroscopy and IR, we can get the Mutual Exclusion rule [5], which says that for a molecule with a center of symmetry, no mode can be both IR and Raman Spectroscopy active. So, if we find a strong bond which is both IR and Raman Spectroscopy active, the molecule doesn't have a center of symmetry.

Pictured below are the IR and Raman spectra of chloroform, $CHCl_3$, as an example of the complementarity of IR and Raman spectroscopy.



Non-classical Raman Spectroscopy

Although classical Raman Spectroscopy has been successfully applied in chemistry, this technique has some major limitations as follows[5]:

1. The probability for photon to undergo Raman Scattering is much lower than that of Rayleigh scattering, which causes low sensitivity of Raman Spectroscopy technique. Thus, for low concentration samples, we have to choose other kinds of techniques.
2. For some samples which are very easily to generate fluorescence, the fluorescence signal may totally obscure the Raman signal. We should consider the competition between the Raman Scattering and fluorescence.
3. In some point groups, such as C_6 , D_6 , D_{6h} , C_{4h} , D_{2h}, there are some vibrational modes that is neither Raman or IR active.
4. The resolution of the classical Raman Spectroscopy is limited by the resolution of the monochromator.

In order to overcome the limitations, special techniques are used to modify the classical Raman Spectroscopy. These non-classical Raman Spectroscopy includes: Resonance Raman Spectroscopy, surface enhanced Raman Spectroscopy, and nonlinear coherent Raman techniques, such as hyper Raman spectroscopy

Resonance Raman Scattering (RRS)

The resonance effect is observed when the photon energy of the exciting laser beam is equal to the energy of the allowed electronic transition. Since only the allowed transition is affected, (in terms of group theory, these are the totally symmetric vibrational ones.), only a few Raman bands are enhanced (by a factor of 10^6). As a result, RRS can increase the resolution of the classical Raman Spectroscopy, which makes the detection of dilution solution possible (concentrations as low as 10^{-3} M). RRS is extensively used

for biological molecules because of its ability to selectively study the local environment. As an example, the Resonance Raman labels are used to study the biologically active sites on the bond ligand. RRS can also be used to study the electronic excited state. For example, the excitation profile which is the Raman intensity as a function of incident laser intensity can tell the interaction between the electronic states and the vibrational modes. Also, it can be used to measure the atomic displacement between the ground state and the excited state.

Surface Enhanced Raman Scattering (SERS)

At 1974, Fleischmann discovered that pyridine adsorbed onto silver electrodes showed enhanced Raman signals. This phenomenon is now called surface enhanced Raman Scattering (SERS). Although the mechanism of SERS is not yet fully understood, it is believed to result from an enhancement either of transition polarizability, α,or the electric field, E, by the interaction with the rough metallic support.

Unlike RRS, SERS enhances every band in the Raman spectrum and has a high sensitivity. Due to the high enhancement (by a factor of $10^{10\sim11}$), the SERS results in a rich spectrum and is an ideal tool for trace analysis and in situ study of interfacial process. Also, it is a better tool to study highly diluted solutions. A concentration of $4x10^{-12}$ M was reported by Kneipp using SERS. [5]

Nonlinear Raman Spectroscopy

In a nonlinear process, the output is not linearly proportional to its input. This happens when the perturbation become large enough that the response to the perturbation doesn't follows the perturbation's magnitude. Nonlinear Raman Spectroscopy includes: Hyper Raman spectroscopy, coherent anti-Stokes Raman Spectroscopy, coherent Stokes Raman spectroscopy, stimulated Raman gain and inverse Ramen spectroscopy. Nonlinear Raman spectroscopy is more sensitive than classical Raman spectroscopy and can effectively reduce/remove the influence of fluorescence. The following paragraph will focus on the most useful nonlinear Raman spectroscopy---coherent anti-Stokes Raman Spectroscopy (CARS):

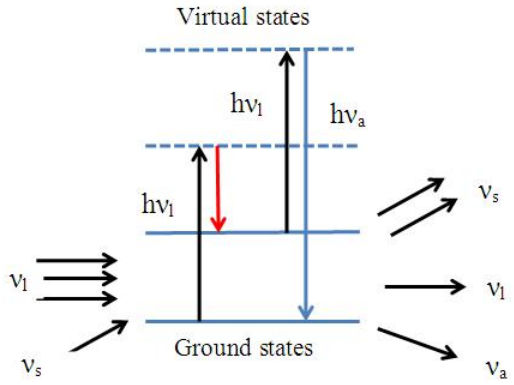


Figure 3: Schematic representation of coherent anti-Stoke Raman. ν is the frequency of the light. The subscript1 represents laser light; s represents Stoke scattering light; a represents anti-Stoke scattering light.

Figure 3) The two laser sources generate a coherent beam at frequency ν_3. There is a signal enhancement when ν_3 equal the anti-Stokes scattering (ν_a), and the vibrational transition equals to the energy difference between two light sources. Since CARS signal is at anti-Stoke region (a higher energy region than fluorescence), the influence of fluorescence is eliminated. Thus, CARS is very useful for molecules with high fluorescence effect, for example, some biological molecules. Another important advantage of CARS is that the resolution is no longer limited by the monochromator as in classical Raman because only the anti-Stokes frequency is studied in CARS. High-resolution CARS has been developed as a tool for small-time scale process, such as photochemical analysis and chemical kinetics studies. [6]

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Problems

1. What are the advantages and disadvantages for Raman spectroscopy, comparing with IR spectroscopy?
2. Please briefly explain the mutual exclusive principle in Raman and IR spectroscopy.

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SECTION OVERVIEW

11.2: Introduction to Lasers

Learning Objectives

The basic theory of lasers will be presented with emphasis on:

- laser radiation properties
- laser components and design
- laser light generation
- common laser types

This module discusses basic concepts related to Lasers. Lasers are light sources that produce electromagnetic radiation through the process of stimulated emission. Laser light has properties different from more common light sources, such as incandescent bulbs and fluorescent lamps. Typically, laser radiation spans a small range of wavelengths and is emitted in a beam that is spatially narrow. The word laser is an acronym for Light Amplification by Stimulated Emission of Radiation. Lasers are ubiquitous in our lives and are broadly applied in areas that include scientific research, medicine, engineering, telecommunications, industry and business (see the Applications page for examples). This module is aimed at presenting the most basic principles of lasers and discussing aspects of common types. Properties of laser radiation and laser optical components are introduced.

Topic hierarchy

11.2.1: History

11.2.2: Basic Principles

11.2.2.1: Laser Radiation Properties

11.2.2.2: Laser Operation and Components

11.2.3: Types of Lasers

11.2.3.1: Gas Lasers

11.2.3.2: Solid State Lasers

11.2.3.3: Diode Lasers

11.2.3.4: Dye Lasers

11.2.4: References

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11.2.1: History

- Laser development has an exciting history and includes a fair bit of controversy, some of which remains unresolved [1-4].
- Charles Townes [5] laid groundwork for the laser in the 1950s by demonstrating amplification of electromagnetic waves by stimulated emission. He was awarded the Nobel Prize in Physics in 1964.
- The first working laser was demonstrated in 1960 by Theodore Maiman [6] at Hughes Research Laboratories.
- The first laser was constructed from a small ruby rod. It was excited by an intense xenon lamp and emitted light at 694.3 nm [1,4].
- The development of gas and semiconductor lasers followed soon after [2,4].

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SECTION OVERVIEW

11.2.2: Basic Principles

Topic hierarchy

11.2.2.1: Laser Radiation Properties

11.2.2.2: Laser Operation and Components

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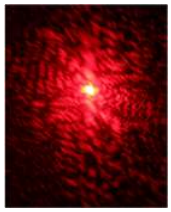
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11.2.2.1: Laser Radiation Properties

Laser Radiation Properties I

- Laser radiation is *nearly monochromatic*. Monochromatic refers to a single wavelength, or "one color" of light. Laser radiation contains a narrow band of wavelengths and can be produced closer to monochromatic than light from other sources.
- Laser radiation is highly *directional*. The radiation is produced in a beam that is spatially narrow and has low divergence relative to other light sources.
- Laser radiation is highly *coherent*, which means the waves of light emitted have a constant relative phase. The waves of light in a laser beam are thought of as in phase with one another at every point. The degree of coherence is proportional to the range of wavelengths in the light beam, or the beam's monochromaticity. Laser radiation has both *spatial* and *temporal* coherence, characterized by the *coherence length* and the *coherence time*.

Coherence

- 
- *Temporal coherence* is the ability of light to maintain a *constant phase* at one point in space at two different times, separated by delay τ. Temporal coherence characterizes how well a wave can interfere with itself at two different times and increases as a source becomes more monochromatic.
- A *coherence time* (τ_{cor}) and *coherence length* ($c \times \tau_{cor}$, where c is the speed of light) can be calculated from the spread of wavelengths ($\Delta\lambda$), or frequencies ($\Delta\nu$), in a beam. Expressed in terms of $\Delta\nu$, or *"bandwidth":*

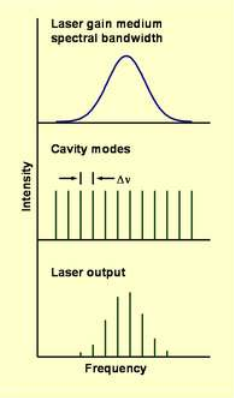
$$\tau_{cor} = \frac{1}{2\pi\Delta\nu} \tag{11.2.2.1.1}$$

Laser Radiation Properties II

- Laser radiation has high *brightness*, a quantity defined as the power emitted per unit surface area per unit solid angle. Because laser light is emitted as a narrow beam with small divergence, the brightness of a 1 mW laser pointer, for example, is > 1,000 ×'s greater than that of the sun, which emits more than 10^{25} W of radiant power [a].
- Laser output can be continuous or pulsed. Continuous wave (CW) lasers are characterized by their *average power*, whereas *peak power*, *energy per pulse* and *pulse repetition rate* are figures of merit that apply to pulsed lasers. *Pulse widths* in the ns-ps range are employed more routinely than fs pulses, and attosecond pulses can be generated. A 10 fs pulse with only 10 mJ energy has a peak power of 10^{12} W, or 1 TW!

Laser Radiation Properties III

- The narrow range of frequencies, or wavelengths, emitted is referred to as the laser *bandwidth*. This output is determined by the spectral emission properties of the gain medium and the modes supported by the cavity.
- When the bandwidth of the gain medium is larger than the cavity mode spacing, the laser output consists of a series of narrow spectral bands (see the following figure and "Laser Radiation Properties IV" below).



- *Cavity modes* develop as a consequence of the properties of light reflection and interference. In the simplest case of a cavity formed by two flat mirrors, the allowable axial modes have wavelength $\lambda = 2L/q$, where L is the cavity length and q is an integer. The frequency spacing ($\Delta\nu$) between modes is given by $\Delta\nu = c/(2L)$, where c is the speed of light. Parabolic mirrors produce more complex cavity modes leading to a *Gaussian beam* [b].

Laser Radiation Properties IV

Laser bandwidth frequency ($\Delta\nu$) and wavelength ($\Delta\lambda$) are related as follows:

$$\Delta\lambda \approx \left(\frac{\lambda_0^2}{c}\right)\Delta\nu \qquad (11.2.2.1.2)$$

where λ_o is the band center wavelength and c is the speed of light. A HeNe laser operating at 632.8 nm has a gain bandwidth of 1.5 GHz, or 0.002 nm. When the gain medium bandwidth is *smaller* than the cavity mode spacing, the laser output consists of a single mode and operates as a *single frequency laser* [c].

- A HeNe laser with 20 cm cavity length has mode spacings of $\Delta\nu$ = 750 MHz, or $\Delta\lambda$ = .001 nm. HeNe lasers are often equipped for and use *single frequency operation* [c, d].
- *Mode locking* [e] produces a fixed phase relationship between laser cavity modes and results in pulsed output. See Refs [2,7,8,9] for more details on mode locking and methods for producing *ultra-short laser pulses* and other aspects of single frequency laser operation.

References

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d. http://www.rp-photonics.com/single_f...operation.html
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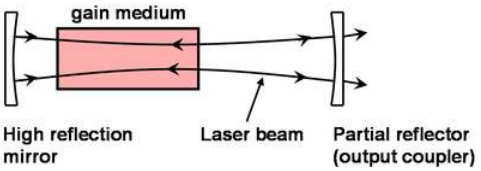
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11.2.2.2: Laser Operation and Components

Laser Operation and Components

- The process of light *stimulated emission* is fundamental to laser operation.
- Laser light is produced by an *active medium*, or *gain medium* inside the *laser optical cavity*. The active medium is a collection of atoms, or molecules that can undergo stimulated emission. The active medium can be in a gaseous, liquid or solid form.
- For lasing to take place, the active medium must be pumped into an *excited state* capable of undergoing stimulated emission. The energy required for excitation is often supplied by an electric current or an intense light source, such as a flashlamp.
- To induce stimulated emission, the laser cavity must provide a means to reflect, or *feedback* emitted light into the gain medium.
- A laser must have an *output coupler* to allow a portion of the laser light to leave the optical cavity.

Laser Optical Cavity



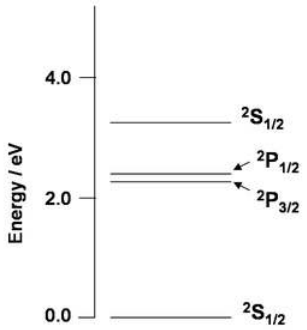
Sketch showing the main components of a laser optical (or resonator) cavity. The optical cavity is formed by a pair of mirrors that surround the gain medium and enable feedback of light into the medium. The output coupler is a partially reflective mirror that allows a portion of the laser radiation to leave the cavity. The gain medium is excited by an external source (not shown), such as a flash lamp, electric current or another laser. The light trapped between the mirrors forms standing wave structures called modes. Although beyond the scope of this discussion, the reader interested in cavity modes can consult References 7-10 and the "Laser Radiation Properties" section.

Stimulated Emission [7-10, 12, 13]

- *Stimulated emission* occurs when a photon of light induces an atom or molecule to lose energy by producing a second photon. The second photon has the same phase, frequency, direction of travel and polarization state as the stimulating photon.
- Since from one photon a second identical photon is produced, stimulated emission leads to *light amplification*.
- Stimulated emission can be understood from an *energy level diagram* within the context of the competing optical processes of *stimulated absorption* and *spontaneous emission*.
- For stimulated emission to take place, a *population inversion* must be created in the laser gain medium.
- For more on stimulated emission, see energy level diagrams and subsequent sections.

Energy Level Diagrams

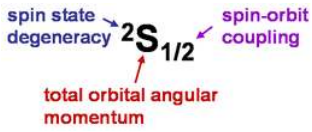
- An energy level diagram displays states of an atom, molecule or material as levels ordered vertically according to energy.
- The states contain contributions from several sources, as appropriate for the matter considered. Sources include the orbital and spin angular momentum of electrons, vibrations of nuclei, molecular rotations, and spin contributions from nuclei.
- The lowest energy level is called the *ground state*.
- Absorption and emission of energy occurs when matter undergoes *transitions between states*.



Energy level diagram showing states of a sodium atom. Each state is labeled by a *term symbol* and includes effects of electron orbital and spin angular momentum.

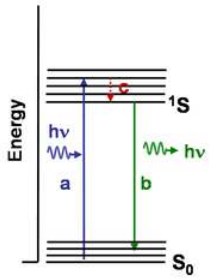
Term Symbols

- Term symbols are a shorthand for describing the angular momentum and coupling interactions among electrons in atoms and molecules.
- As a starting point for understanding a term symbol, write the electron configuration for the state considered. For Na, the electron configuration of the ground state is: $1s^2 2s^2 2p^6 3s^1$
- The central letter describes the total orbital angular momentum. Only the valence electrons need to be considered. For Na, there is one valence electron, and it occupies an s-orbital. The angular momentum quantum number for an s-orbital is l = 0. The total orbital angular momentum for ground state Na is L = l = 0. Symbols are assigned to the values of L as follows: L = 0 (S), L = 1 (P), L = 2 (D), etc.
- The left superscript reflects the coupling of valence electron spin angular momentum and gives the degeneracy of spin states. For Na, s = 1/2 for the valence electron; therefore, the total spin, S = 1/2 and the degeneracy = (2 S + 1) = 2.
- The right subscript reflects the coupling between spin and orbital angular momentum. For ground state Na, J = L + S = 1/2.
- For a detailed discussion of term symbols, see Ref 11.



Absorption and Emission Processes and Transitions Between Energy States

- *Stimulated absorption* (a) occurs when light, or a photon of light (hν), excites matter to a higher energy (or *excited*) state.
- *Spontaneous emission* (b) is a process whereby energy is spontaneously released from matter as light.
- Molecules typically transition to vibrationally excited levels within the excited electronic state.
- Following excitation, the vibrational energy is quickly released by *non-radiative* pathways (c).
- In molecules, spontaneous emission known as *fluorescence* (b) occurs by transition from the lowest level in the excited electronic state, to upper vibrational levels of the lower electronic state.

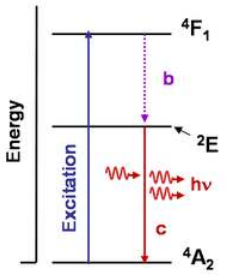


Energy level diagram for a typical dye molecule. The vibrational levels of each electronic state, labeled by S_0 and S_1, are included.

Stimulated Emission - Details [7-10, 12, 13]

- Laser radiation is produced when energy in atoms or molecules is released as *stimulated emission* (c).
- Stimulated emission requires a *population inversion* in the laser gain medium.
- A *population inversion* occurs when the number of atoms or molecules in an excited state exceeds the number in lower levels (usually the ground state).
- To create the population inversion, the gain medium must transition to a *metastable state*, which is long lived relative to spontaneous emission.
- The three-level diagram (below) shows excitation followed by *non-radiative (nr) decay* (b) to ^2E states. The ^2E states are long lived, because the transition to $^4A_2$ requires a change in the electron spin state.
- A photon of the same energy as the $^2E \rightarrow {}^4A_2$ transition can stimulate the emission of a second photon (c), leading to *light amplification*, or lasing.

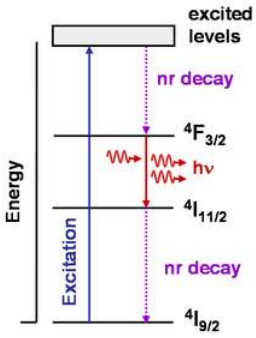
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Three-level energy diagram. Simplified diagram showing transitions for Cr^{3+} in a ruby laser.

Three and Four Level Lasers [7-10, 12, 13]

- Three-level lasers require intense pumping to maintain the population inversion, because the lasing transition re-populates the ground state.
- Lasers based on transitions between four energy levels (see below), can be more efficiently pumped, because the lower level of the lasing transition is not the ground state.
- Only four-level lasers provide continuous output. HeNe and Nd:YAG are common four-level lasers.
- A population inversion is necessary for lasing, because without one, the photon inducing stimulated emission would instead have a greater probability of undergoing absorption in the gain medium.
- For more in depth information about laser transitions and population inversion, Refs 7-10, 12 (pg 96) and 13 can be consulted.



Four-level energy diagram. Simplified diagram showing transitions for Nd^{3+} in a Nd:YAG laser.

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SECTION OVERVIEW

11.2.3: Types of Lasers

Topic hierarchy

11.2.3.1: Gas Lasers

11.2.3.2: Solid State Lasers

11.2.3.3: Diode Lasers

11.2.3.4: Dye Lasers

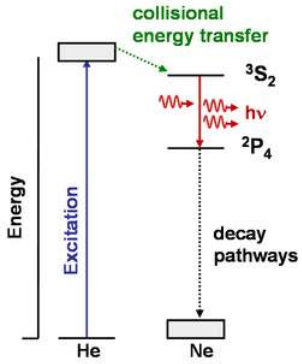
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See Refs. 2, 7-10 and 12-14 for more in depth coverage of the above systems, and others including excimer, free-electron, chemical and X-ray lasers.

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11.2.3.1: Gas Lasers

- Examples of gas lasers include helium-neon (HeNe), nitrogen and argon-ion
- The gain medium in these lasers is a gas-filled tube
- Excitation of gas molecules is achieved by the passage of an electric current or discharge through the gas
- In a HeNe laser, an electric discharge excites He atoms to excited levels. Collisions between He and Ne atoms transfer energy and produce excited Ne atoms. Lasing occurs when Ne atoms attain a population inversion.
- The lasing transition in a HeNe laser procudes light at 632.8 nm.



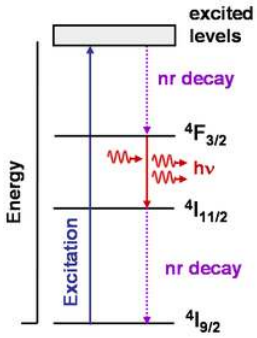
Simplified energy level diagram showing HeNe laser transitions.

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11.2.3.2: Solid State Lasers

- Nd:YAG and Ruby are examples of solid-state laser systems
- A flashlamp is used to excite (or “pump”) the gain medium in these lasers
- Cr^{3+} ions in ruby undergo transitions to produce lasing in a Ruby laser
- Nd^{3+} ions in a yttrium aluminum garnet (YAG) matrix are the optically active species in a Nd:YAG laser
- Transitions in ruby occur mainly between three levels, whereas Nd:YAG is referred to as a 4-level system (see the energy level diagram, below).
- The fundamental lasing transitions are at 694 nm and 1064 nm for Ruby and Nd:YAG lasers, respectively.



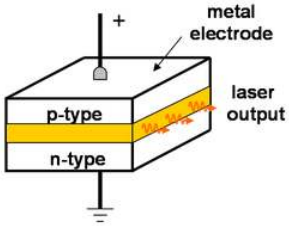
Four-level energy diagram. Simplified diagram showing transitions for Nd^{3+} in a Nd:YAG laser.

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11.2.3.3: Diode Lasers

- Semiconductor materials layered to form a *diode* [2, a] serve as the gain medium.
- Excitation is achieved by the passage of electric current (forward biased) through the diode *p-n junction*, which forms at the interface between semiconductors with different electronic doping levels.
- Light emission occurs when electrons and holes in the vicinity of the p-n junction *recombine* following excitation.
- The layered structure and high *refractive index* of semiconductor materials enables the laser optical cavity to be formed on the diode (see drawing, right).
- Diode lasers are finding application in a wide range of areas, such as communications, medicine and chemical analysis (see Ref 2, 14, a, b).



Edge- emitting diode laser

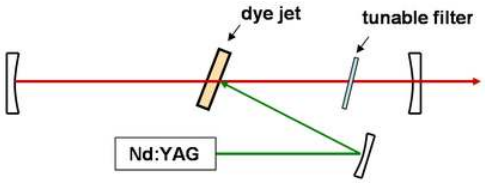
a. http://www.rp-photonics.com/laser_diodes.html
b. www1.union.edu/newmanj/lasers...ctorLasers.htm

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11.2.3.4: Dye Lasers

- The gain medium in a dye laser consists of organic dye molecules dissolved in a solvent.
- Light, sometimes another laser, is used to excite the gain medium
- Because fluorescence emission from organic dye molecules occurs across a broad range of wavelengths, dye lasers can be scanned (or “tuned”) to select a narrow band of emission light from across a wide spectral range



Sketch of a Nd:YAG pumped dye laser.

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Additional references are included in the "Applications" section.

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11.3: Resonant vs. Nonresonant Raman Spectroscopy

Raman spectroscopy is a chemical instrumentation technique that exploits molecular vibrations. It does not require large sample sizes and is non-destructive to samples. It is capable of qualitative analysis of samples and the intensity of spectral bands produced assist in quantitative analysis as well. Raman spectroscopy is even being used in areas outside of physical science (i.e. archeology and art preservation) due to the characteristics mentioned above.

Introduction

Raman spectroscopy is based on scattering of radiation (Raman scattering), which is a phenomenon discovered in 1928 by physicist Sir C. V. Raman. The field of Raman spectroscopy was greatly enhanced by the advent of laser technology during the 1960s.[1] Resonance Raman also helped to advance the field. This technique is more selective compared to non-resonance Raman spectroscopy. It works by exciting the analyte with incident radiation corresponding to the electronic absorption bands.[2] This causes an augmentation of the emission up to a factor of 10^6 in comparison to non-resonance Raman.[2,3]

In this section readers will be introduced to the theory behind resonance and non-resonance Raman spectroscopy. Each technique has its share of advantages and challenges. Each of these aspects will be explored.

Theory

Raman scattering is the basis of the two Raman techniques. A molecule must have polarizability to Raman scatter and its symmetry must be even (or gerade) for it to have polarizability[2]. Furthermore, the more electrons a molecule has gererally increases its polarizability. Polarizability (α) is a measure of an applied electronic field's (E) ability to generate a dipole moment (μ) in the molecule.[5] In other words, it is an alteration of a molecule's electron cloud. Mathematically, this can be determined by the following equation:

$$\mu = \alpha E \tag{11.3.1}$$

To help provide a better visualization of how Raman spectroscopy works, a generic diagram can be seen in Figure 2. A sample is irradiated with monochromatic laser light; which is then scattered by the sample. The scattered light passes through a filter to remove any stray light that may have also been scattered by the sample.[2] The filtered light is then dispersed by the diffraction grating and collected on the detector. This set-up works for both the non-resonance and resonance Raman techniques.

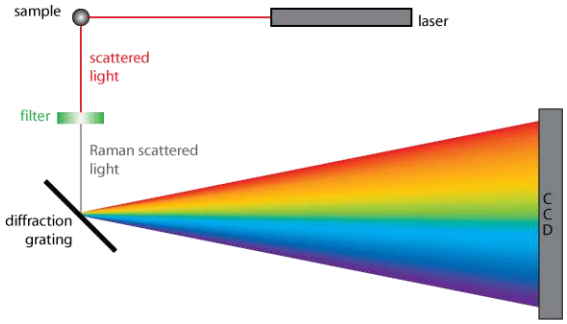


Figure 1: Simplified diagram of how a Raman spectrometer works. Images used with permission from www.doitpoms.ac.uk

Non-resonance Raman scattering occurs when the radiation interacts with a molecule resulting in polarization of the molecule's electrons.[4] The increase in energy from the radiation excites the electrons to an unstable virtual state; therefore, the interaction is almost immediately discontinued and the radiation is emitted (scattered) at a slightly different energy than the incident radiation.[4]

Resonance Raman scattering occurs in a similar fashion. However, the incident radiation is at a frequency near the frequency of an electronic transition of the molecule of interest. This provides enough energy to excite the electrons to a higher electronic state. Figure 1 provides a visual depiction of what non-resonance and resonance Raman scattering looks like in terms of energy levels.

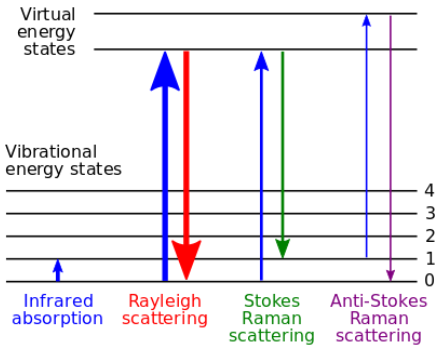


Figure 2: Diagram depicting the different processes for non-resonance and resonance Raman spectroscopy. Notice that fluorescence is more likely to be a problem with resonance Raman than with non-resonance. Image used with permisison from Wikipedia (Credit: Moxfyre).

Advantages of Non-Resonance and Resonance Raman

Instrumental techniques each have certain strengths that make them better suited for some jobs as oppose to others. Non-resonance is a good example of this notion. It is considered better suited for analyzing water containing samples due to water's low polarizability. Non-resonance and resonance Raman each have the capability to analyze samples in the gaseous, liquid, or solid state. Their non-destructive nature makes it a great candidate for doing analysis of delicate materials. Archeologists and art historians even find resonance Raman spectroscopy useful for studying and authentication of artifacts and artwork.[4]

Monochromatic light in the ultraviolet or near-infrared regions is generally used for both resonance and non-resonance Raman spectroscopy. A tunable laser is preferred for resonance Raman and can be an advantage. That is because only one laser is necessary to do analyses of multiples samples in which each one requires a different excitation wavelength.[4] This allows the user to switch out samples without having to switch out the lasers as well. It becomes a matter of just changing the setting on the tunable laser. If the laboratory is not equipped with a tunable laser, any laser that is available can be used to achieve the enhancement of the Raman signal. The only stipulation being that the laser available must have a frequency as near as possible to one of the analyte's electronic transitions.[2] Therefore, researchers conducting resonance Raman spectroscopy without a tunable laser are at the mercy of whatever laser they do have in the laboratory.

Resonance Raman spectroscopy has greater sensitivity compared to its non-resonance counterpart. It is capable of analyzing samples with concentrations as low as 10^{-8} M. Non-resonance Raman can analyze samples with concentrations no lower than 0.1 M. Resonance Raman spectroscopy produces a spectrum with relatively few lines. The reason being that the technique only augments Raman signals affiliated with chromophores in the analyte.[2,4] This makes the technique particularly useful for analysis of larger molecules like biomolecules.

Fluorescence Disadvantage

Fluorescence is a problem for both Resonance Raman techniques, particularly when using sources in the visible range.[2] Non-resonance Raman signals are generally weak and can be easily overwhelmed by fluorescence signals.[6] In addition, fluorescence has a longer excited state lifetime compared to Raman scattering, causing an inability to detect Raman signals.[2,6] Even when the analyte is not a fluorescent molecule, the signal could be a result of the sample matrix content (i.e. solvent or contaminants). Resonance Raman is particularly at risk of inducing fluorescence because it uses sources at frequencies near to that of a molecule's electronic transition. The radiation is more likely to absorb resulting in fluorescence as a possible mechanism for the electrons return to the ground state. Thus, highly fluorescent molecules should be avoided when using Raman spectroscopy; especially resonance Raman. Figure 3 is a general illustration of how a fluorescence signal can overwhelm Raman signals.

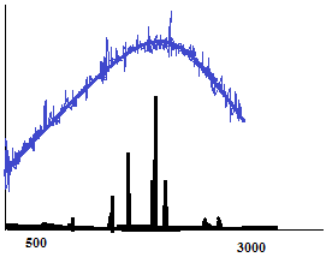


***Figure 3**: Two generic Raman spectra overlaid. The blue Raman spectrum represents one obtained via excitation source in the visible range. The black Raman spectrum represents one obtained via excitation source in the near-infrared range. The black Raman signals are free of fluorescence interference.*

There are techniques that spectroscopists use to avoid fluorescence interference. For instance, background subtraction could be done. Another example is to use near-infrared radiation to excite the sample as a means to overcome fluorescence.[6] A more elaborate method was used by Matousek et al. They took advantage of the differences in excitation lifetimes for Raman and fluorescence. It required implementing shifted excitation Raman difference spectroscopy (SERDS) in conjunction with a device known as a Kerr gate to successfully obtain a resonance Raman spectrum of the rhodamine 6G dye.[6] SERDS is a technique that uses two excitation wavelengths to produce two Raman spectra. The excitation wavelengths have a difference in value that corresponds to the bandwidth of the Raman signal.[6] The two spectra are subtracted from each other and the difference spectra is recreated by means of mathematical processes.[6]

A Kerr gate can be used to remove fluorescence from a Raman signal based on their different lifetimes.[6] The device consists of a couple of crossed polarizers, Kerr medium, and an additional laser to provide a gating pulse.[7] Now consider a sample that has been irradiated resulting in fluorescence and Raman scattering. The fluorescence and Raman scatter would pass through the crossed polarizer and then through the Kerr medium (Matousek et al. used carbon disulfide as the Kerr medium). The Kerr gate is referred to as being open when a laser pulse (the gating pulse) strikes the Kerr medium as the fluorescence and Raman scattered light pass through.[7] Furthermore, the gate remains open for a length of time corresponding to the lifetime of the Raman scatter,[7] The interaction of the gating pulse with the Kerr medium causes the light to become anisotropic and transmit beyond the Kerr medium.[7] The light then goes from being polarized in a linear direction to elliptical polarization. However, Raman scattered light can be selectively switched back to linear polarization by selecting the appropriate propagation length for Kerr medium transmission; or by altering the degree of anisotropy.[7] The Raman scattered light is then allowed to pass through the second crossed polarizer and on to the spectrometer. Any fluorescence that passes through the Kerr medium is prevented from entering the spectrometer due to its inability to transmit through the second crossed polarizer on account of its new elliptical polarization. Figure 4 should assist with the visualization of such process.

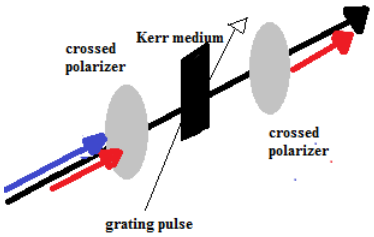


Figure 4: This is an illustration of a Kerr gate. The thick black arrow represents the direction of the signal produced. The blue and red arrows are fluorescence and Raman scattering, respectively. All other components are labeled in the figure.

Conclusions

This module was meant to provide an introduction to the similarities and differences between non-resonance and resonance Raman spectroscopy. Notice that they each have their own advantages making both of them powerful analytical techniques. Non-resonance Raman is more advantageous when compared to IR spectroscopy. However, resonance Raman appears to have the upper hand when compared to its non-resonance counterpart. The important thing is to choose the technique that is most appropriate for the work to be done.

Problems

1. What does Dr. Nivens' quote mean and what can be done to avoid the problem?
2. Archeology and art preservation were mentioned as fields in which Raman spectroscopy is useful due to the following characteristics: non-destructive to sample, qualitative analysis, and quantitative analysis. Name an additional area/field that could benefit from Raman due to these characteristics and why.
3. Does non-resonance Raman or resonance Raman have a better limit of detection?
4. What is polarizability?
5. You are working on a project that is trying to determine if lycopene is absorbed better via supplements or diet. You only have a Raman spectrometer available to conduct your analysis. Thus you decide to determine the amount absorbed in the body by subtracting the amount excreted in urine from the total intake. In general, would it be better to detect lycopene in your biological sample using non-resonance or resonance Raman spectroscopy and why?

References

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Solutions

1. "Fluorescence is the enemy of Raman" refers to the fact that fluorescence induced during Raman spectroscopy will inhibit detection of Raman signals. One way to avoid such a problem is to use an excitation source that is in the ultra-violet or near-infrared range.
2. Answers will vary, but here is an example: Forensic science is a field that could benefit for the indicated characteristics of Raman spectroscopy. The non-destructive nature will insure that precious evidence is not damage or destroyed during analysis. The evidence can then be preserved for additional testing. The qualitative and quantitative factors could provide information to investigators as to what is present in their evidence (i.e. bodily fluids, drugs, accelerants, etc.).
3. Resonance Raman is capable of analyte detection at concentrations as low as 10^{-8} M. The limit of detection is much higher for non-resonance Raman.
4. Polarizability measures the capability of an applied electric field to cause a dipole moment.
5. The sample will most likely contain a multitude of things because it is biological. Resonance Raman would be the better choice because lycopene contains chromophores which are targeted for excitation in that technique. Thus the spectra will not be cluttered by all the other contents in the biological sample.

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11.4: Raman Spectroscopy - Review with a few questions

Learning Objectives

After completing this unit the student will be able to:

- Determine whether the molecular vibrations of a triatomic molecule are Raman active.
- Explain the difference between Stokes and anti-Stokes lines in a Raman spectrum.
- Justify the difference in intensity between Stokes and anti-Stokes lines.
- Draw the Stokes and anti-Stokes lines in a Raman spectrum of a compound when given the energies of the different transitions.

Raman spectroscopy is an alternative way to get information about the infrared transitions within a molecule. In order for a vibrational transition to be Raman active, the molecule must undergo a change in polarizability during the vibration. Polarizability refers to the ease of distorting electrons from their original position. The polarizability of a molecule decreases with increasing electron density, increasing bond strength, and decreasing bond length.

Consider the molecular vibrations of carbon dioxide and determine whether or not they are Raman active.

The symmetric stretch of carbon dioxide is not IR active because there is no change in the net molecular dipole (Figure **11.4.1**). Since both bonds are stretched (i.e., lengthened), both bonds are more easily polarizable. The overall molecular polarizability changes and the symmetric stretch is Raman active.

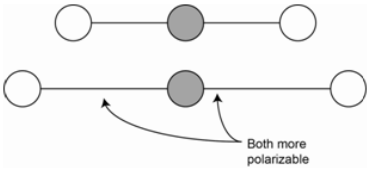


Figure **11.4.1**: Representation of the Raman active symmetric stretch of carbon dioxide.

The asymmetric stretch of carbon dioxide is IR active because there is a change in the net molecular dipole (Figure **11.4.2**). In the asymmetric stretch, one bond is stretched and is now more polarizable while the other bond is compressed and is less polarizable. The change in polarizability of the longer bond is exactly offset by the change in the shorter bond such that the overall polarizability of the molecule does not change. Therefore, the asymmetric stretch is not Raman active.

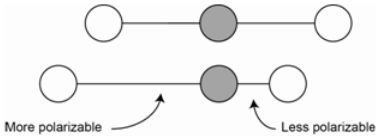


Figure **11.4.2**: Representation of the Raman inactive asymmetric stretch of carbon dioxide.

The bending motion of carbon dioxide is IR active because there is a change in the net molecular dipole (Figure **11.4.3**). Since the bending motion involves no changes in bond length, there is no change in the polarizability of the molecule. Therefore, the bending motion is not Raman active.

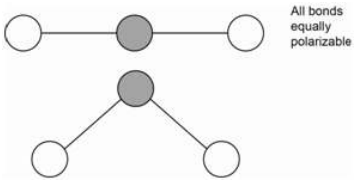


Figure **11.4.3**: Representation of the Raman inactive bending vibration of carbon dioxide.

Note that the IR active vibrations of carbon dioxide (asymmetric stretch, bend) are Raman inactive and the IR inactive vibration (symmetric stretch) is Raman active. This does not occur with all molecules, but often times, the IR and Raman spectra provide

complementary information about many of the vibrations of molecular species. Raman spectra are usually less complex than IR spectra.

An intriguing aspect of Raman spectroscopy is that information about the vibrational transitions is obtained using visible radiation. The process involves shining monochromatic visible radiation on the sample. The visible radiation interacts with the molecule and creates something that is known as a **virtual state**. From this virtual state it is possible to have a modulated scatter known as Raman scatter. Raman scatter occurs when there is a momentary distortion of the electrons in a bond of a molecule. The momentary distortion means that the molecule has an induced dipole and is temporarily polarized. As the bond returns to its normal state, the radiation is reemitted as Raman scatter.

One form of the modulated scatter produces **Stokes lines**. The other produces **anti-Stokes lines**. Stokes lines are scattered photons that are reduced in energy relative to the incident photons that interacted with the molecule. The reductions in energy of the scatter photons are proportional to the energies of the vibrational levels of the molecule. Anti-Stokes lines are scattered photons that are increased in energy relative to the incident photons that interacted with the molecule. The increases in energy of the scatter photons are proportional to the energies of the vibrational levels of the molecule.

The energy level diagram in Figure **11.4.4** shows representations for IR absorption, Rayleigh scatter, Stokes Raman scatter and anti-Stokes Raman scatter. For Stokes lines, the incident photons interact with a ground state molecule and form a virtual state. The scattered photons come from molecules that end up in excited vibrational states of the ground state, thereby explaining why they are lower in energy than the incident photons. For anti-Stokes lines, the incident photons interact with a molecule that is vibrationally excited. The virtual state produced by this interaction has more energy than the virtual state produced when the incident photon interacted with a ground state molecule. The scattered photons come from molecules that end up in the ground state, thereby explaining why they are higher in energy than the incident photons.

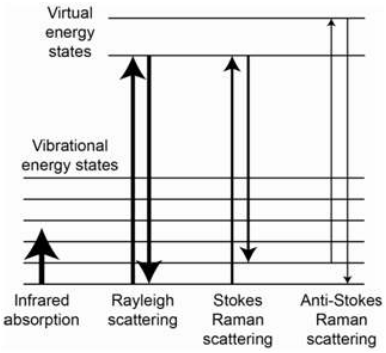


Figure **11.4.4**: Energy level diagram showing the origin or infrared absorption, Rayleigh scatter, Stokes Raman scatter, and anti-Stokes Raman scatter.

It is important to recognize that, while the processes in Figure **11.4.4** responsible for Raman scatter might look similar to the process of fluorescence, the process in Raman spectroscopy involves a modulated scatter that is different from fluorescence. How do we know this? One reason is that Raman scatter occurs when the incident radiation has energy well away from any absorption band of the molecule. Therefore, the molecule is not excited to some higher electronic state but instead exists in a virtual state that corresponds to a high energy vibrational state of the ground state. Another is that Raman scatter has a lifetime of 10^{-14} second, which is much faster than fluorescent emission.

Which set of lines, Stokes or anti-Stokes, is weaker?

The anti-Stokes lines will be much weaker than the Stokes lines because there are many more molecules in the ground state than in excited vibrational states.

What effect would raising the temperature have on the intensity of Stokes and anti-Stokes lines?

Raising the temperature would decrease the population of the ground state and increase the population of higher energy vibrational states. Therefore, with increased temperature, the intensity of the Stokes lines would decrease and the intensity of the anti-Stokes lines would increase. However, the Stokes lines would still have higher intensity than the anti-Stokes lines.

Because scatter occurs in all directions, the scattered photons are measured at 90^{o} to the incident radiation. Also, Raman scatter is generally a rather unfavorable process resulting in a weak signal.

What would be the ideal source to use for measuring Raman spectra?

The more incident photons sent in to the sample, the more chance there is to produce molecules in the proper virtual state to produce Raman scattering. Since the signal is measured over no background, this suggests that we want a high power source. That means that a laser would be preferable as a source for measuring Raman spectra. The highly monochromatic emission from a laser also means that we can more accurately measure the frequency of the Stokes lines in the resulting spectrum. Also an array detector is preferable as it enables the simultaneous measurement of all of the scattered radiation.

The molecule carbon tetrachloride (CCl_4) has three Raman-active absorptions that occur at 218, 314 and 459 cm^{-1} away from the laser line. Draw a representation of the Raman spectrum of CCl_4 that includes both the Stokes and anti-Stokes lines.

The spectrum in Figure **11.4.5** shows a representation of the complete Raman spectrum for carbon tetrachloride and includes the Stokes and anti-Stokes lines. The laser line undergoes an elastic scattering known as Rayleigh scatter and a complete spectrum has a peak at the laser line that is far more intense than the Raman scatter. Note that the anti-Stokes lines are lower in intensity and higher in energy than the Stokes lines. Note as well that the two spectra appear as mirror images of each other with regards to the placement of the bands at 218, 314 and 459 cm^{-1} away from the Rayleigh scatter peak.

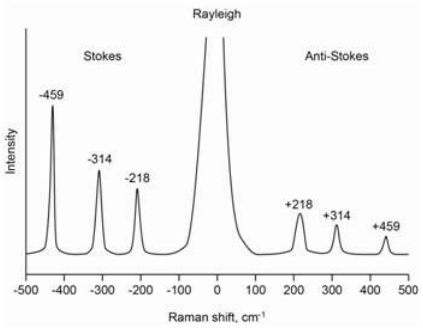


Figure **11.4.5**: Complete Raman spectrum of carbon tetrachloride (CCl_4).

The energy level diagram in Figure **11.4.6** shows the origin of all of the lines and inspection of it should rationalize why the placement of the Stokes and anti-Stokes lines are mirror images of each other. The relative intensity of the three Stokes lines depends on the probability of each scatter process and is something we could not readily predict ahead of time.

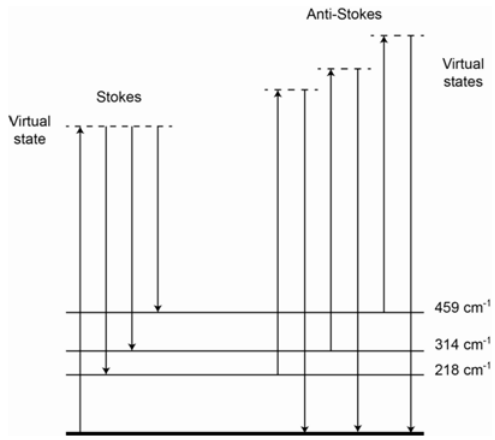


Figure **11.4.6**: Energy level diagram showing the origin of Stokes and anti-Stokes lines in the Raman spectrum of carbon tetrachloride (CCl_4).

Why do the anti-Stokes lines of carbon tetrachloride have the following order of intensity: 219 > 314 > 459 cm^{-1}?

The intensity of the three anti-Stokes lines drops going from the 218 to 314 to 459 cm^{-1} band. Anti-Stokes scatter requires an interaction of the incident photon with vibrationally excited molecules. Heat in the system causes some molecules to be vibrationally excited. The drop in intensity is predictable because, as the vibrational levels increase in energy, they would have lower populations and therefore fewer molecules to produce Raman scatter at that transition.

Raman spectroscopy is an important tool used in the characterization of many compounds. As we have already seen, because the selection rules for Raman (change in polarizability) are different than infrared (change in the dipole moment) spectroscopy, there are some vibrations that are active in one technique but not the other. Water is a weak Raman scatterer and, unlike infrared spectroscopy, where water has strong absorptions, water can be used as a solvent. Glass cells can be used with the visible laser radiation, which is more convenient that the salt plates that need to be used in infrared spectroscopy. Because Raman spectroscopy involves the measurement of vibrational energy states with visible light, it is especially useful for measurements of vibrational processes that occur in the far IR portion of the spectrum. Finally, since Raman spectroscopy involves a scattering process, it can be used for remote monitoring such as atmospheric monitoring. A pulsed laser can be passed through the atmosphere or effluent from a smoke stack and Raman scattered radiation measured by remote detectors.

One disadvantage of Raman spectroscopy is that Raman scatter is an unfavorable process and the signals are weak compared to many other spectroscopic methods. There are two strategies that have been found to significantly increase the probability of Raman scatter and lower the detection limits.

One is a technique known as **surface-enhanced Raman spectroscopy (SERS)**. It is observed that compounds on surfaces consisting of roughened silver, gold or copper have much higher probability of producing Raman scatter. The other involves the use of **resonance Raman spectroscopy**. If the molecule is excited using a laser line close to an electronic absorption band, large enhancements in the Raman bands of symmetrical vibrations occur. As noted earlier, the lifetime of 10^{-14} second of Raman scatter indicates that the increased signal is not from a fluorescent transition.

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11.5: Problems/Questions

1. At what wavelength in nanometers would the Stokes and anti-Stokes Raman lines for carbon tetrachloride (218, 314, 459, 762, and 790 cm^{-1}) appear if the source was (a) a helium/neon laser at 632.8 nm, (b) solid state laser at 785 nm , and (c) a nitrogen laser at 337 nm.

2. Why does the ratio of anti-Stokes to Stokes intensities increase with sample temperature?

3. What is a virtual state?

4. Why is fluorescence such a problem in Raman Spectroscopy?

5. Of the three laser sources described in Problem 1, which will yield the strongest Raman signals (has the best scattering power) and which is most likely to give problems due to fluorescence ?

6. Suggest two or three ways in which one might distinguish whether an observed peak obtained on laser excitation is caused by fluorescence or Raman scattering.

7. For vibrational states, the Boltzmann equation can be written as N_1/N_0 = exp (-delta E/kt) where N_0 and N_1 are the populations of the lower and upper energy states, respectively. delta E is the energy difference between the states, k is the Boltzmann constant (1.38066×10^{-23} J/K = 0.695035 cm^{-1}/K) and T is the temperature is Kelvins. For temperatures of 25 degree Celsius and 100 degree Celsius, calculate the ratio of the intensities of the Stokes and anti-Stokes lines for CCl_4 at (a) 218 cm^{-1}, (b) 459 cm^{-1}, and (c) 790 cm^{-1}.

8. Do a brief search of the web for notch filters for Raman spectroscopy and identify what is a notch filter and why are they useful in Raman instruments

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CHAPTER OVERVIEW

12: An Introduction to Chromatographic Separations

12.1: Overview of Analytical Separations

12.2: General Theory of Column Chromatography

12.3: Optimizing Chromatographic Separations

12.4: Problems

Thumbnail: Separation of black ink on a thin layer chromatography plate. Image used with permission (CC BY-SA 3.0; Natrij).

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12.1: Overview of Analytical Separations

In your Organic Chemistry Laboratory class you examined several methods for separating an analyte from potential interferents. For example, in a liquid–liquid extraction the analyte and interferent initially are present in a single liquid phase. We add a second, immiscible liquid phase and thoroughly mix them by shaking. During this process the analyte and interferents partition between the two phases to different extents, effecting their separation. After allowing the phases to separate, we draw off the phase enriched in analyte. Despite the power of liquid–liquid extractions, there are significant limitations.

Two Limitations of Liquid-Liquid Extractions

Suppose we have a sample that contains an analyte in a matrix that is incompatible with our analytical method. To determine the analyte's concentration we first separate it from the matrix using a simple liquid–liquid extraction. If we have several analytes, we may need to complete a separate extraction for each analyte. For a complex mixture of analytes this quickly becomes a tedious process. This is one limitation to a liquid–liquid extraction.

A more significant limitation is that the extent of a separation depends on the distribution ratio of each species in the sample. If the analyte's distribution ratio is similar to that of another species, then their separation becomes impossible. For example, let's assume that an analyte, *A*, and an interferent, *I*, have distribution ratios of, respectively, 5 and 0.5. If we use a liquid–liquid extraction with equal volumes of sample and extractant, then it is easy to show that a single extraction removes approximately 83% of the analyte and 33% of the interferent. Although we can remove 99% of the analyte with three extractions, we also remove 70% of the interferent. In fact, there is no practical combination of number of extractions or volumes of sample and extractant that produce an acceptable separation.

Bsed on our experience with extraction we can define the distribution ratio, *D*, for a solute, *S*, is

$$D = \frac{[S]_{\text{ext}}}{[S]_{\text{samp}}}$$

where $[S]_{\text{ext}}$ is its equilibrium concentration in the extracting phase and $[S]_{\text{samp}}$ is its equilibrium concentration in the sample. We can use the distribution ratio to calculate the fraction of *S* that remains in the sample, q_{samp}, after an extraction

$$q_{\text{samp}} = \frac{V_{\text{samp}}}{DV_{\text{ext}} + V_{\text{samp}}}$$

where V_{samp} is the volume of sample and V_{ext} is the volume of the extracting phase. For example, if D = 10, V_{samp} = 20, and V_{ext} = 5, the fraction of *S* remaining in the sample after the extraction is

$$q_{\text{samp}} = \frac{20}{10 \times 5 + 20} = 0.29$$

or 29%. The remaining 71% of the analyte is in the extracting phase.

A Better Way to Separate Mixtures

The problem with a liquid–liquid extraction is that the separation occurs in one direction only: from the sample to the extracting phase. Let's take a closer look at the liquid–liquid extraction of an analyte and an interferent with distribution ratios of, respectively, 5 and 0.5. Figure **12.1.1** shows that a single extraction using equal volumes of sample and extractant transfers 83% of the analyte and 33% of the interferent to the extracting phase. If the original concentrations of *A* and *I* are identical, then their concentration ratio in the extracting phase after one extraction is

$$\frac{[A]}{[I]} = \frac{0.83}{0.33} = 2.5$$

A single extraction, therefore, enriches the analyte by a factor of **2.5×**. After completing a second extraction (Figure **12.1.1**) and combining the two extracting phases, the separation of the analyte and the interferent, surprisingly, is less efficient.

$$\frac{[A]}{[I]} = \frac{0.97}{0.55} = 1.8$$

Figure 12.1.1 makes it clear why the second extraction results in a poorer overall separation: the second extraction actually favors the interferent!

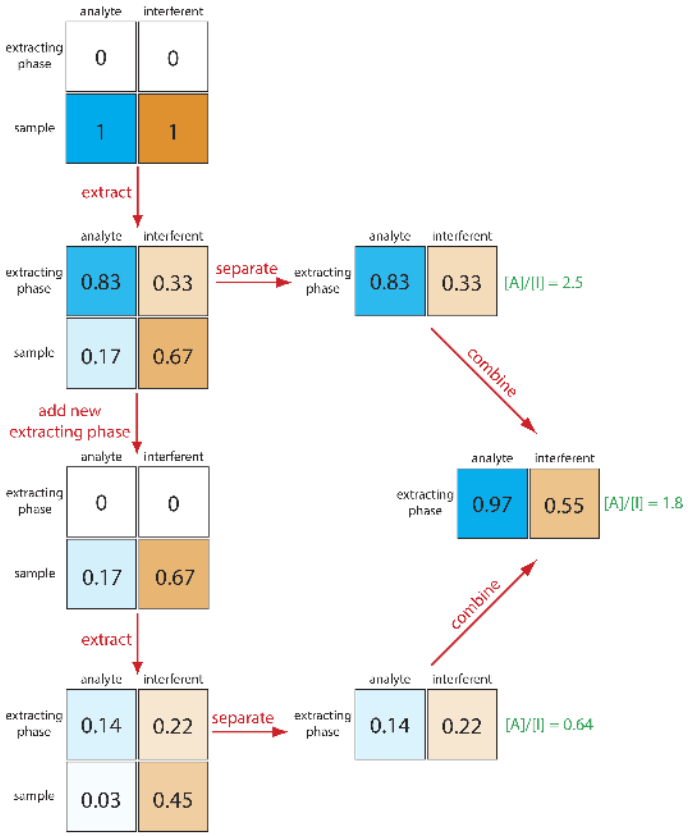


Figure 12.1.1. Progress of a traditional liquid–liquid extraction using two identical extractions of a single sample using fresh portions of the extractant. The numbers give the fraction of analyte and interferent in each phase assuming equal volumes of sample and extractant and distribution ratios of 5 and 0.5 for the analyte and the interferent, respectively. The opacity of the colors equals the fraction of analyte and interferent present.

We can improve the separation by first extracting the solutes from the sample into the extracting phase and then extracting them back into a fresh portion of solvent that matches the sample's matrix (Figure 12.1.2). Because the analyte has the larger distribution ratio, more of it moves into the extractant during the first extraction and less of it moves back to the sample phase during the second extraction. In this case the concentration ratio in the extracting phase after two extractions is significantly greater.

$$\frac{[A]}{[I]} = \frac{0.69}{0.11} = 6.3$$

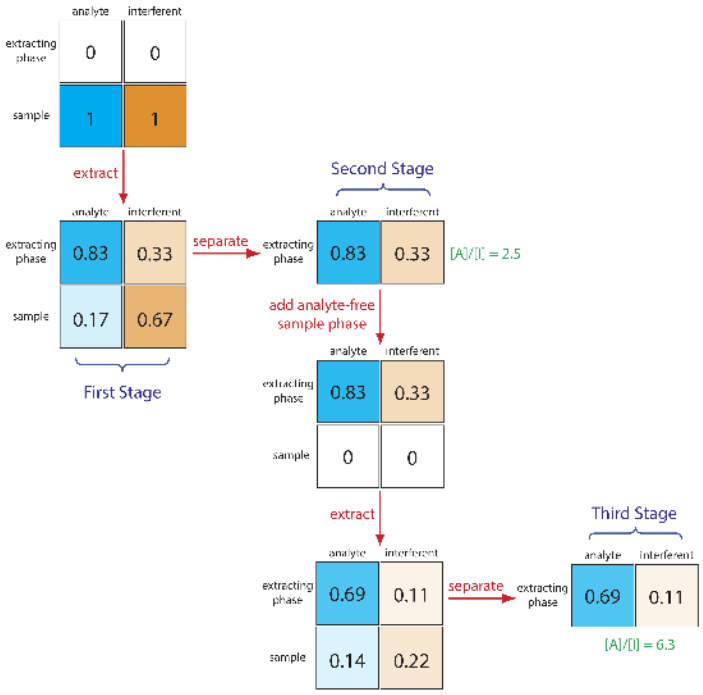


Figure 12.1.2. Progress of a liquid–liquid extraction in which we first extract the solutes into the extracting phase and then extract them back into an analyte-free portion of the sample's phase. The numbers give the fraction of analyte and interferent in each phase assuming equal volumes of sample and extractant and distribution ratios of 5 and 0.5 for the analyte and the interferent, respectively. The opacity of the colors equals the fraction of analyte and interferent present.

Not shown in Figure 12.2 is that we can add a fresh portion of the extracting phase to the sample that remains after the first extraction (the bottom row of the first stage in Figure 12.2, beginning the process anew. As we increase the number of extractions, the analyte and the interferent each spread out in space over a series of stages. Because the interferent's distribution ratio is smaller than the analyte's, the interferent lags behind the analyte. With a sufficient number of extractions—that is, a sufficient number of stages—a complete separation of the analyte and interferent is possible. This process of extracting the solutes back and forth between fresh portions of the two phases, which we call a ***countercurrent extraction***, was developed by Craig in the 1940s [Craig, L. C. *J. Biol. Chem.* **1944**, *155*, 519–534]. The same phenomenon forms the basis of modern chromatography.

Chromatographic Separations

In ***chromatography*** we pass a sample-free phase, which we call the ***mobile phase***, over a second sample-free ***stationary phase*** that remains fixed in space (Figure **12.1.3**). We inject or place the sample into the mobile phase. As the sample moves with the mobile phase, its components partition between the mobile phase and the stationary phase. A component whose distribution ratio favors the stationary phase requires more time to pass through the system. Given sufficient time and sufficient stationary and mobile phase, we can separate solutes even if they have similar distribution ratios.

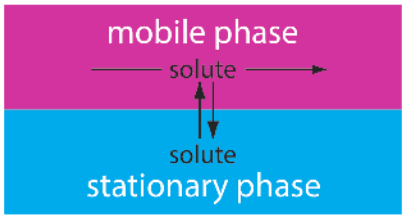


Figure12.1.3. In chromatography we pass a mobile phase over a stationary phase. When we inject a sample into the mobile phase, the sample's components both move with the mobile phase and partition into the stationary phase. The solute that spends the most time in the stationary phase takes the longest time to move through the system.

There are many ways in which we can identify a chromatographic separation: by describing the physical state of the mobile phase and the stationary phase; by describing how we bring the stationary phase and the mobile phase into contact with each other; or by describing the chemical or physical interactions between the solute and the stationary phase. Let's briefly consider how we might use each of these classifications.

> We can trace the history of chromatography to the turn of the century when the Russian botanist Mikhail Tswett used a column packed with calcium carbonate and a mobile phase of petroleum ether to separate colored pigments from plant extracts. As the sample moved through the column, the plant's pigments separated into individual colored bands. After effecting the separation, the calcium carbonate was removed from the column, sectioned, and the pigments recovered. Tswett named the technique chromatography, combining the Greek words for "color" and "to write." There was little interest in Tswett's technique until Martin and Synge's pioneering development of a theory of chromatography (see Martin, A. J. P.; Synge, R. L. M. "A New Form of Chromatogram Employing Two Liquid Phases," *Biochem. J.* **1941**, *35*, 1358–1366). Martin and Synge were awarded the 1952 Nobel Prize in Chemistry for this work.

Types of Mobile Phases and Stationary Phases

The mobile phase is a liquid or a gas, and the stationary phase is a solid or a liquid film coated on a solid substrate. We often name chromatographic techniques by listing the type of mobile phase followed by the type of stationary phase. In gas–liquid chromatography, for example, the mobile phase is a gas and the stationary phase is a liquid film coated on a solid substrate. If a technique's name includes only one phase, as in gas chromatography, it is the mobile phase.

Contact Between the Mobile Phase and the Stationary Phase

There are two common methods for bringing the mobile phase and the stationary phase into contact. In ***column chromatography*** we pack the stationary phase into a narrow column and pass the mobile phase through the column using gravity or by applying pressure. The stationary phase is a solid particle or a thin liquid film coated on either a solid particulate packing material or on the column's walls.

In ***planar chromatography*** the stationary phase is coated on a flat surface—typically, a glass, metal, or plastic plate. One end of the plate is placed in a reservoir that contains the mobile phase, which moves through the stationary phase by capillary action. In paper chromatography, for example, paper is the stationary phase.

Interaction Between the Solute and the Stationary Phase

The interaction between the solute and the stationary phase provides a third method for describing a separation (Figure **12.1.4**). In ***adsorption chromatography***, solutes separate based on their ability to adsorb to a solid stationary phase. In ***partition chromatography***, the stationary phase is a thin liquid film on a solid support. Separation occurs because there is a difference in the equilibrium partitioning of solutes between the stationary phase and the mobile phase. A stationary phase that consists of a solid support with covalently attached anionic (e.g., $-SO_3^-$) or cationic (e.g., $-N(CH_3)_3^+$) functional groups is the basis for ion-exchange chromatography in which ionic solutes are attracted to the stationary phase by electrostatic forces. In size-exclusion chromatography the stationary phase is a porous particle or gel, with separation based on the size of the solutes. Larger solutes are unable to penetrate as deeply into the porous stationary phase and pass more quickly through the column.

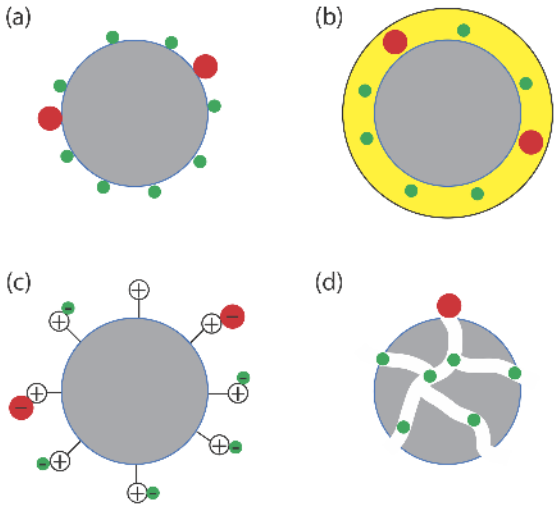


Figure 12.1.4. Four examples of interactions between a solute and the stationary phase: (a) adsorption on a solid surface, (b) partitioning into a liquid phase, (c) ion-exchange, and (d) size exclusion. For each example, the smaller, green solute is more strongly retained than the larger, red solute.

There are other interactions that can serve as the basis of a separation. In affinity chromatography the interaction between an antigen and an antibody, between an enzyme and a substrate, or between a receptor and a ligand forms the basis of a separation. See this chapter's additional resources for some suggested readings.

Electrophoretic Separations

In chromatography, a separation occurs because there is a difference in the equilibrium partitioning of solutes between the mobile phase and the stationary phase. Equilibrium partitioning, however, is not the only basis for effecting a separation. In an electrophoretic separation, for example, charged solutes migrate under the influence of an applied potential. A separation occurs because of differences in the charges and the sizes of the solutes (Figure **12.1.5**).

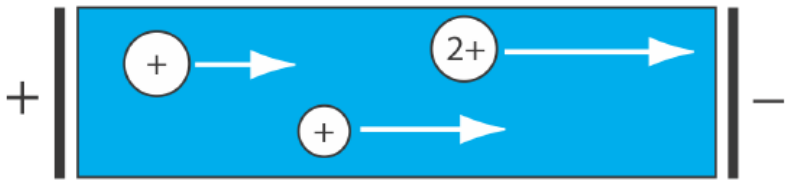


Figure 12.1.5. Movement of charged solutes under the influence of an applied potential. The lengths of the arrows indicate the relative speed of the solutes. In general, a larger solute moves more slowly than a smaller solute of equal charge, and a solute with a larger charge move more quickly than a solute with a smaller charge.

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12.2: General Theory of Column Chromatography

Of the two methods for bringing the stationary phase and the mobile phases into contact, the most important is column chromatography. In this section we develop a general theory that we may apply to any form of column chromatography.

Figure 12.2.1 provides a simple view of a liquid–solid column chromatography experiment. The sample is introduced as a narrow band at the top of the column. Ideally, the solute's initial concentration profile is rectangular (Figure 12.2.2 a). As the sample moves down the column, the solutes begin to separate (Figure 12.2.1 b,c) and the individual solute bands begin to broaden and develop a Gaussian profile (Figure 12.2.2 b,c). If the strength of each solute's interaction with the stationary phase is sufficiently different, then the solutes separate into individual bands (Figure 12.2.1 d and Figure 12.2.2 d).

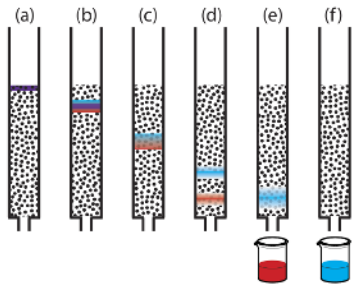


Figure 12.2.1 . Progress of a column chromatographic separation of a two-component mixture. In (a) the sample is layered on top of the stationary phase. As mobile phase passes through the column, the sample separates into two solute bands (b–d). In (e) and (f), we collect each solute as it elutes from the column.

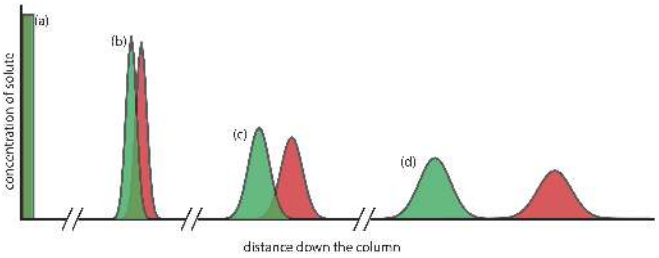


Figure 12.2.2 . An alternative view of the separation in Figure 12.2.1 showing the concentration of each solute as a function of distance down the column.

We can follow the progress of the separation by collecting fractions as they elute from the column (Figure 12.2.1 e,f), or by placing a suitable detector at the end of the column. A plot of the detector's response as a function of elution time, or as a function of the volume of mobile phase, is known as a ***chromatogram*** (Figure 12.2.3), and consists of a peak for each solute.

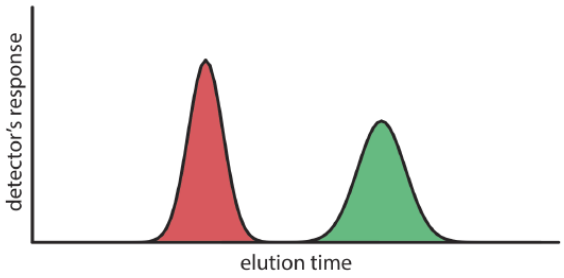


Figure 12.2.3 . Chromatogram for the separation shown in Figure 12.2.1 and Figure 12.2.2 , showing the detector's response as a function of the elution time.

> There are many possible detectors that we can use to monitor the separation. Later sections of this chapter describe some of the most popular.

We can characterize a chromatographic peak's properties in several ways, two of which are shown in Figure 12.2.4 . ***Retention time***, t_r, is the time between the sample's injection and the maximum response for the solute's peak. A chromatographic peak's ***baseline width***, w, as shown in Figure 12.2.4 , is determined by extending tangent lines from the inflection points on either side of the peak through the baseline. Although usually we report t_r and w using units of time, we can report them using units of volume by multiplying each by the mobile phase's velocity, or report them in linear units by measuring distances with a ruler.

> For example, a solute's retention volume, V_r, is $\boldsymbol{t_r \times u}$ where u is the mobile phase's velocity through the column.

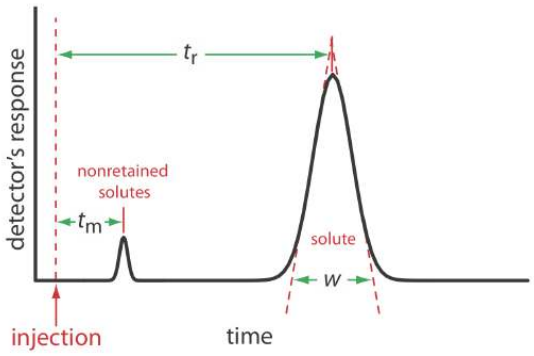


Figure 12.2.4 . Chromatogram showing a solute's retention time, t_r, and baseline width, w, and the column's void time, t_m, for nonretained solutes.

In addition to the solute's peak, Figure 12.2.4 also shows a small peak that elutes shortly after the sample is injected into the mobile phase. This peak contains all ***nonretained solutes***, which move through the column at the same rate as the mobile phase. The time required to elute the nonretained solutes is called the column's ***void time***, t_m.

Chromatographic Resolution

The goal of chromatography is to separate a mixture into a series of chromatographic peaks, each of which constitutes a single component of the mixture. The ***resolution*** between two chromatographic peaks, R_{AB}, is a quantitative measure of their separation, and is defined as

$$R_{AB} = \frac{t_{t,B} - t_{t,A}}{0.5\,(w_B + w_A)} = \frac{2\Delta t_r}{w_B + w_A} \tag{12.2.1}$$

where B is the later eluting of the two solutes. As shown in Figure 12.2.5 , the separation of two chromatographic peaks improves with an increase in R_{AB}. If the areas under the two peaks are identical—as is the case in Figure 12.2.5 —then a resolution of 1.50 corresponds to an overlap of only 0.13% for the two elution profiles. Because resolution is a quantitative measure of a separation's success, it is a useful way to determine if a change in experimental conditions leads to a better separation.

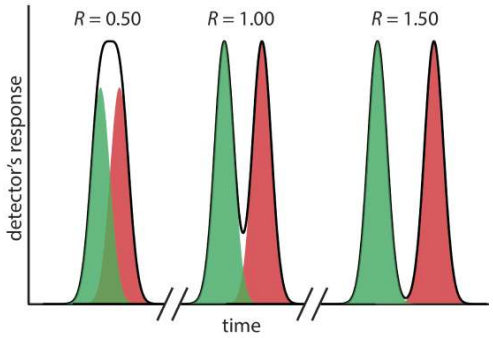


Figure 12.2.5 . Three examples that show the relationship between resolution and the separation of a two component mixture. The green peak and the red peak are the elution profiles for the two components. The chromatographic peak— which is the sum of the two elution profiles—is shown by the solid black line.

Example 12.2.1

In a chromatographic analysis of lemon oil a peak for limonene has a retention time of 8.36 min with a baseline width of 0.96 min. γ-Terpinene elutes at 9.54 min with a baseline width of 0.64 min. What is the resolution between the two peaks?

Solution

Using Equation 12.2.1 we find that the resolution is

$$R_{AB} = \frac{2\Delta t_r}{w_B + w_A} = \frac{2(9.54\text{ min} - 8.36\text{ min})}{0.64\text{ min} + 0.96\text{ min}} = 1.48$$

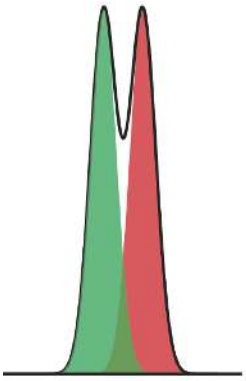


Figure 12.2.6 . Chromatogram for Exercise 12.2.1 .

Exercise 12.2.1

Figure 12.2.6 shows the separation of a two-component mixture. What is the resolution between the two components? Use a ruler to measure Δt_r, w_A, and w_B in millimeters.

Answer

Because the relationship between elution time and distance is proportional, we can measure Δt_r, w_A, and w_B using a ruler. My measurements are 8.5 mm for Δt_r, and 12.0 mm each for w_A and w_B. Using these values, the resolution is

$$R_{AB} = \frac{2\Delta t_t}{w_A + w_B} = \frac{2(8.5\text{ mm})}{12.0\text{ mm} + 12.0\text{ mm}} = 0.70$$

Your measurements for Δt_r, w_A, and w_B will depend on the relative size of your monitor or printout; however, your value for the resolution should be similar to the answer above.

Equation 12.2.1 suggests that we can improve resolution by increasing Δt_r, or by decreasing w_A and w_B (Figure 12.2.7). To increase Δt_r we can use one of two strategies. One approach is to adjust the separation conditions so that both solutes spend less time in the mobile phase—that is, we increase each *solute's retention factor*—which provides more time to effect a separation. A second approach is to increase *selectivity* by adjusting conditions so that only one solute experiences a significant change in its retention time. The baseline width of a solute's peak depends on the solutes movement within and between the mobile phase and the stationary phase, and is governed by several factors that collectively we call *column efficiency*. We will consider each of these approaches for improving resolution in more detail, but first we must define some terms.

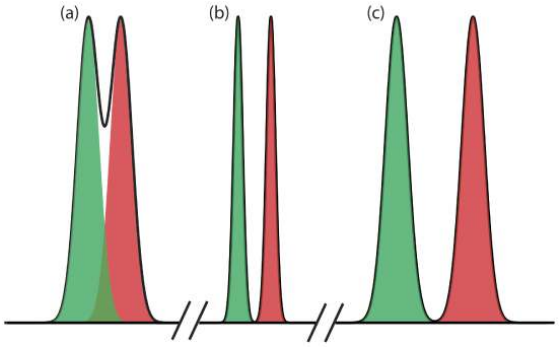


Figure 12.2.7 . Two method for improving chromatographic resolution: (a) original chromatogram; (b) chromatogram after decreasing w_A and w_B by 4×; and (c) chromatogram after increasing Δt_r by 2×.

Solute Retention Factor

Let's assume we can describe a solute's distribution between the mobile phase and stationary phase using the following equilibrium reaction

$$S_m \rightleftharpoons S_s$$

where S_m is the solute in the mobile phase and S_s is the solute in the stationary phase. Following the same approach we used in Chapter 7.7 for liquid–liquid extractions, the equilibrium constant for this reaction is an equilibrium partition coefficient, K_D.

$$K_D = \frac{[S_s]}{[S_m]}$$

This is not a trivial assumption. In this section we are, in effect, treating the solute's equilibrium between the mobile phase and the stationary phase as if it is identical to the equilibrium in a liquid–liquid extraction. You might question whether this is a reasonable assumption. There is an important difference between the two experiments that we need to consider. In a liquid–liquid extraction, which takes place in a separatory funnel, the two phases remain in contact with each other at all times, allowing for a true equilibrium. In chromatography, however, the mobile phase is in constant motion. A solute that moves into the stationary phase from the mobile phase will equilibrate back into a different portion of the mobile phase; this does not describe a true equilibrium.

So, we ask again: Can we treat a solute's distribution between the mobile phase and the stationary phase as an equilibrium process? The answer is yes, if the mobile phase velocity is slow relative to the kinetics of the solute's movement back and forth between the two phase. In general, this is a reasonable assumption.

In the absence of any additional equilibrium reactions in the mobile phase or the stationary phase, K_D is equivalent to the distribution ratio, D,

$$D = \frac{[S_0]}{[S_m]} = \frac{(\text{mol S})_s / V_s}{(\text{mol S})_m / V_m} = K_D \tag{12.2.2}$$

where V_s and V_m are the volumes of the stationary phase and the mobile phase, respectively.

A conservation of mass requires that the total moles of solute remain constant throughout the separation; thus, we know that the following equation is true.

$$(\text{mol S})_{tot} = (\text{mol S})_m + (\text{mol S})_s \tag{12.2.3}$$

Solving Equation 12.2.3 for the moles of solute in the stationary phase and substituting into Equation 12.2.2 leaves us with

$$D = \frac{\{(\text{mol S})_{tot} - (\text{mol S})_m\} / V_s}{(\text{mol S})_m / V_m}$$

Rearranging this equation and solving for the fraction of solute in the mobile phase, f_m, gives

$$f_m = \frac{(\text{mol S})_m}{(\text{mol S})_{tot}} = \frac{V_m}{DV_s + V_m} \tag{12.2.4}$$

which is identical to the result for a liquid-liquid extraction (see Chapter 7). Because we may not know the exact volumes of the stationary phase and the mobile phase, we simplify Equation 12.2.4 by dividing both the numerator and the denominator by V_m; thus

$$f_m = \frac{V_m / V_m}{DV_s / V_m + V_m / V_m} = \frac{1}{DV_s / V_m + 1} = \frac{1}{1 + k} \tag{12.2.5}$$

where k

$$k = D \times \frac{V_s}{V_m} \tag{12.2.6}$$

is the solute's ***retention factor***. Note that the larger the retention factor, the more the distribution ratio favors the stationary phase, leading to a more strongly retained solute and a longer retention time.

Other (older) names for the retention factor are capacity factor, capacity ratio, and partition ratio, and it sometimes is given the symbol k'. Keep this in mind if you are using other resources. Retention factor is the approved name from the IUPAC Gold Book.

We can determine a solute's retention factor from a chromatogram by measuring the column's void time, t_m, and the solute's retention time, t_r (see Figure 12.2.4). Solving Equation 12.2.5 for k, we find that

$$k = \frac{1 - f_m}{f_m} \tag{12.2.7}$$

Earlier we defined f_m as the fraction of solute in the mobile phase. Assuming a constant mobile phase velocity, we also can define f_m as

$$f_m = \frac{\text{time spent in the mobile phase}}{\text{time spent in the stationary phase}} = \frac{t_m}{t_r}$$

Substituting back into Equation 12.2.7 and rearranging leaves us with

$$k = \frac{1 - \frac{t_m}{t_t}}{\frac{t_m}{t_r}} = \frac{t_t - t_m}{t_m} = \frac{t'_r}{t_m} \tag{12.2.8}$$

where t'_r is the ***adjusted retention time***.

Example 12.2.2

In a chromatographic analysis of low molecular weight acids, butyric acid elutes with a retention time of 7.63 min. The column's void time is 0.31 min. Calculate the retention factor for butyric acid.

Solution

$$k_{but} = \frac{t_r - t_m}{t_m} = \frac{7.63 \text{ min} - 0.31 \text{ min}}{0.31 \text{ min}} = 23.6$$

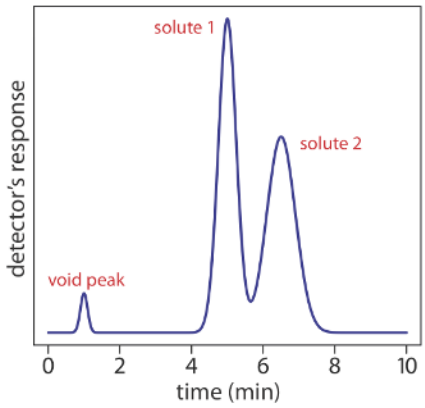


Figure 12.2.8 . Chromatogram for Exercise 12.2.2 .

Exercise 12.2.2

Figure 12.2.8 is the chromatogram for a two-component mixture. Determine the retention factor for each solute assuming the sample was injected at time $t = 0$.

Answer

Because the relationship between elution time and distance is proportional, we can measure t_m, $t_{r,1}$, and $t_{r,2}$ using a ruler. My measurements are 7.8 mm, 40.2 mm, and 51.5 mm, respectively. Using these values, the retention factors for solute A and solute B are

$$k_1 = \frac{t_{r1} - t_m}{t_m} = \frac{40.2 \text{ mm} - 7.8 \text{ mm}}{7.8 \text{ mm}} = 4.15$$

$$k_2 = \frac{t_{r2} - t_m}{t_m} = \frac{51.5 \text{ mm} - 7.8 \text{ mm}}{7.8 \text{ mm}} = 5.60$$

Your measurements for t_m, $t_{r,1}$, and $t_{r,2}$ will depend on the relative size of your monitor or printout; however, your value for the resolution should be similar to the answer above.

Selectivity

Selectivity is a relative measure of the retention of two solutes, which we define using a selectivity factor, α

$$\alpha = \frac{k_B}{k_A} = \frac{t_{r,B} - t_m}{t_{r,A} - t_m} \tag{12.2.9}$$

where solute *A* has the smaller retention time. When two solutes elute with identical retention time, $\alpha = 1.00$; for all other conditions $\alpha > 1.00$.

Example 12.2.3

In the chromatographic analysis for low molecular weight acids described in Example 12.2.2 , the retention time for isobutyric acid is 5.98 min. What is the selectivity factor for isobutyric acid and butyric acid?

Solution

First we must calculate the retention factor for isobutyric acid. Using the void time from Example 12.2.2 we have

$$k_{iso} = \frac{t_r - t_m}{t_m} = \frac{5.98 \text{ min} - 0.31 \text{ min}}{0.31 \text{ min}} = 18.3$$

The selectivity factor, therefore, is

$$\alpha = \frac{k_{but}}{k_{iso}} = \frac{23.6}{18.3} = 1.29$$

Exercise 12.2.3

Determine the selectivity factor for the chromatogram in Exercise 12.2.2 .

Answer

Using the results from Exercise 12.2.2 , the selectivity factor is

$$\alpha = \frac{k_2}{k_1} = \frac{5.60}{4.15} = 1.35$$

Your answer may differ slightly due to differences in your values for the two retention factors.

Column Efficiency

Suppose we inject a sample that has a single component. At the moment we inject the sample it is a narrow band of finite width. As the sample passes through the column, the width of this band continually increases in a process we call ***band broadening***. Column efficiency is a quantitative measure of the extent of band broadening.

See Figure 12.2.1 and Figure 12.2.2 . When we inject the sample it has a uniform, or rectangular concentration profile with respect to distance down the column. As it passes through the column, the band broadens and takes on a Gaussian concentration profile.

In their original theoretical model of chromatography, Martin and Synge divided the chromatographic column into discrete sections, which they called theoretical plates. Within each theoretical plate there is an equilibrium between the solute present in the stationary phase and the solute present in the mobile phase [Martin, A. J. P.; Synge, R. L. M. *Biochem. J.* **1941**, *35*, 1358–1366]. They described column efficiency in terms of the number of ***theoretical plates***, N,

$$N = \frac{L}{H} \tag{12.2.10}$$

where L is the column's length and H is the height of a theoretical plate. For any given column, the column efficiency improves—and chromatographic peaks become narrower—when there are more theoretical plates.

If we assume that a chromatographic peak has a Gaussian profile, then the extent of band broadening is given by the peak's variance or standard deviation. The height of a theoretical plate is the peak's variance per unit length of the column

$$H = \frac{\sigma^2}{L} \tag{12.2.11}$$

where the standard deviation, σ, has units of distance. Because retention times and peak widths usually are measured in seconds or minutes, it is more convenient to express the standard deviation in units of time, τ, by dividing σ by the solute's average linear velocity, $\overline{u}$, which is equivalent to dividing the distance it travels, L, by its retention time, t_r.

$$\tau = \frac{\sigma}{\overline{u}} = \frac{\sigma t_r}{L} \tag{12.2.12}$$

For a Gaussian peak shape, the width at the baseline, w, is four times its standard deviation, τ.

$$w = 4\tau \tag{12.2.13}$$

Combining Equation 12.2.11, Equation 12.2.12, and Equation 12.2.13 defines the height of a theoretical plate in terms of the easily measured chromatographic parameters t_r and w.

$$H = \frac{Lw^2}{16t_r^2} \tag{12.2.14}$$

Combing Equation 12.2.14 and Equation 12.2.10 gives the number of theoretical plates.

$$N = 16\frac{t_r^2}{w^2} = 16\left(\frac{t_r}{w}\right)^2 \tag{12.2.15}$$

Example 12.2.4

A chromatographic analysis for the chlorinated pesticide Dieldrin gives a peak with a retention time of 8.68 min and a baseline width of 0.29 min. Calculate the number of theoretical plates? Given that the column is 2.0 m long, what is the height of a theoretical plate in mm?

Solution

Using Equation 12.2.15, the number of theoretical plates is

$$N = 16\frac{t_r^2}{w^2} = 16 \times \frac{(8.68\text{ min})^2}{(0.29\text{ min})^2} = 14300\text{ plates}$$

Solving Equation 12.2.10 for H gives the average height of a theoretical plate as

$$H = \frac{L}{N} = \frac{2.00\text{ m}}{14300\text{ plates}} \times \frac{1000\text{ mm}}{\text{m}} = 0.14\text{ mm/plate}$$

Exercise 12.2.4

For each solute in the chromatogram for Exercise 12.2.2 , calculate the number of theoretical plates and the average height of a theoretical plate. The column is 0.5 m long.

Answer

Because the relationship between elution time and distance is proportional, we can measure $t_{r,1}$, $t_{r,2}$, w_1, and w_2 using a ruler. My measurements are 40.2 mm, 51.5 mm, 8.0 mm, and 13.5 mm, respectively. Using these values, the number of theoretical plates for each solute is

$$N_1 = 16\frac{t_{r,1}^2}{w_1^2} = 16 \times \frac{(40.2\text{ mm})^2}{(8.0\text{ mm})^2} = 400\text{ theoretical plates}$$

$$N_2 = 16\frac{t_{r,2}^2}{w_2^2} = 16 \times \frac{(51.5\text{ mm})^2}{(13.5\text{ mm})^2} = 233\text{ theoretical plates}$$

The height of a theoretical plate for each solute is

$$H_1 = \frac{L}{N_1} = \frac{0.500\text{ m}}{400\text{ plates}} \times \frac{1000\text{ mm}}{\text{m}} = 1.2\text{ mm/plate}$$

$$H_2 = \frac{L}{N_2} = \frac{0.500\text{ m}}{233\text{ plates}} \times \frac{1000\text{ mm}}{\text{m}} = 2.15\text{ mm/plate}$$

Your measurements for $t_{r,1}$, $t_{r,2}$, w_1, and w_2 will depend on the relative size of your monitor or printout; however, your values for N and for H should be similar to the answer above.

It is important to remember that a theoretical plate is an artificial construct and that a chromatographic column does not contain physical plates. In fact, the number of theoretical plates depends on both the properties of the column and the solute. As a result, the number of theoretical plates for a column may vary from solute to solute.

Peak Capacity

One advantage of improving column efficiency is that we can separate more solutes with baseline resolution. One estimate of the number of solutes that we can separate is

$$n_c = 1 + \frac{\sqrt{N}}{4}\ln\frac{V_{max}}{V_{min}} \tag{12.2.16}$$

where n_c is the column's ***peak capacity***, and V_{min} and V_{max} are the smallest and the largest volumes of mobile phase in which we can elute and detect a solute [Giddings, J. C. *Unified Separation Science*, Wiley-Interscience: New York, 1991]. A column with 10 000 theoretical plates, for example, can resolve no more than

$$n_c = 1 + \frac{\sqrt{10000}}{4}\ln\frac{30\text{mL}}{1\text{mL}} = 86\text{ solutes}$$

if V_{min} and V_{max} are 1 mL and 30 mL, respectively. This estimate provides an upper bound on the number of solutes and may help us exclude from consideration a column that does not have enough theoretical plates to separate a complex mixture. Just because a column's theoretical peak capacity is larger than the number of solutes, however, does not mean that a separation is feasible. In most situations the practical peak capacity is less than the theoretical peak capacity because the retention characteristics of some solutes are so similar that a separation is impossible. Nevertheless, columns with more theoretical plates, or with a greater range of possible elution volumes, are more likely to separate a complex mixture.

The smallest volume we can use is the column's void volume. The largest volume is determined either by our patience—the maximum analysis time we can tolerate—or by our inability to detect solutes because there is too much band broadening.

Asymmetric Peaks

Our treatment of chromatography in this section assumes that a solute elutes as a symmetrical Gaussian peak, such as that shown in Figure 12.2.4 . This ideal behavior occurs when the solute's partition coefficient, K_D

$$K_D = \frac{[S_s]}{[S_m]}$$

is the same for all concentrations of solute. If this is not the case, then the chromatographic peak has an asymmetric peak shape similar to those shown in Figure 12.2.9 . The chromatographic peak in Figure 12.2.9 a is an example of ***peak tailing***, which occurs when some sites on the stationary phase retain the solute more strongly than other sites. Figure 12.2.9 b, which is an example of ***peak fronting*** most often is the result of overloading the column with sample.

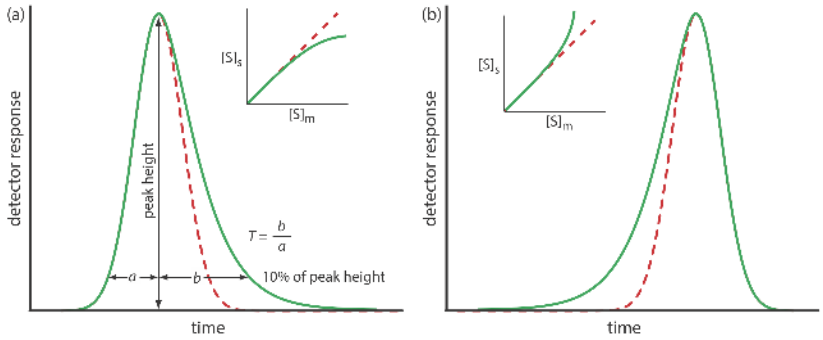


Figure 12.2.9 . Examples of asymmetric chromatographic peaks showing (a) peak tailing and (b) peak fronting. For both (a) and (b) the green chromatogram is the asymmetric peak and the red dashed chromatogram shows the ideal, Gaussian peak shape. The insets show the relationship between the concentration of solute in the stationary phase, $[S]_s$, and its concentration in the mobile phase, $[S]_m$. The dashed red lines show ideal behavior (K_D is constant for all conditions) and the green lines show nonideal behavior (K_D decreases or increases for higher total concentrations of solute). A quantitative measure of peak tailing, T, is shown in (a).

As shown in Figure 12.2.9 a, we can report a peak's asymmetry by drawing a horizontal line at 10% of the peak's maximum height and measuring the distance from each side of the peak to a line drawn vertically through the peak's maximum. The asymmetry factor, T, is defined as

$$T = \frac{b}{a}$$

The number of theoretical plates for an asymmetric peak shape is approximately

$$N \approx \frac{41.7 \times \frac{t_r^2}{(w_{0.1})^2}}{T + 1.25} = \frac{41.7 \times \frac{t_r^2}{(a+b)^2}}{T + 1.25}$$

where $w_{0.1}$ is the width at 10% of the peak's height [Foley, J. P.; Dorsey, J. G. *Anal. Chem.* **1983**, *55*, 730–737].

Asymmetric peaks have fewer theoretical plates, and the more asymmetric the peak the smaller the number of theoretical plates. For example, the following table gives values for N for a solute eluting with a retention time of 10.0 min and a peak width of 1.00 min.

b	a	T	N
0.5	0.5	1.00	1850
0.6	0.4	1.50	1520
0.7	0.3	2.33	1160
0.8	0.2	4.00	790

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12.3: Optimizing Chromatographic Separations

Now that we have defined the solute retention factor, selectivity, and column efficiency we are able to consider how they affect the resolution of two closely eluting peaks. Because the two peaks have similar retention times, it is reasonable to assume that their peak widths are nearly identical.

> If the number of theoretical plates is the same for all solutes—not strictly true, but not a bad assumption—then from equation 11.2.15, the ratio t_r/w is a constant. If two solutes have similar retention times, then their peak widths must be similar.

Equation 11.2.1, therefore, becomes

$$R_{AB} = \frac{t_{r,B} - t_{r,A}}{0.5\,(w_B + w_A)} \approx \frac{t_{r,B} - t_{r,A}}{0.5\,(2w_B)} = \frac{t_{r,B} - t_{r,A}}{w_B} \tag{12.3.1}$$

where B is the later eluting of the two solutes. Solving equation 12.2.15 for w_B and substituting into equation 12.3.1 leaves us with the following result.

$$R_{AB} = \frac{\sqrt{N_B}}{4} \times \frac{t_{r,B} - t_{r,A}}{t_{r,B}} \tag{12.3.2}$$

Rearranging equation 11.2.8 provides us with the following equations for the retention times of solutes A and B.

$$t_{r,A} = k_A t_m + t_m \quad \text{and} \quad t_{r,B} = k_B t_m + t_m$$

After substituting these equations into equation 12.3.2 and simplifying, we have

$$R_{AB} = \frac{\sqrt{N_B}}{4} \times \frac{k_B - k_A}{1 + k_B}$$

Finally, we can eliminate solute A's retention factor by substituting in equation 11.2.9. After rearranging, we end up with the following equation for the resolution between the chromatographic peaks for solutes A and B.

$$R_{AB} = \frac{\sqrt{N_B}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_B}{1 + k_B} \tag{12.3.3}$$

In addition to resolution, another important factor in chromatography is the amount of time needed to elute a pair of solutes, which we can approximate using the retention time for solute B.

$$t_{r,s} = \frac{16 R_{AB}^2 H}{u} \times \left(\frac{\alpha}{\alpha - 1}\right)^2 \times \frac{(1 + k_B)^3}{k_B^2} \tag{12.3.4}$$

where u is the mobile phase's velocity.

> Although equation 12.3.3 is useful for considering how a change in N, α, or k qualitatively affects resolution—which suits our purpose here—it is less useful for making accurate quantitative predictions of resolution, particularly for smaller values of N and for larger values of R. For more accurate predictions use the equation
>
> $$R_{AB} = \frac{\sqrt{N}}{4} \times (\alpha - 1) \times \frac{k_B}{1 + k_{avg}}$$
>
> where k_{avg} is $(k_A + k_B)/2$. For a derivation of this equation and for a deeper discussion of resolution in column chromatography, see Foley, J. P. "Resolution Equations for Column Chromatography," *Analyst*, **1991**, *116*, 1275-1279.

Equation ??? and equation ??? contain terms that correspond to column efficiency, selectivity, and the solute retention factor. We can vary these terms, more or less independently, to improve resolution and analysis time. The first term, which is a function of the number of theoretical plates (for equation ???) or the height of a theoretical plate (for equation ???), accounts for the effect of column efficiency. The second term is a function of α and accounts for the influence of column selectivity. Finally, the third term in both equations is a function of k_B and accounts for the effect of solute B's retention factor. A discussion of how we can use these parameters to improve resolution is the subject of the remainder of this section.

Using the Retention Factor to Optimize Resolution

One of the simplest ways to improve resolution is to adjust the retention factor for solute *B*. If all other terms in equation ??? remain constant, an increase in k_B will improve resolution. As shown by the green curve in Figure **12.3.1**, however, the improvement is greatest if the initial value of k_B is small. Once k_B exceeds a value of approximately 10, a further increase produces only a marginal improvement in resolution. For example, if the original value of k_B is 1, increasing its value to 10 gives an 82% improvement in resolution; a further increase to 15 provides a net improvement in resolution of only 87.5%.

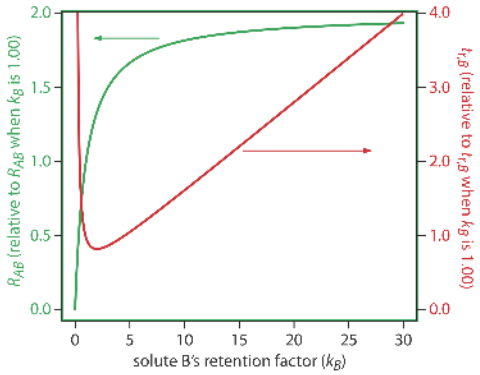


Figure 12.3.1. Effect of k_B on the resolution for a pair of solutes, R_{AB}, and the retention time for the later eluting solute, $t_{r,B}$. The *y*-axes display the resolution and retention time relative to their respective values when k_B is 1.00.

Any improvement in resolution from increasing the value of k_B generally comes at the cost of a longer analysis time. The red curve in Figure **12.3.1** shows the relative change in the retention time for solute *B* as a function of its retention factor. Note that the minimum retention time is for $k_B = 2$. Increasing k_B from 2 to 10, for example, approximately doubles solute B's retention time.

> The relationship between retention factor and analysis time in Figure **12.3.1** works to our advantage if a separation produces an acceptable resolution with a large k_B. In this case we may be able to decrease k_B with little loss in resolution and with a significantly shorter analysis time.

To increase k_B without changing selectivity, α, any change to the chromatographic conditions must result in a general, nonselective increase in the retention factor for both solutes. In gas chromatography, we can accomplish this by decreasing the column's temperature. Because a solute's vapor pressure is smaller at lower temperatures, it spends more time in the stationary phase and takes longer to elute. In liquid chromatography, the easiest way to increase a solute's retention factor is to use a mobile phase that is a weaker solvent. When the mobile phase has a lower solvent strength, solutes spend proportionally more time in the stationary phase and take longer to elute.

Adjusting the retention factor to improve the resolution between one pair of solutes may lead to unacceptably long retention times for other solutes. For example, suppose we need to analyze a four-component mixture with baseline resolution and with a run-time of less than 20 min. Our initial choice of conditions gives the chromatogram in Figure **12.3.2**a. Although we successfully separate components 3 and 4 within 15 min, we fail to separate components 1 and 2. Adjusting conditions to improve the resolution for the first two components by increasing k_2 provides a good separation of all four components, but the run-time is too long (Figure **12.3.2** b). This problem of finding a single set of acceptable operating conditions is known as the ***general elution problem***.

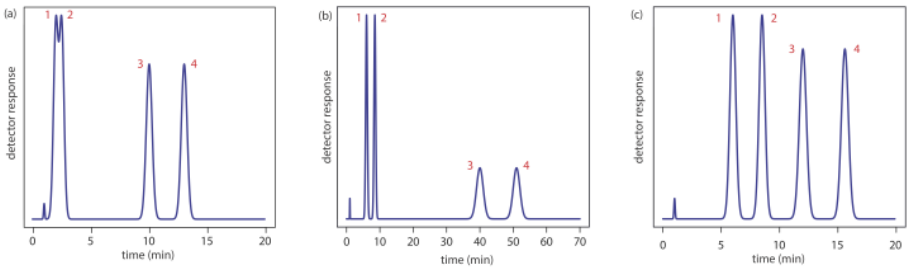


Figure 12.3.2. Example showing the general elution problem in chromatography. See text for details.

One solution to the general elution problem is to make incremental adjustments to the retention factor as the separation takes place. At the beginning of the separation we set the initial chromatographic conditions to optimize the resolution for early eluting solutes. As the separation progresses, we adjust the chromatographic conditions to decrease the retention factor—and, therefore, to decrease the retention time—for each of the later eluting solutes (Figure **12.3.2**c). In gas chromatography this is accomplished by temperature programming. The column's initial temperature is selected such that the first solutes to elute are resolved fully. The temperature is then increased, either continuously or in steps, to bring off later eluting components with both an acceptable resolution and a reasonable analysis time. In liquid chromatography the same effect is obtained by increasing the solvent's eluting strength. This is known as a gradient elution. We will have more to say about each of these in later sections of this chapter.

Using Selectivity to Optimize Resolution

A second approach to improving resolution is to adjust the selectivity, α. In fact, for $\alpha \approx 1$ usually it is not possible to improve resolution by adjusting the solute retention factor, k_B, or the column efficiency, N. A change in α often has a more dramatic effect on resolution than a change in k_B. For example, changing α from 1.1 to 1.5, while holding constant all other terms, improves resolution by 267%. In gas chromatography, we adjust α by changing the stationary phase; in liquid chromatography, we change the composition of the mobile phase to adjust α.

To change α we need to selectively adjust individual solute retention factors. Figure **12.3.3** shows one possible approach for the liquid chromatographic separation of a mixture of substituted benzoic acids. Because the retention time of a compound's weak acid form and its weak base form are different, its retention time will vary with the pH of the mobile phase, as shown in Figure **12.3.3**a. The intersections of the curves in Figure **12.3.3**a show pH values where two solutes co-elute. For example, at a pH of 3.8 terephthalic acid and *p*-hydroxybenzoic acid elute as a single chromatographic peak.

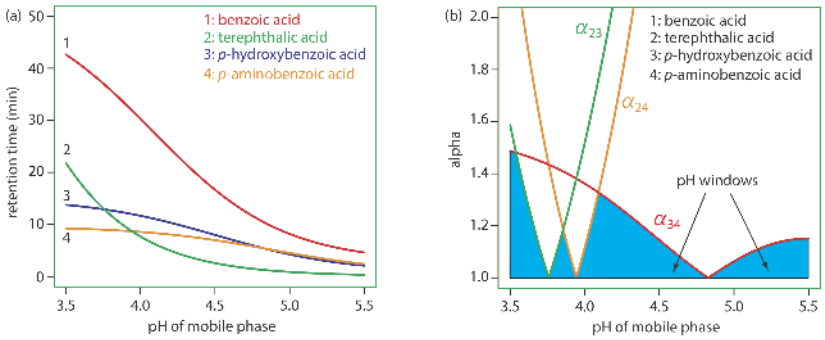


Figure 12.3.3. Example showing how the mobile phase pH in liquid chromatography affects selectivity: (a) retention times for four substituted benzoic acids as a function of the mobile phase's pH; (b) alpha values for three pairs of solutes that are difficult to separate. See text for details. The mobile phase is an acetic acid/sodium acetate buffer and the stationary phase is a nonpolar hydrocarbon. Data from Harvey, D. T.; Byerly, S.; Bowman, A.; Tomlin, J. "Optimization of HPLC and GC Separations Using Response Surfaces," *J. Chem. Educ.* **1991**, *68*, 162–168.

Figure **12.3.3**a shows that there are many pH values where some separation is possible. To find the optimum separation, we plot a for each pair of solutes. The red, green, and orange curves in Figure **12.3.3**b show the variation in a with pH for the three pairs of solutes that are hardest to separate (for all other pairs of solutes, $\alpha > 2$ at all pH levels). The blue shading shows windows of pH values in which at least a partial separation is possible—this figure is sometimes called a window diagram—and the highest point in each window gives the optimum pH within that range. The best overall separation is the highest point in any window, which, for this example, is a pH of 3.5. Because the analysis time at this pH is more than 40 min (Figure **12.3.3**a), choosing a pH between 4.1–4.4 might produce an acceptable separation with a much shorter analysis time.

Let's use benzoic acid, C_6H_5COOH, to explain why pH can affect a solute's retention time. The separation uses an aqueous mobile phase and a nonpolar stationary phase. At lower pHs, benzoic acid predominately is in its weak acid form, C_6H_5COOH, and partitions easily into the nonpolar stationary phase. At more basic pHs, however, benzoic acid is in its weak base form, $C_6H_5COO^-$. Because it now carries a charge, its solubility in the mobile phase increases and its solubility in the nonpolar stationary phase decreases. As a result, it spends more time in the mobile phase and has a shorter retention time.

Although the usual way to adjust pH is to change the concentration of buffering agents, it also is possible to adjust pH by changing the column's temperature because a solute's pK_a value is pH-dependent; for a review, see Gagliardi, L. G.; Tascon, M.; Castells, C. B. "Effect of Temperature on Acid–Base Equilibria in Separation Techniques: A Review," *Anal. Chim. Acta*, **2015**, *889*, 35–57.

Using Column Efficiency to Optimize Resolution

A third approach to improving resolution is to adjust the column's efficiency by increasing the number of theoretical plates, *N*. If we have values for k_B and α, then we can use equation 12.3.3 to calculate the number of theoretical plates for any resolution. Table 12.3.1 provides some representative values. For example, if α = 1.05 and k_B = 2.0, a resolution of 1.25 requires approximately 24 800 theoretical plates. If our column provides only 12 400 plates, half of what is needed, then a separation is not possible. How can we double the number of theoretical plates? The easiest way is to double the length of the column, although this also doubles the analysis time. A better approach is to cut the height of a theoretical plate, *H*, in half, providing the desired resolution without changing the analysis time. Even better, if we can decrease *H* by more than 50%, it may be possible to achieve the desired resolution with an even shorter analysis time by also decreasing k_B or α.

Table 12.3.1. Minimum Number of Theoretical Plates to Achieve Desired Resolution for Selected Values of k_B and α

	R_{AB} = 1.00		R_{AB} = 1.25		R_{AB} = 1.50	
k_B	$\alpha = 1.05$	$\alpha = 1.10$	$\alpha = 1.05$	$\alpha = 1.10$	$\alpha = 1.05$	$\alpha = 1.10$
0.5	63500	17400	99200	27200	143000	39200
1.0	28200	7740	44100	12100	63500	17400
1.5	19600	5380	30600	8400	44100	12100
2.0	15900	4360	24800	6810	35700	9800
3.0	12500	3440	19600	5380	28200	7740
5.0	10200	2790	15900	4360	22900	6270
10.0	8540	2340	13300	3660	19200	5270

To decrease the height of a theoretical plate we need to understand the experimental factors that affect band broadening. There are several theoretical treatments of band broadening. We will consider one approach that considers four contributions: variations in path lengths, longitudinal diffusion, mass transfer in the stationary phase, and mass transfer in the mobile phase.

Multiple Paths: Variations in Path Length

As solute molecules pass through the column they travel paths that differ in length. Because of this difference in path length, two solute molecules that enter the column at the same time will exit the column at different times. The result, as shown in Figure 12.3.4, is a broadening of the solute's profile on the column. The contribution of ***multiple paths*** to the height of a theoretical plate, H_p, is

$$H_p = 2\lambda d_p \tag{12.3.5}$$

where d_p is the average diameter of the particulate packing material and λ is a constant that accounts for the consistency of the packing. A smaller range of particle sizes and a more consistent packing produce a smaller value for λ. For a column without packing material, H_p is zero and there is no contribution to band broadening from multiple paths.

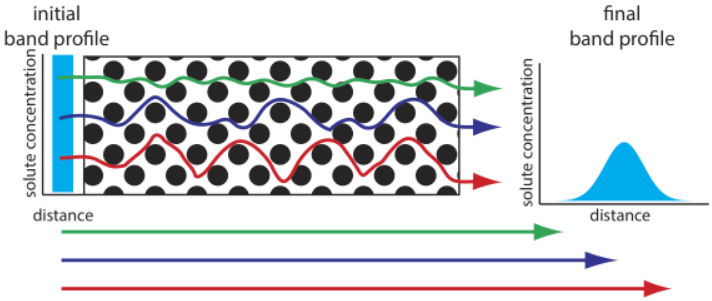


Figure 12.3.4. The effect of multiple paths on a solute's band broadening. The solute's initial band profile is rectangular. As this band travels through the column, individual solute molecules travel different paths, three of which are shown by the meandering colored paths (the actual lengths of these paths are shown by the straight arrows at the bottom of the figure). Most solute molecules travel paths with lengths similar to that shown in blue, with a few traveling much shorter paths (green) or much longer paths (red). As a result, the solute's band profile at the end of the column is broader and Gaussian in shape.

An inconsistent packing creates channels that allow some solute molecules to travel quickly through the column. It also can creates pockets that temporarily trap some solute molecules, slowing their progress through the column. A more uniform packing minimizes these problems.

Longitudinal Diffusion

The second contribution to band broadening is the result of the solute's ***longitudinal diffusion*** in the mobile phase. Solute molecules are in constant motion, diffusing from regions of higher solute concentration to regions where the concentration of solute is smaller. The result is an increase in the solute's band width (Figure **12.3.5**). The contribution of longitudinal diffusion to the height of a theoretical plate, H_d, is

$$H_d = \frac{2\gamma D_m}{u} \tag{12.3.6}$$

where D_m is the solute's diffusion coefficient in the mobile phase, u is the mobile phase's velocity, and γ is a constant related to the efficiency of column packing. Note that the effect of H_d on band broadening is inversely proportional to the mobile phase velocity: a higher velocity provides less time for longitudinal diffusion. Because a solute's diffusion coefficient is larger in the gas phase than in a liquid phase, longitudinal diffusion is a more serious problem in gas chromatography.

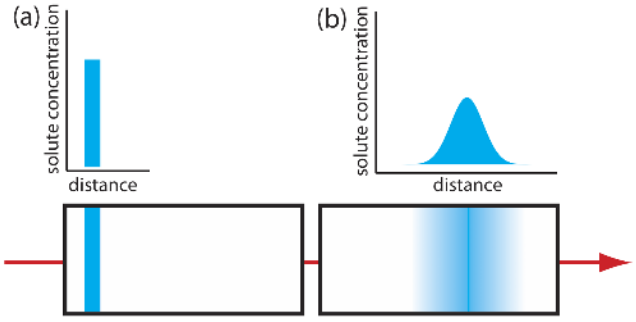


Figure 12.3.5. The effect of longitudinal diffusion on a solute's band broadening. Two horizontal cross-sections through the column and the corresponding concentration versus distance profiles are shown, with (a) being earlier in time. The red arrow shows the direction in which the mobile phase is moving.

Mass Transfer

As the solute passes through the column it moves between the mobile phase and the stationary phase. We call this movement between phases ***mass transfer***. As shown in Figure **12.3.6**, band broadening occurs if the solute's movement within the mobile phase or within the stationary phase is not fast enough to maintain an equilibrium in its concentration between the two phases. On average, a solute molecule in the mobile phase moves down the column before it passes into the stationary phase. A solute molecule in the stationary phase, on the other hand, takes longer than expected to move back into the mobile phase. The

contributions of mass transfer in the stationary phase, H_s, and mass transfer in the mobile phase, H_m, are given by the following equations

$$H_s = \frac{qkd_f^2}{(1+k)^2 D_s} u \tag{12.3.7}$$

$$H_m = \frac{fn\left(d_p^2, d_c^2\right)}{D_m} u \tag{12.3.8}$$

where d_f is the thickness of the stationary phase, d_c is the diameter of the column, D_s and D_m are the diffusion coefficients for the solute in the stationary phase and the mobile phase, k is the solute's retention factor, and q is a constant related to the column packing material. Although the exact form of H_m is not known, it is a function of particle size and column diameter. Note that the effect of H_s and H_m on band broadening is directly proportional to the mobile phase velocity because a smaller velocity provides more time for mass transfer.

> The abbreviation *fn* in equation 12.3.7 means "is a function of."

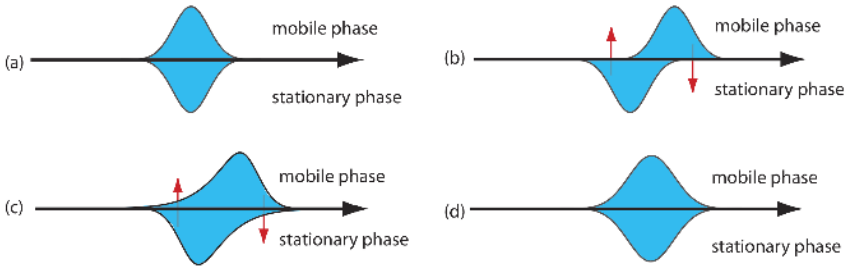


Figure 12.3.6. Effect of mass transfer on band broadening: (a) Ideal equilibrium Gaussian profiles for the solute in the mobile phase and in the stationary phase. (b, c) If we allow the solute's band to move a small distance down the column, an equilibrium between the two phases no longer exits. The red arrows show the movement of solute—what we call the mass transfer of solute—from the stationary phase to the mobile phase, and from the mobile phase to the stationary phase. (d) Once equilibrium is reestablished, the solute's band is now broader.

Putting It All Together

The height of a theoretical plate is a summation of the contributions from each of the terms affecting band broadening.

$$H = H_p + H_d + H_s + H_m \tag{12.3.9}$$

An alternative form of this equation is the ***van Deemter equation***

$$H = A + \frac{B}{u} + Cu \tag{12.3.10}$$

which emphasizes the importance of the mobile phase's velocity. In the van Deemter equation, A accounts for the contribution of multiple paths (H_p), B/u accounts for the contribution of longitudinal diffusion (H_d), and Cu accounts for the combined contribution of mass transfer in the stationary phase and in the mobile phase (H_s and H_m).

There is some disagreement on the best equation for describing the relationship between plate height and mobile phase velocity [Hawkes, S. J. *J. Chem. Educ.* **1983**, *60*, 393–398]. In addition to the van Deemter equation, other equations include

$$H = \frac{B}{u} + (C_s + C_m)\, u$$

where C_s and C_m are the mass transfer terms for the stationary phase and the mobile phase and

$$H = Au^{1/3} + \frac{B}{u} + Cu$$

All three equations, and others, have been used to characterize chromatographic systems, with no single equation providing the best explanation in every case [Kennedy, R. T.; Jorgenson, J. W. *Anal. Chem.* **1989**, *61*, 1128–1135].

To increase the number of theoretical plates without increasing the length of the column, we need to decrease one or more of the terms in equation 12.3.9. The easiest way to decrease H is to adjust the velocity of the mobile phase. For smaller mobile phase velocities, column efficiency is limited by longitudinal diffusion, and for higher mobile phase velocities efficiency is limited by the two mass transfer terms. As shown in Figure 12.3.7—which uses the van Deemter equation—the optimum mobile phase velocity is the minimum in a plot of H as a function of u.

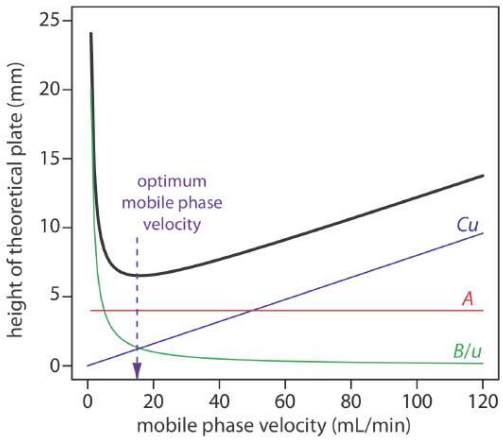


Figure 12.3.7. Plot showing the relationship between the height of a theoretical plate, H, and the mobile phase's velocity, u, based on the van Deemter equation.

The remaining parameters that affect the terms in equation ??? are functions of the column's properties and suggest other possible approaches to improving column efficiency. For example, both H_p and H_m are a function of the size of the particles used to pack the column. Decreasing particle size, therefore, is another useful method for improving efficiency.

> For a more detailed discussion of ways to assess the quality of a column, see Desmet, G.; Caooter, D.; Broeckhaven, K. "Graphical Data Represenation Methods to Assess the Quality of LC Columns," *Anal. Chem.* **2015**, *87*, 8593–8602.

Perhaps the most important advancement in chromatography columns is the development of open-tubular, or ***capillary columns***. These columns have very small diameters ($d_c \approx$ 50–500 μm) and contain no packing material ($d_p = 0$). Instead, the capillary column's interior wall is coated with a thin film of the stationary phase. Plate height is reduced because the contribution to H from H_p (equation ???) disappears and the contribution from H_m (equation ???) becomes smaller. Because the column does not contain any solid packing material, it takes less pressure to move the mobile phase through the column, which allows for longer columns. The combination of a longer column and a smaller height for a theoretical plate increases the number of theoretical plates by approximately 100×. Capillary columns are not without disadvantages. Because they are much narrower than packed columns, they require a significantly smaller amount of sample, which may be difficult to inject reproducibly. Another approach to improving resolution is to use thin films of stationary phase, which decreases the contribution to H from H_s (equation ???).

> The smaller the particles, the more pressure is needed to push the mobile phase through the column. As a result, for any form of chromatography there is a practical limit to particle size.

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12.4: Problems

1. The following data were obtained for four compounds separated on a 20-m capillary column.

compound	t_r (min)	w (min)
A	8.04	0.15
B	8.26	0.15
C	8.43	0.16

(a) Calculate the number of theoretical plates for each compound and the average number of theoretical plates for the column, in mm.

(b) Calculate the average height of a theoretical plate.

(c) Explain why it is possible for each compound to have a different number of theoretical plates.

2. Using the data from Problem 1, calculate the resolution and the selectivity factors for each pair of adjacent compounds. For resolution, use both equation 12.2.1 and equation 12.3.3, and compare your results. Discuss how you might improve the resolution between compounds B and C. The retention time for an nonretained solute is 1.19 min.

3. Use the chromatogram in Figure 12.8.1 , obtained using a 2-m column, to determine values for t_r, w, t'_r, k, N, and H.

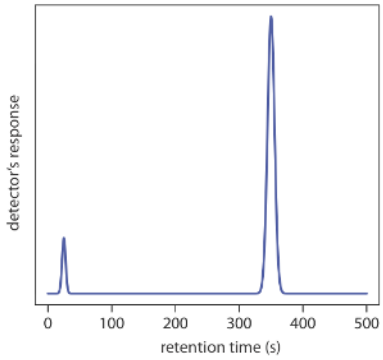


Figure 12.8.1 . Chromatogram for Problem 3.

4. Use the partial chromatogram in Figure 12.8.2 to determine the resolution between the two solute bands.

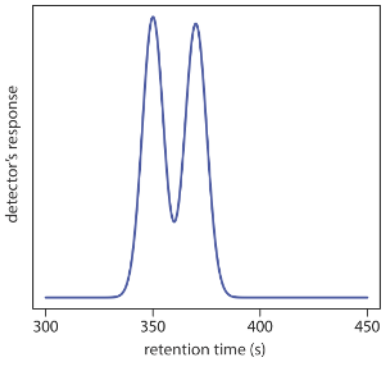


Figure 12.8.2 . Chromatogram for Problem 4.

5. The chromatogram in Problem 4 was obtained on a 2-m column with a column dead time of 50 s. Suppose you want to increase the resolution between the two components to 1.5. Without changing the height of a theoretical plate, what length column do you need? What height of a theoretical plate do you need to achieve a resolution of 1.5 without increasing the column's length?

6. Complete the following table.

N_B	α	k_B	R
100000	1.05	0.50	
10000	1.10		1.50
10000		4.0	1.00
	1.05	3.0	1.75

7. Moody studied the efficiency of a GC separation of 2-butanone on a dinonyl phthalate packed column [Moody, H. W. *J. Chem. Educ.* **1982**, *59*, 218–219]. Evaluating plate height as a function of flow rate gave a van Deemter equation for which *A* is 1.65 mm, *B* is 25.8 mm•mL min^{-1}, and *C* is 0.0236 mm•min mL^{-1}.

(a) Prepare a graph of *H* versus *u* for flow rates between 5 –120 mL/min.

(b) For what range of flow rates does each term in the Van Deemter equation have the greatest effect?

(c) What is the optimum flow rate and the corresponding height of a theoretical plate?

(d) For open-tubular columns the *A* term no longer is needed. If the *B* and *C* terms remain unchanged, what is the optimum flow rate and the corresponding height of a theoretical plate?

(e) Compared to the packed column, how many more theoretical plates are in the open-tubular column?

8. Hsieh and Jorgenson prepared 12–33 μm inner diameter HPLC columns packed with 5.44-μm spherical stationary phase particles [Hsieh, S.; Jorgenson, J. W. *Anal. Chem.* **1996**, *68*, 1212–1217]. To evaluate these columns they measured reduced plate height, *h*, as a function of reduced flow rate, *v*,

$$h = \frac{H}{d_p} \quad v = \frac{ud_p}{D_m}$$

where d_p is the particle diameter and D_m is the solute's diffusion coefficient in the mobile phase. The data were analyzed using van Deemter plots. The following table contains a portion of their results for norepinephrine.

internal diameter (μm)	A	B	C
33	0.63	1.32	0.10
33	0.67	1.30	0.08
23	0.40	1.34	0.09
23	0.58	1.11	0.09
17	0.31	1.47	0.11
17	0.40	1.41	0.11
12	0.22	1.53	0.11
12	0.19	1.27	0.12

(a) Construct separate van Deemter plots using the data in the first row and in the last row for reduced flow rates in the range 0.7–15. Determine the optimum flow rate and plate height for each case given d_p = 5.44 μm and $D_m = \mathbf{6.23 \times 10^{-6}}$ cm^2 s^{-1}.

(b) The *A* term in the van Deemter equation is strongly correlated with the column's inner diameter, with smaller diameter columns providing smaller values of *A*. Offer an explanation for this observation. *Hint: consider how many particles can fit across a capillary of each diameter.*

When comparing columns, chromatographers often use dimensionless, reduced parameters. By including particle size and the solute's diffusion coefficient, the reduced plate height and reduced flow rate correct for differences between the packing material, the solute, and the mobile phase.

9. A mixture of *n*-heptane, tetrahydrofuran, 2-butanone, and *n*-propanol elutes in this order when using a polar stationary phase such as Carbowax. The elution order is exactly the opposite when using a nonpolar stationary phase such as polydimethyl siloxane. Explain the order of elution in each case.

10. The analysis of trihalomethanes in drinking water is described in Representative Method 12.4.1. A single standard that contains all four trihalomethanes gives the following results.

compound	concentration (ppb)	peak area
$CHCl_3$	1.30	1.35×10^4
$CHCl_2Br$	0.90	6.12×10^4
$CHClBr_2$	4.00	1.71×10^4
$CHBr_3$	1.20	1.52×10^4

Analysis of water collected from a drinking fountain gives areas of 1.56×10^4, 5.13×10^4, 1.49×10^4, and 1.76×10^4 for, respectively, $CHCl_3$, $CHCl_2Br$, $CHClBr_2$, and $CHBr_3$. All peak areas were corrected for variations in injection volumes using an internal standard of 1,2-dibromopentane. Determine the concentration of each of the trihalomethanes in the sample of water.

11. Zhou and colleagues determined the %w/w H_2O in methanol by capillary column GC using a polar stationary phase and a thermal conductivity detector [Zhou, X.; Hines, P. A.; White, K. C.; Borer, M. W. *Anal. Chem.* **1998**, *70*, 390–394]. A series of calibration standards gave the following results.

%w/w H_2O	peak height (arb. units)
0.00	1.15
0.0145	2.74
0.0472	6.33
0.0951	11.58
0.1757	20.43
0.2901	32.97

(a) What is the %w/w H_2O in a sample that has a peak height of 8.63?

(b) The %w/w H_2O in a freeze-dried antibiotic is determined in the following manner. A 0.175-g sample is placed in a vial along with 4.489 g of methanol. Water in the vial extracts into the methanol. Analysis of the sample gave a peak height of 13.66. What is the %w/w H_2O in the antibiotic?

12. Loconto and co-workers describe a method for determining trace levels of water in soil [Loconto, P. R.; Pan, Y. L.; Voice, T. C. *LC•GC* **1996**, *14*, 128–132]. The method takes advantage of the reaction of water with calcium carbide, CaC_2, to produce acetylene gas, C_2H_2. By carrying out the reaction in a sealed vial, the amount of acetylene produced is determined by sampling the headspace. In a typical analysis a sample of soil is placed in a sealed vial with CaC_2. Analysis of the headspace gives a blank corrected signal of 2.70×10^5. A second sample is prepared in the same manner except that a standard addition of 5.0 mg H_2O/g soil is added, giving a blank-corrected signal of 1.06×10^6. Determine the milligrams H_2O/g soil in the soil sample.

13. Van Atta and Van Atta used gas chromatography to determine the %v/v methyl salicylate in rubbing alcohol [Van Atta, R. E.; Van Atta, R. L. *J. Chem. Educ.* **1980**, *57*, 230–231]. A set of standard additions was prepared by transferring 20.00 mL of rubbing

alcohol to separate 25-mL volumetric flasks and pipeting 0.00 mL, 0.20 mL, and 0.50 mL of methyl salicylate to the flasks. All three flasks were diluted to volume using isopropanol. Analysis of the three samples gave peak heights for methyl salicylate of 57.00 mm, 88.5 mm, and 132.5 mm, respectively. Determine the %v/v methyl salicylate in the rubbing alcohol.

14. The amount of camphor in an analgesic ointment is determined by GC using the method of internal standards [Pant, S. K.; Gupta, P. N.; Thomas, K. M.; Maitin, B. K.; Jain, C. L. *LC•GC* **1990**, *8*, 322–325]. A standard sample is prepared by placing 45.2 mg of camphor and 2.00 mL of a 6.00 mg/mL internal standard solution of terpene hydrate in a 25-mL volumetric flask and diluting to volume with CCl_4. When an approximately 2-µL sample of the standard is injected, the FID signals for the two components are measured (in arbitrary units) as 67.3 for camphor and 19.8 for terpene hydrate. A 53.6-mg sample of an analgesic ointment is prepared for analysis by placing it in a 50-mL Erlenmeyer flask along with 10 mL of CCl_4. After heating to 50°C in a water bath, the sample is cooled to below room temperature and filtered. The residue is washed with two 5-mL portions of CCl_4 and the combined filtrates are collected in a 25-mL volumetric flask. After adding 2.00 mL of the internal standard solution, the contents of the flask are diluted to volume with CCl_4. Analysis of an approximately 2-µL sample gives FID signals of 13.5 for the terpene hydrate and 24.9 for the camphor. Report the %w/w camphor in the analgesic ointment.

15. The concentration of pesticide residues on agricultural products, such as oranges, is determined by GC-MS [Feigel, C. *Varian GC/MS Application Note*, Number 52]. Pesticide residues are extracted from the sample using methylene chloride and concentrated by evaporating the methylene chloride to a smaller volume. Calibration is accomplished using anthracene-d_{10} as an internal standard. In a study to determine the parts per billion heptachlor epoxide on oranges, a 50.0-g sample of orange rinds is chopped and extracted with 50.00 mL of methylene chloride. After removing any insoluble material by filtration, the methylene chloride is reduced in volume, spiked with a known amount of the internal standard and diluted to 10 mL in a volumetric flask. Analysis of the sample gives a peak–area ratio ($A_{analyte}/A_{intstd}$) of 0.108. A series of calibration standards, each containing the same amount of anthracene-d_{10} as the sample, gives the following results.

ppb heptachlor epoxide	$A_{analyte}/A_{intstd}$
20.0	0.065
60.0	0.153
200.0	0.637
500.0	1.554
1000.0	3.198

Report the nanograms per gram of heptachlor epoxide residue on the oranges.

16. The adjusted retention times for octane, toluene, and nonane on a particular GC column are 15.98 min, 17.73 min, and 20.42 min, respectively. What is the retention index for each compound?

17. The following data were collected for a series of normal alkanes using a stationary phase of Carbowax 20M.

alkane	t_r' (min)
pentane	0.79
hexane	1.99
heptane	4.47
octane	14.12
nonane	33.11

What is the retention index for a compound whose adjusted retention time is 9.36 min?

18. The following data were reported for the gas chromatographic analysis of *p*-xylene and methylisobutylketone (MIBK) on a capillary column [Marriott, P. J.; Carpenter, P. D. *J. Chem. Educ.* **1996**, *73*, 96–99].

injection mode	compound	t_r (min)	peak area (arb. units)	peak width (min)
split	MIBK	1.878	54285	0.028
	p-xylene	5.234	123483	0.044
splitless	MIBK	3.420	2493005	1.057
	p-xylene	5.795	3396656	1.051

Explain the difference in the retention times, the peak areas, and the peak widths when switching from a split injection to a splitless injection.

19. Otto and Wegscheider report the following retention factors for the reversed-phase separation of 2-aminobenzoic acid on a C_{18} column when using 10% v/v methanol as a mobile phase [Otto, M.; Wegscheider, W. *J. Chromatog.* **1983**, *258*, 11–22].

pH	*k*
2.0	10.5
3.0	16.7
4.0	15.8
5.0	8.0
6.0	2.2
7.0	1.8

Explain the effect of pH on the retention factor for 2-aminobenzene.

20. Haddad and associates report the following retention factors for the reversed-phase separation of salicylamide and caffeine [Haddad, P.; Hutchins, S.; Tuffy, M. *J. Chem. Educ.* **1983**, *60*, 166-168].

%v/v methanol	30%	35%	40%	45%	50%	55%
k_{sal}	2.4	1.6	1.6	1.0	0.7	0.7
k_{caff}	4.3	2.8	2.3	1.4	1.1	0.9

(a) Explain the trends in the retention factors for these compounds.

(b) What is the advantage of using a mobile phase with a smaller %v/v methanol? Are there any disadvantages?

21. Suppose you need to separate a mixture of benzoic acid, aspartame, and caffeine in a diet soda. The following information is available.

	t_r in aqueous mobile phase of pH			
compound	3.0	3.5	4.0	4.5
benzoic acid	7.4	7.0	6.9	4.4
aspartame	5.9	6.0	7.1	8.1
caffeine	3.6	3.7	4.1	4.4

(a) Explain the change in each compound's retention time.

(b) Prepare a single graph that shows retention time versus pH for each compound. Using your plot, identify a pH level that will yield an acceptable separation.

22. The composition of a multivitamin tablet is determined using an HPLC with a diode array UV/Vis detector. A 5-μL standard sample that contains 170 ppm vitamin C, 130 ppm niacin, 120 ppm niacinamide, 150 ppm pyridoxine, 60 ppm thiamine, 15 ppm folic acid, and 10 ppm riboflavin is injected into the HPLC, giving signals (in arbitrary units) of, respectively, 0.22, 1.35, 0.90, 1.37, 0.82, 0.36, and 0.29. The multivitamin tablet is prepared for analysis by grinding into a powder and transferring to a 125-mL Erlenmeyer flask that contains 10 mL of 1% v/v NH_3 in dimethyl sulfoxide. After sonicating in an ultrasonic bath for 2 min, 90 mL of 2% acetic acid is added and the mixture is stirred for 1 min and sonicated at 40°C for 5 min. The extract is then filtered through a 0.45-μm membrane filter. Injection of a 5-μL sample into the HPLC gives signals of 0.87 for vitamin C, 0.00 for niacin, 1.40 for niacinamide, 0.22 for pyridoxine, 0.19 for thiamine, 0.11 for folic acid, and 0.44 for riboflavin. Report the milligrams of each vitamin present in the tablet.

23. The amount of caffeine in an analgesic tablet was determined by HPLC using a normal calibration curve. Standard solutions of caffeine were prepared and analyzed using a 10-μL fixed-volume injection loop. Results for the standards are summarized in the following table.

concentration (ppm)	signal (arb. units)
50.0	8.354
100.0	16925
150.0	25218
200.0	33584
250.0	42002

The sample is prepared by placing a single analgesic tablet in a small beaker and adding 10 mL of methanol. After allowing the sample to dissolve, the contents of the beaker, including the insoluble binder, are quantitatively transferred to a 25-mL volumetric flask and diluted to volume with methanol. The sample is then filtered, and a 1.00-mL aliquot transferred to a 10-mL volumetric flask and diluted to volume with methanol. When analyzed by HPLC, the signal for caffeine is found to be 21 469. Report the milligrams of caffeine in the analgesic tablet.

24. Kagel and Farwell report a reversed-phase HPLC method for determining the concentration of acetylsalicylic acid (ASA) and caffeine (CAF) in analgesic tablets using salicylic acid (SA) as an internal standard [Kagel, R. A.; Farwell, S. O. *J. Chem. Educ.* **1983**, *60*, 163–166]. A series of standards was prepared by adding known amounts of ace- tylsalicylic acid and caffeine to 250-mL Erlenmeyer flasks and adding 100 mL of methanol. A 10.00-mL aliquot of a standard solution of salicylic acid was then added to each. The following results were obtained for a typical set of standard solutions.

	milligrams of		peak height ratios for	
standard	ASA	CAF	ASA/SA	CAF/SA
1	200.0	20.0	20.5	10.6
2	250.0	40.0	25.1	23.0
3	300.0	60.0	30.9	36.8

A sample of an analgesic tablet was placed in a 250-mL Erlenmeyer flask and dissolved in 100 mL of methanol. After adding a 10.00-mL portion of the internal standard, the solution was filtered. Analysis of the sample gave a peak height ratio of 23.2 for ASA and of 17.9 for CAF.

(a) Determine the milligrams of ASA and CAF in the tablet.

(b) Why is it necessary to filter the sample?

(c) The directions indicate that approximately 100 mL of methanol is used to dissolve the standards and samples. Why is it not necessary to measure this volume more precisely?

(d) In the presence of moisture, ASA decomposes to SA and acetic acid. What complication might this present for this analysis? How might you evaluate whether this is a problem?

25. Bohman and colleagues described a reversed-phase HPLC method for the quantitative analysis of vitamin A in food using the method of standard additions Bohman, O.; Engdahl, K. A.; Johnsson, H. *J. Chem. Educ.* **1982**, *59*, 251–252]. In a typical example, a 10.067-g sample of cereal is placed in a 250-mL Erlenmeyer flask along with 1 g of sodium ascorbate, 40 mL of ethanol, and 10 mL of 50% w/v KOH. After refluxing for 30 min, 60 mL of ethanol is added and the solution cooled to room temperature. Vitamin A is extracted using three 100-mL portions of hexane. The combined portions of hexane are evaporated and the residue containing vitamin A transferred to a 5-mL volumetric flask and diluted to volume with methanol. A standard addition is prepared in a similar manner using a 10.093-g sample of the cereal and spiking with 0.0200 mg of vitamin A. Injecting the sample and standard addition into the HPLC gives peak areas of, respectively, 6.77×10^3 and 1.32×10^4. Report the vitamin A content of the sample in milligrams/100 g cereal.

26. Ohta and Tanaka reported on an ion-exchange chromatographic method for the simultaneous analysis of several inorganic anions and the cations Mg^{2+} and Ca^{2+} in water [Ohta, K.; Tanaka, K. *Anal. Chim. Acta* **1998**, *373*, 189–195]. The mobile phase includes the ligand 1,2,4-benzenetricarboxylate, which absorbs strongly at 270 nm. Indirect detection of the analytes is possible because its absorbance decreases when complexed with an anion.

(a) The procedure also calls for adding the ligand EDTA to the mobile phase. What role does the EDTA play in this analysis?

(b) A standard solution of 1.0 mM $NaHCO_3$, 0.20 mM $NaNO_2$, 0.20 mM $MgSO_4$, 0.10 mM $CaCl_2$, and 0.10 mM $Ca(NO_3)_2$ gives the following peak areas (arbitrary units).

ion	HCO_3^-	Cl^-	NO_2^-	NO_3^-
peak area	373.5	322.5	264.8	262.7
ion	Ca^{2+}	Mg^{2+}	SO_4^{2-}	
peak area	458.9	352.0	341.3	

Analysis of a river water sample (pH of 7.49) gives the following results.

ion	HCO_3^-	Cl^-	NO_2^-	NO_3^-
peak area	310.0	403.1	3.97	157.6
ion	Ca^{2+}	Mg^{2+}	SO_4^{2-}	
peak area	734.3	193.6	324.3	

Determine the concentration of each ion in the sample.

(c) The detection of HCO_3^- actually gives the total concentration of carbonate in solution ($[CO_3^{2-}]+[HCO_3^-]+[H_2CO_3]$). Given that the pH of the water is 7.49, what is the actual concentration of HCO_3^-?

(d) An independent analysis gives the following additional concentrations for ions in the sample: $[Na^+]$ = 0.60 mM; $[NH_4^+]$ = 0.014 mM; and $[K^+]$ = 0.046 mM. A solution's ion balance is defined as the ratio of the total cation charge to the total anion charge. Determine the charge balance for this sample of water and comment on whether the result is reasonable.

27. The concentrations of Cl^-, NO_2^-, and SO_4^{2-} are determined by ion chromatography. A 50-µL standard sample of 10.0 ppm Cl^-, 2.00 ppm NO_2^-, and 5.00 ppm SO_4^{2-} gave signals (in arbitrary units) of 59.3, 16.1, and 6.08 respectively. A sample of effluent from a wastewater treatment plant is diluted tenfold and a 50-µL portion gives signals of 44.2 for Cl^-, 2.73 for NO_2^-, and 5.04 for SO_4^{2-}. Report the parts per million for each anion in the effluent sample.

28. A series of polyvinylpyridine standards of different molecular weight was analyzed by size-exclusion chromatography, yielding the following results.

formula weight	retention volume (mL)
600000	6.42
100000	7.98
30000	9.30
3000	10.94

When a preparation of polyvinylpyridine of unknown formula weight is analyzed, the retention volume is 8.45 mL. Report the average formula weight for the preparation.

29. Diet soft drinks contain appreciable quantities of aspartame, benzoic acid, and caffeine. What is the expected order of elution for these compounds in a capillary zone electrophoresis separation using a pH 9.4 buffer given that aspartame has pK_a values of 2.964 and 7.37, benzoic acid has a pK_a of 4.2, and the pK_a for caffeine is less than 0. Figure 12.8.3 provides the structures of these compounds.

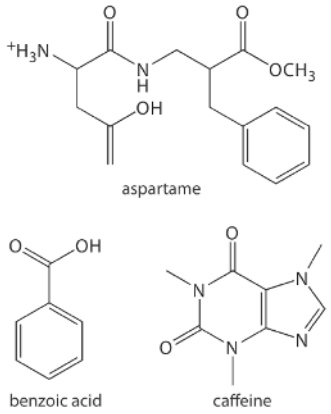


Figure 12.8.3
. Structures for the compounds in Problem 29.

30. Janusa and coworkers describe the determination of chloride by CZE [Janusa, M. A.; Andermann, L. J.; Kliebert, N. M.; Nannie, M. H. *J. Chem. Educ.* **1998**, *75*, 1463–1465]. Analysis of a series of external standards gives the following calibration curve.

$$\text{area} = -883 + 5590 \times \text{ppm } Cl^-$$

A standard sample of 57.22% w/w Cl^- is analyzed by placing 0.1011-g portions in separate 100-mL volumetric flasks and diluting to volume. Three unknowns are prepared by pipeting 0.250 mL, 0.500 mL, an 0.750 mL of the bulk unknown in separate 50-mL volumetric flasks and diluting to volume. Analysis of the three unknowns gives areas of 15 310, 31 546, and 47 582, respectively. Evaluate the accuracy of this analysis.

31. The analysis of NO_3^- in aquarium water is carried out by CZE using IO_4^- as an internal standard. A standard solution of 15.0 ppm NO_3^- and 10.0 ppm IO_4^- gives peak heights (arbitrary units) of 95.0 and 100.1, respectively. A sample of water from an aquarium is diluted 1:100 and sufficient internal standard added to make its concentration 10.0 ppm in IO_4^-. Analysis gives signals of 29.2 and 105.8 for NO_3^- and IO_4^-, respectively. Report the ppm NO_3^- in the sample of aquarium water.

32. Suggest conditions to separate a mixture of 2-aminobenzoic acid (pK_{a1} = 2.08, pK_{a2} = 4.96), benzylamine (pK_a = 9.35), and 4-methylphenol (pK_{a2} = 10.26) by capillary zone electrophoresis. Figure ***PageIndex4*** provides the structures of these compounds.

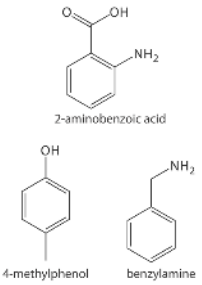


Figure 12.8.4 . Structures for the compounds in Problem 32.

33. McKillop and associates examined the electrophoretic separation of some alkylpyridines by CZE [McKillop, A. G.; Smith, R. M.; Rowe, R. C.; Wren, S. A. C. *Anal. Chem.* **1999**, *71*, 497–503]. Separations were carried out using either 50-µm or 75-µm inner diameter capillaries, with a total length of 57 cm and a length of 50 cm from the point of injection to the detector. The run buffer was a pH 2.5 lithium phosphate buffer. Separations were achieved using an applied voltage of 15 kV. The electroosmotic mobility, μ_{eof}, as measured using a neutral marker, was found to be $\mathbf{6.398 \times 10^{-5}}$ $cm^2\ V^{-1}\ s^{-1}$. The diffusion coefficient for alkylpyridines is $\mathbf{1.0 \times 10^{-5}}$ $cm^2\ s^{-1}$.

(a) Calculate the electrophoretic mobility for 2-ethylpyridine given that its elution time is 8.20 min.

(b) How many theoretical plates are there for 2-ethylpyridine?

(c) The electrophoretic mobilities for 3-ethylpyridine and 4-ethylpyridine are $\mathbf{3.366 \times 10^{-4}}$ $cm^2\ V^{-1}\ s^{-1}$ and $\mathbf{3.397 \times 10^{-4}}$ $\mathbf{cm^2\ V^{-1}\ s^{-1}}$, respectively. What is the expected resolution between these two alkylpyridines?

(d) Explain the trends in electrophoretic mobility shown in the following table.

alkylpyridine	μ_{ep} ($cm^2\ V^{-1}\ s^{-1}$)
2-methylpyridine	$\mathbf{3.581 \times 10^{-4}}$
2-ethylpyridine	$\mathbf{3.222 \times 10^{-4}}$
2-propylpyridine	$\mathbf{2.923 \times 10^{-4}}$
2-pentylpyridine	$\mathbf{2.534 \times 10^{-4}}$
2-hexylpyridine	$\mathbf{2.391 \times 10^{-4}}$

(e) Explain the trends in electrophoretic mobility shown in the following table.

alkylpyridine	μ_{ep} ($cm^2\ V^{-1}\ s^{-1}$)
2-ethylpyridine	$\mathbf{3.222 \times 10^{-4}}$
3-ethylpyridine	$\mathbf{3.366 \times 10^{-4}}$
4-ethylpyridine	$\mathbf{3.397 \times 10^{-4}}$

(f) The pK_a for pyridine is 5.229. At a pH of 2.5 the electrophoretic mobility of pyridine is $\mathbf{4.176 \times 10^{-4}}$ $cm^2\ V^{-1}\ s^{-1}$. What is the expected electrophoretic mobility if the run buffer's pH is 7.5?

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CHAPTER OVERVIEW

13: Gas Chromatography

Gas chromatography (GC) is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. https://chem.libretexts.org/Under_Co...otography_(GC)

13.1: Gas Chromatography

13.2: Advances in GC

13.3: Problems

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13.1: Gas Chromatography

In ***gas chromatography*** (GC) we inject the sample, which may be a gas or a liquid, into an gaseous mobile phase (often called the carrier gas). The mobile phase carries the sample through a packed or a capillary column that separates the sample's components based on their ability to partition between the mobile phase and the stationary phase. Figure **13.1.1** shows an example of a typical gas chromatograph, which consists of several key components: a supply of compressed gas for the mobile phase; a heated injector, which rapidly volatilizes the components in a liquid sample; a column, which is placed within an oven whose temperature we can control during the separation; and a detector to monitor the eluent as it comes off the column. Let's consider each of these components.



Figure 13.1.1. Example of a typical gas chromatograph with insets showing the heated injection ports—note the symbol indicating that it is hot—and the oven that houses the column. This particular instrument is equipped with an autosampler for injecting samples, a capillary column, and a mass spectrometer (MS) as the detector. Note that the carrier gas is supplied by a tank of compressed gas.

Mobile Phase

The most common mobile phases for gas chromatography are He, Ar, and N_2, which have the advantage of being chemically inert toward both the sample and the stationary phase. The nature of the carrier gas has no significant influence on K, the partition coefficient, but it does have an effect on the solutes dispersion (*has an effect on N_{eff} and LOD*). The choice of carrier gas often is determined by the needs of instrument's detector. For a packed column the mobile phase velocity usually is 25–150 mL/min. The typical flow rate for a capillary column is 1–25 mL/min.

Oven

The oven should be able to reach temperatures up to 400 ° with a thermal stability to ± 0.1 ° C. The oven should also have a weak thermal inertia to allow heating rates up to 100 °C/min for temperature programming.

Chromatographic Columns

There are two broad classes of chromatographic columns: packed columns and capillary columns. In general, a packed column can handle larger samples and a capillary column can separate more complex mixtures.

Packed Columns

Packed columns are constructed from glass, stainless steel, copper, or aluminum, and typically are 2–6 m in length with internal diameters of 2–4 mm. The column is filled with a particulate solid support, with particle diameters ranging from 37–44 µm to 250–354 µm. Figure **13.1.2** shows a typical example of a packed column.

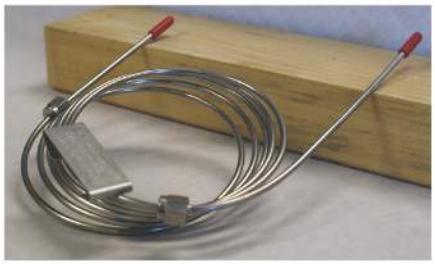


Figure 13.1.2. Typical example of a packed column for gas chromatography. This column is made from stainless steel and is 2 m long with an internal diameter of 3.2 mm. The packing material in this column has a particle diameter of 149–177 μm. To put this in perspective, beach sand has a typical diameter of 700 μm and the diameter of fine grained sand is 250 μm.

The most widely used particulate support is diatomaceous earth, which is composed of the silica skeletons of diatoms. These particles are very porous, with surface areas ranging from 0.5–7.5 m^2/g, which provides ample contact between the mobile phase and the stationary phase. Two high resolution images of diatomaceous earth are shown in Figure **13.1.3**. When hydrolyzed, the surface of a diatomaceous earth contains silanol groups (–SiOH), that serve as active sites for absorbing solute molecules in gas-solid chromatography (GSC).

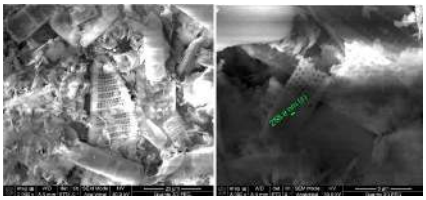


Figure **13.1.3**: Two scanning electron microcopy images of diatomaceous earth revealing the porous, high surface area nature of this widely used packing material. "File:1-s2.0-S0272884217313470-gr4 lrg.jpg" by Zirconia1980 is licensed under CC BY-SA 4.0

In ***gas-liquid chromatography*** (GLC), we coat the packing material with a liquid mobile phase. To prevent uncoated packing material from adsorbing solutes, which degrades the quality of the separation, surface silanols are deactivated by reacting them with dimethyldichlorosilane and rinsing with an alcohol—typically methanol—before coating the particles with stationary phase.

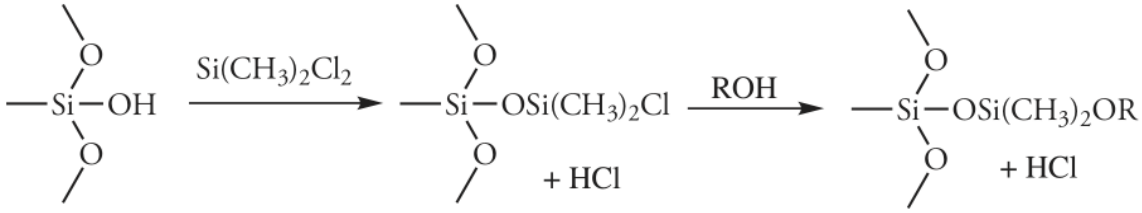


Figure 13.1.4, for example, has approximately 1800 plates/m, or a total of approximately 3600 theoretical plates. If we assume a $V_{max}/V_{min} \approx 50$, then it has a peak capacity (equation 12.2.16) of

$$n_c = 1 + \frac{\sqrt{3600}}{4}\ln(50) \approx 60$$

Capillary Columns

A capillary, or ***open tubular column*** is constructed from fused silica and is coated with a protective polymer coating. Columns range from 15–100 m in length with an internal diameter of approximately 150–300 μm. Figure **13.1.5** shows an example of a typical capillary column.



Figure 13.1.5. Typical example of a capillary column for gas chromatography. This column is 30 m long with an internal diameter of 247 μm. The interior surface of the capillary has a 0.25 μm coating of the liquid phase.

Capillary columns are of three principal types. In a ***wall-coated open tubular column*** (WCOT) a thin layer of stationary phase, typically 0.25 nm thick, is coated on the capillary's inner wall. In a ***porous-layer open tubular column*** (PLOT), a porous solid support—alumina, silica gel, and molecular sieves are typical examples—is attached to the capillary's inner wall. A ***support-coated open tubular column*** (SCOT) is a PLOT column that includes a liquid stationary phase. Figure **13.1.6** shows the differences between these types of capillary columns.

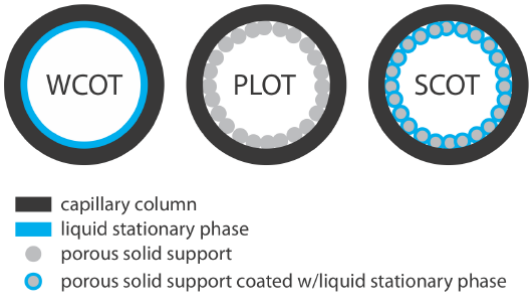


Figure 13.1.6. Cross sections through the three types of capillary columns.

As shown in Figure **13.1.7** an open tube or wall coated open tube capillary column provides a significant improvement in separation efficiency because it has more theoretical plates per meter and is longer than a packed column. For example, the capillary column in Figure **13.1.5** has almost 4300 plates/m, or a total of 129 000 theoretical plates. If we assume a $V_{max}/V_{min} \approx 50$, then it has a peak capacity of approximately 350. On the other hand, a packed column can handle a larger sample. Because of its smaller diameter, a capillary column requires a smaller sample, typically less than 10^{-2} μL and a sensitive detector.

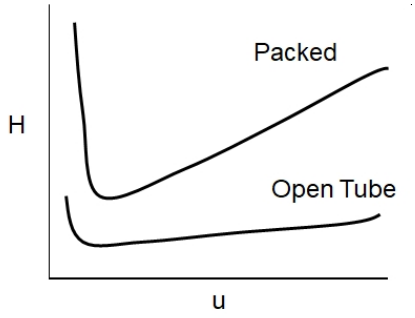


Figure **13.1.7**: A sketch of the improved separation efficiency for an open tube column versus a packed column as revealed in a van Deemter plot. In this figure H is the height equivalent of the theoretical plate and u is the linear flow velocity.

Stationary Phases for Gas-Liquid Chromatography

Elution order in gas–liquid chromatography depends on two factors: the boiling point of the solutes, and the interaction between the solutes and the stationary phase. If a mixture's components have significantly different boiling points, then the choice of stationary phase is less critical. If two solutes have similar boiling points, then a separation is possible only if the stationary phase selectively interacts with one of the solutes. As a general rule, nonpolar solutes are separated more easily when using a nonpolar stationary phase, and polar solutes are easier to separate when using a polar stationary phase.

There are several important criteria for choosing a stationary phase: it must not react with the solutes, it must be thermally stable, it must have a low volatility, and it must have a polarity that is appropriate for the sample's components. Table **13.1.1** summarizes the properties of several popular stationary phases.

Table **13.1.1**. Selected Examples of Stationary Phases for Gas-Liquid Chromatography

stationary phase	polarity	trade name	temperature limit (^{o}C)	representative applications
squalane	nonpolar	Squalane	150	low-boiling aliphatics hydrocarbons
Apezion L	nonpolar	Apezion L	300	amides, fatty acid methyl esters, terpenoids
polydimethyl siloxane	slightly polar	SE-30	300–350	alkaloids, amino acid derivatives, drugs, pesticides, phenols, steroids
phenylmethyl polysiloxane (50% phenyl, 50% methyl)	moderately polar	OV-17	375	alkaloids, drugs, pesticides, polyaromatic hydrocarbons, polychlorinated biphenyls
trifluoropropylmethyl polysiloxane (50% trifluoropropyl, 50% methyl)	moderately polar	OV-210	275	alkaloids, amino acid derivatives, drugs, halogenated compounds, ketones
cyanopropylphenylmethyl polysiloxane (50%cyanopropyl, 50% phenylmethyl)	polar	OV-225	275	nitriles, pesticides, steroids
polyethylene glycol	polar	Carbowax 20M	225	aldehydes, esters, ethers, phenols

Many stationary phases have the general structure shown in Figure **13.1.8**a. A stationary phase of polydimethyl siloxane, in which all the –R groups are methyl groups, $–CH_3$, is nonpolar and often makes a good first choice for a new separation. The order of elution when using polydimethyl siloxane usually follows the boiling points of the solutes, with lower boiling solutes eluting first. Replacing some of the methyl groups with other substituents increases the stationary phase's polarity and provides greater selectivity. For example, replacing 50% of the $–CH_3$ groups with phenyl groups, $–C_6H_5$, produces a slightly polar stationary phase. Increasing polarity is provided by substituting trifluoropropyl, $–C_3H_6CF$, and cyanopropyl, $–C_3H_6CN$, functional groups, or by using a stationary phase of polyethylene glycol (Figure **13.1.8**b).

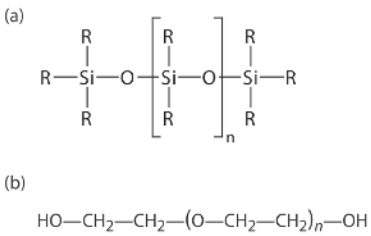


Figure 13.1.8. General structures of common stationary phases: (a) substituted polysiloxane; (b) polyethylene glycol.

An important problem with all liquid stationary phases is their tendency to elute, or ***bleed*** from the column when it is heated. The temperature limits in Table **13.1.1** minimize this loss of stationary phase. Capillary columns with bonded or cross-linked stationary

phases provide superior stability. A bonded stationary phase is attached chemically to the capillary's silica surface. Cross-linking, which is done after the stationary phase is in the capillary column, links together separate polymer chains to provide greater stability.

Another important consideration is the thickness of the stationary phase. From equation 12.3.7 we know that separation efficiency improves with thinner films of stationary phase. The most common thickness is 0.25 μm, although a thicker films is useful for highly volatile solutes, such as gases, because it has a greater capacity for retaining such solutes. Thinner films are used when separating low volatility solutes, such as steroids.

A few stationary phases take advantage of chemical selectivity. The most notable are stationary phases that contain chiral functional groups, which are used to separate enantiomers [Hinshaw, J. V. *LC .GC* **1993**, *11*, 644–648]. α- and β- cyclodextrins are commonly used chiral selectors for the separation of enantiomers.

Sample Introduction

Three factors determine how we introduce a sample to the gas chromatograph. First, all of the sample's constituents must be volatile. Second, the analytes must be present at an appropriate concentration. Finally, the physical process of injecting the sample must not degrade the separation. Each of these needs is considered in this section.

Preparing a Volatile Sample

Not every sample can be injected directly into a gas chromatograph. To move through the column, the sample's constituents must be sufficiently volatile. A solute of low volatility, for example, may be retained by the column and continue to elute during the analysis of subsequent samples. A nonvolatile solute will condense at the top of the column, degrading the column's performance.

We can separate a sample's volatile analytes from its nonvolatile components using any of the extraction techniques described in Chapter 7. A liquid–liquid extraction of analytes from an aqueous matrix into methylene chloride or another organic solvent is a common choice. Solid-phase extractions also are used to remove a sample's nonvolatile components.

An attractive approach to isolating analytes is a ***solid-phase microextraction*** (SPME). In one approach, which is illustrated in Figure **13.1.6**, a fused-silica fiber is placed inside a syringe needle. The fiber, which is coated with a thin film of an adsorbent material, such as polydimethyl siloxane, is lowered into the sample by depressing a plunger and is exposed to the sample for a predetermined time. After withdrawing the fiber into the needle, it is transferred to the gas chromatograph for analysis.

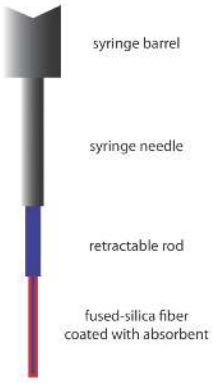


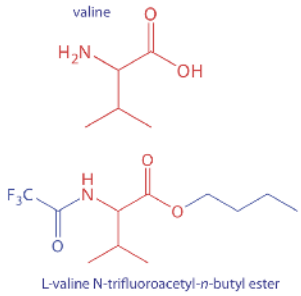
Figure 13.1.6. Schematic diagram of a solid-phase microextraction device. The absorbent is shown in red.

Two additional methods for isolating volatile analytes are a purge-and-trap and headspace sampling. In a ***purge-and-trap***, we bubble an inert gas, such as He or N_2, through the sample, releasing—or purging—the volatile compounds. These compounds are carried by the purge gas through a trap that contains an absorbent material, such as Tenax, where they are retained. Heating the trap and back-flushing with carrier gas transfers the volatile compounds to the gas chromatograph. In ***headspace sampling*** we place the sample in a closed vial with an overlying air space. After allowing time for the volatile analytes to equilibrate between the sample and the overlying air, we use a syringe to extract a portion of the vapor phase and inject it into the gas chromatograph. Alternatively, we can sample the headspace with an SPME.

Thermal desorption is a useful method for releasing volatile analytes from solids. We place a portion of the solid in a glass-lined, stainless steel tube. After purging with carrier gas to remove any O_2 that might be present, we heat the sample. Volatile analytes are swept from the tube by an inert gas and carried to the GC. Because volatilization is not a rapid process, the volatile analytes often are concentrated at the top of the column by cooling the column inlet below room temperature, a process known as ***cryogenic focusing***. Once volatilization is complete, the column inlet is heated rapidly, releasing the analytes to travel through the column.

> The reason for removing O_2 is to prevent the sample from undergoing an oxidation reaction when it is heated.

To analyze a nonvolatile analyte we must convert it to a volatile form. For example, amino acids are not sufficiently volatile to analyze directly by gas chromatography. Reacting an amino acid, such as valine, with 1-butanol and acetyl chloride produces an esterified amino acid. Subsequent treatment with trifluoroacetic acid gives the amino acid's volatile N-trifluoroacetyl-*n*-butyl ester derivative.



L-valine N-trifluoroacetyl-*n*-butyl ester

Adjusting the Analyte's Concentration

In an analyte's concentration is too small to give an adequate signal, then we must concentrate the analyte before we inject the sample into the gas chromatograph. A side benefit of many extraction methods is that they often concentrate the analytes. Volatile organic materials isolated from an aqueous sample by a purge-and-trap, for example, are concentrated by as much as **1000×**.

If an analyte is too concentrated, it is easy to overload the column, resulting in peak fronting (see Figure 12.2.7) and a poor separation. In addition, the analyte's concentration may exceed the detector's linear response. Injecting less sample or diluting the sample with a volatile solvent, such as methylene chloride, are two possible solutions to this problem.

Injecting the Sample

In Chapter 12.3 we examined several explanations for why a solute's band increases in width as it passes through the column, a process we called band broadening. We also introduce an additional source of band broadening if we fail to inject the sample into the minimum possible volume of mobile phase. There are two principal sources of this precolumn band broadening: injecting the sample into a moving stream of mobile phase and injecting a liquid sample instead of a gaseous sample. The design of a gas chromatograph's injector helps minimize these problems.

An example of a simple injection port for a packed column is shown in Figure **13.1.7**. The top of the column fits within a heated injector block, with carrier gas entering from the bottom. The sample is injected through a rubber septum using a microliter syringe, such as the one shown in in Figure **13.1.8**. Injecting the sample directly into the column minimizes band broadening because it mixes the sample with the smallest possible amount of carrier gas. The injector block is heated to a temperature at least 50°C above the boiling point of the least volatile solute, which ensures a rapid vaporization of the sample's components.

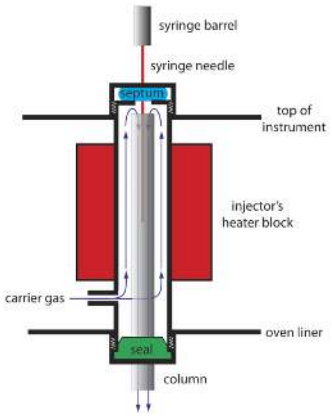


Figure 13.1.7. Schematic diagram of a heated GC injector port for use with packed columns. The needle pierces a rubber septum and enters into the top of the column, which is located within a heater block.



Figure 13.1.8. Example of a syringe for injecting samples into a gas chromatograph. This syringe has a maximum capacity of 10 µL with graduations every 0.1 µL.

Because a capillary column's volume is significantly smaller than that for a packed column, it requires a different style of injector to avoid overloading the column with sample. Figure **13.1.9** shows a schematic diagram of a typical split/splitless injector for use with a capillary column.

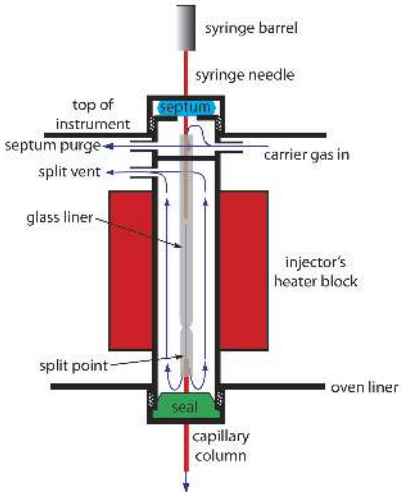


Figure 13.1.9. Schematic diagram of a split/splitless injection port for use with capillary columns. The needle pierces a rubber septum and enters into a glass liner, which is located within a heater block. In a split injection the split vent is open; the split vent is closed for a splitless injection.

In a ***split injection*** we inject the sample through a rubber septum using a microliter syringe. Instead of injecting the sample directly into the column, it is injected into a glass liner where it mixes with the carrier gas. At the split point, a small fraction of the carrier gas and sample enters the capillary column with the remainder exiting through the split vent. By controlling the flow rate of the carrier gas as it enters the injector, and its flow rate through the septum purge and the split vent, we can control the fraction of sample that enters the capillary column, typically 0.1–10%.

> For example, if the carrier gas flow rate is 50 mL/min, and the flow rates for the septum purge and the split vent are 2 mL/min and 47 mL/min, respectively, then the flow rate through the column is 1 mL/min (= 50 – 2 – 47). The ratio of sample entering the column is 1/50, or 2%.

In a ***splitless injection***, which is useful for trace analysis, we close the split vent and allow all the carrier gas that passes through the glass liner to enter the column—this allows virtually all the sample to enters the column. Because the flow rate through the injector is low, significant precolumn band broadening is a problem. Holding the column's temperature approximately 20–25°C below the solvent's boiling point allows the solvent to condense at the entry to the capillary column, forming a barrier that traps the solutes. After allowing the solutes to concentrate, the column's temperature is increased and the separation begins.

For samples that decompose easily, an ***on-column injection*** may be necessary. In this method the sample is injected directly into the column without heating. The column temperature is then increased, volatilizing the sample with as low a temperature as is practical.

Temperature Control

Control of the column's temperature is critical to attaining a good separation when using gas chromatography. For this reason the column is placed inside a thermostated oven (see Figure 13.1.1). In an ***isothermal*** separation we maintain the column at a constant temperature. To increase the interaction between the solutes and the stationary phase, the temperature usually is set slightly below that of the lowest-boiling solute. An alternative "Rule of Thumb" for isothermal separations is toe set the oven temperature equal to or just above the average boiling point of the solutes in a sample.

One difficulty with an isothermal separation is that a temperature that favors the separation of a low-boiling solute may lead to an unacceptably long retention time for a higher-boiling solute. ***Temperature programming*** provides a solution to this problem. At the beginning of the analysis we set the column's initial temperature below that for the lowest-boiling solute. As the separation progresses, we slowly increase the temperature at either a uniform rate or in a series of steps.

An example of a simple generic temperature program is shown in Figure **13.1.10** and a sketch of the effect of raising the oven (and column) temperature is shown in Figure **13.1.11**

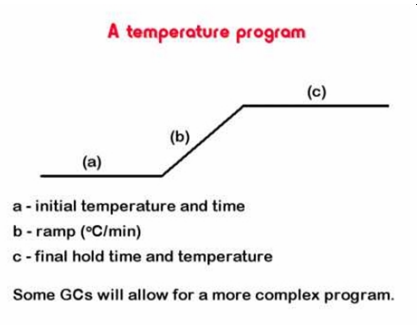


Figure **13.1.10**: A simple hold, ramp and hold temperature program for gas chromatography. Image source currently unknown.

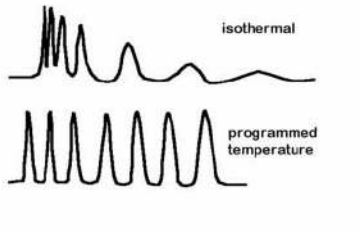


Figure **13.1.11**: An example of the effect of temperature programming. Image source currently unknown.

As one can see in Figure **13.1.11** by raising the temperature during the separation process the retention factor, k, and the peak width for th elate eluting solutes will be reduced resulting in improved resolution, soulte detectability and overall shorter separation times.

Detectors for Gas Chromatography

The final part of a gas chromatograph is the detector. The ideal detector has several desirable features: a low detection limit, a linear response over a wide range of solute concentrations (which makes quantitative work easier), sensitivity for all solutes or

selectivity for a specific class of solutes, and an insensitivity to a change in flow rate or temperature. No single detector satisfies all these characteristics and most instruments have a single detector or at most two types for a dual channel instrument (two injectors, two columns, two detectors all in the same oven and with the same data system).

Thermal Conductivity Detector (TCD)

One of the earliest gas chromatography detectors takes advantage of the mobile phase's thermal conductivity. As the mobile phase exits the column it passes over a tungsten-rhenium wire filament (see Figure **13.1.12**. The filament's electrical resistance depends on its temperature, which, in turn, depends on the thermal conductivity of the mobile phase. Because of its high thermal conductivity, helium is the mobile phase of choice when using a ***thermal conductivity detector*** (TCD).

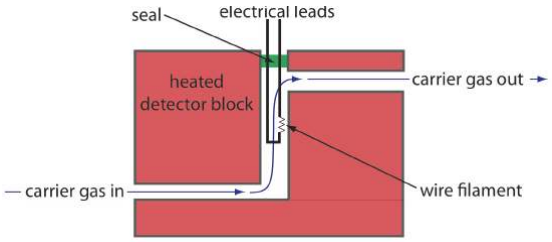


Figure 13.1.12. Schematic diagram of a thermal conductivity detector showing one cell of a matched pair. The sample cell takes the carrier gas as it elutes from the column. A source of carrier gas that bypasses the column passes through a reference cell.

> Thermal conductivity, as the name suggests, is a measure of how easily a substance conducts heat. A gas with a high thermal conductivity moves heat away from the filament—and, thus, cools the filament—more quickly than does a gas with a low thermal conductivity.

When a solute elutes from the column, the thermal conductivity of the mobile phase in the TCD cell decreases and the temperature of the wire filament, and thus it resistance, increases. A reference cell, through which only the mobile phase passes, corrects for any time-dependent variations in flow rate, pressure, or electrical power, all of which affect the filament's resistance.

Because all solutes affect the mobile phase's thermal conductivity, the thermal conductivity detector is a universal detector. Another advantage is the TCD's linear response over a concentration range spanning 10^4–10^5 orders of magnitude. The detector also is non-destructive, which allows us to recover analytes using a postdetector cold trap. One significant disadvantage of the TCD detector is its poor detection limit for most analytes.

Flame Ionization Detector (FID)

The combustion of an organic compound in an H_2/air flame results in a flame that contains electrons and organic cations, presumably CHO^+. Applying a potential of approximately 300 volts across the flame creates a small current of roughly 10^{-9} to 10^{-12} amps. When amplified, this current provides a useful analytical signal. This is the basis of the popular ***flame ionization detector,*** a schematic diagram of which is shown in Figure **13.1.13**.

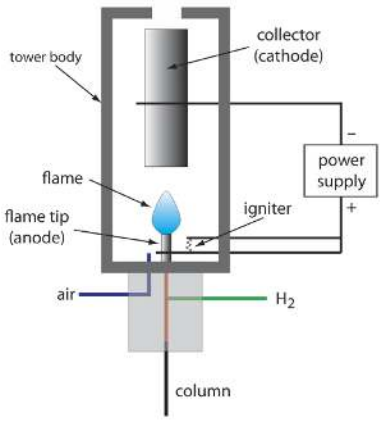


Figure 13.1.13. Schematic diagram of a flame ionization detector. The eluent from the column mixes with H_2 and is burned in the presence of excess air. Combustion produces a flame that contains electrons and the cation $CHO^{+\cdot}$. Applying a potential between the flame's tip and the collector gives a current that is proportional to the concentration of cations in the flame.

Most carbon atoms—except those in carbonyl and carboxylic groups—generate a signal, which makes the FID an almost universal detector for organic compounds. Most inorganic compounds and many gases, such as H_2O and CO_2, are not detected, which makes the FID detector a useful detector for the analysis of organic analytes in atmospheric and aqueous environmental samples. Advantages of the FID include a detection limit that is approximately two to three orders of magnitude smaller than that for a thermal conductivity detector, and a linear response over 10^6–10^7 orders of magnitude in the amount of analyte injected. The sample, of course, is destroyed when using a flame ionization detector.

Electron Capture Detector (ECD)

The ***electron capture detector*** is an example of a selective detector. As shown in Figure **13.1.14**, the detector consists of a β-emitter, such as ^{63}Ni. The emitted electrons ionize the mobile phase, usually N_2, generating a standing current between a pair of electrodes. When a solute with a high affinity for capturing electrons elutes from the column, the current decreases, which serves as the signal. The ECD is highly selective toward solutes with electronegative functional groups, such as halogens and nitro groups, and is relatively insensitive to amines, alcohols, and hydrocarbons. Although its detection limit is excellent, its linear range extends over only about two orders of magnitude.

> A β-particle is an electron.

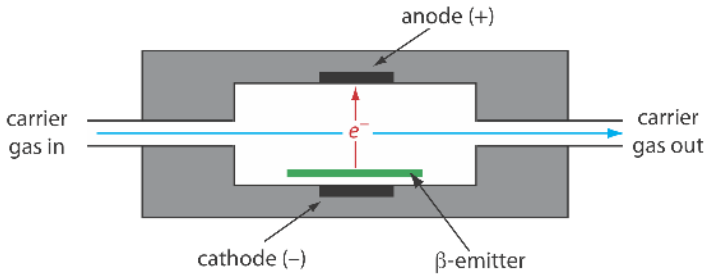


Figure 13.1.14. Schematic diagram showing an electron capture detector.

Nitrogen/Phosphorous Thermionic Detector

The Nitrogen/Phosphorous (NPD) detector is based on ceramic bead containing RbCl or CsCl placed inside a heater coil. As shown in Figure **13.1.15**, the bead is situated above a hydrogen flame. The heated alkali impregnated bead emits electrons by thermionic emission which are collected at the anode and provides background current through the electrode system. When a solute that contains N or P is eluted, the partially combusted N and P materials are adsorbed on the surface of the bead. The adsorbed material reduces the workfunction of the surface and, thus, e- emission is increased and the current collected at the anode rises.

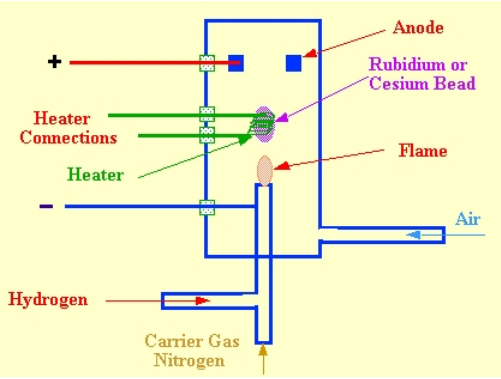


Figure 13.1.15: A sketch of a nitrogen/phosphorous thermionic detector. Image source currently unknown.

The NPD has a very high sensitivity, *i.e.*, about an order of magnitude less than that of the electron capture detector (*ca*.10^{-12} g/ml for phosphorus and 10^{-11} g/ml for nitrogen). Relative to the FID, the NPD is 500x more sensitive for P-bearing species and 50x more sensitive for N-bearing species

Mass Spectrometer (MS)

A ***mass spectrometer*** is an instrument that ionizes a gaseous molecule using sufficient energy that the resulting ion breaks apart into smaller ions. Because these ions have different mass-to-charge ratios, it is possible to separate them using a magnetic field or an electrical field. The resulting mass spectrum contains both quantitative and qualitative information about the analyte. Figure 13.1.16 shows a mass spectrum for toluene.

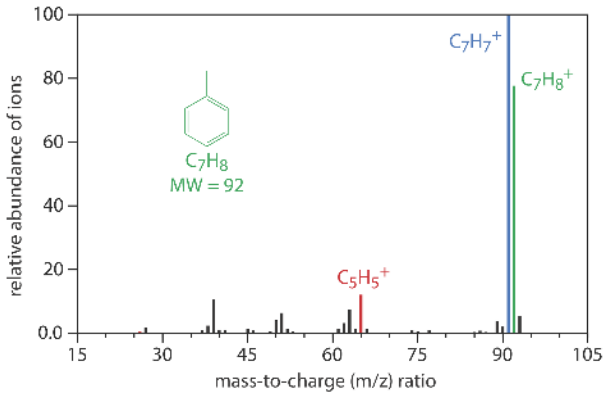


Figure 13.1.16. Mass spectrum for toluene highlighting the molecular ion in green (m/z=92), and two fragment ions in blue (m/z=91) and in red (m/z= 65). A mass spectrum provides both quantitative and qualitative information: the height of any peak is proportional to the amount of toluene in the mass spectrometer and the fragmentation pattern is unique to toluene.

Figure 13.1.17 shows a block diagram of a typical gas chromatography-mass spectrometer (GC–MS) instrument. The effluent from the column enters the mass spectrometer's ion source in a manner that eliminates the majority of the carrier gas. In the ionization chamber the remaining molecules—a mixture of carrier gas, solvent, and solutes—undergo ionization and fragmentation. The mass spectrometer's mass analyzer separates the ions by their mass-to-charge ratio and a detector counts the ions and displays the mass spectrum.

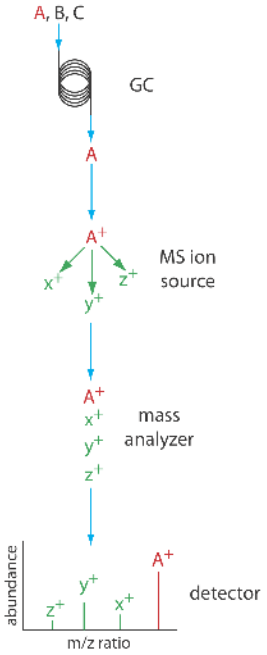


Figure 13.1.17. Block diagram of GC– MS. A three component mixture enters the GC. When component A elutes from the column, it enters the MS ion source and ionizes to form the parent ion and several fragment ions. The ions enter the mass analyzer, which separates them by their mass-to-charge ratio, providing the mass spectrum shown at the detector.

There are several options for monitoring a chromatogram when using a mass spectrometer as the detector. The most common method is to continuously scan the entire mass spectrum and report the total signal for all ions that reach the detector during each scan. This total ion scan provides universal detection for all analytes. We can achieve some degree of selectivity by monitoring one or more specific mass-to-charge ratios, a process called selective-ion monitoring. A mass spectrometer provides excellent detection limits, typically 25 fg to 100 pg, with a linear range of 10^5 orders of magnitude. Because we continuously record the mass spectrum of the column's eluent, we can go back and examine the mass spectrum for any time increment. This is a distinct advantage for GC–MS because we can use the mass spectrum to help identify a mixture's components.

> For more details on mass spectrometry see Introduction to Mass Spectrometry by Michael Samide and Olujide Akinbo, a resource that is part of the Analytical Sciences Digital Library.

Other Detectors

A Fourier transform infrared spectrophotometer (FT–IR) also can serve as a detector. In GC–FT–IR, effluent from the column flows through an optical cell constructed from a 10–40 cm Pyrex tube with an internal diameter of 1–3 mm. The cell's interior surface is coated with a reflecting layer of gold. Multiple reflections of the source radiation as it is transmit- ted through the cell increase the optical path length through the sample. As is the case with GC–MS, an FT–IR detector continuously records the column eluent's spectrum, which allows us to examine the IR spectrum for any time increment.

> See Section 10.3 for a discussion of FT-IR spectroscopy and instrumentation.

An atomic emission detector is an element sensitive detect that relies on the characteristic atomic emission of the element contained a eluting solute. eluting solutes are introduced into a microwave induced plasma generator. The emitted light is collected, dispersed and detected with a CCD type detector.

Quantitative Applications

Gas chromatography is widely used for the analysis of a diverse array of samples in environmental, clinical, pharmaceutical, biochemical, forensic, food science and petrochemical laboratories. Table **13.1.2** provides some representative examples of applications.

Table **13.1.2**. Representative Applications of Gas Chromatography

area	applications
environmental analysis	green house gases (CO_2, CH_4, NO_x) in air pesticides in water, wastewater, and soil vehicle emissions trihalomethanes in drinking water
clinical analysis	drugs blood alcohols
forensic analysis	analysis of arson accelerants detection of explosives
consumer products	volatile organics in spices and fragrances trace organics in whiskey monomers in latex paint
petrochemical and chemical industry	purity of solvents refinery gas composition of gasoline

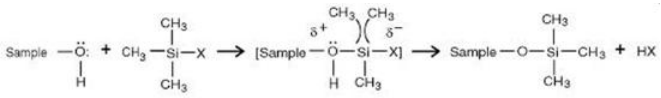
Important requirements for analytes to be suitable for analysis by gas chromatography are that the solutes have a significant vapor pressure at temperatures below 400 °C and that they do not thermally decompose or thermally decompose in a know way (i.e. alcohols that dehydrate upon heating). However, well-established derivatization reactions can be employed to change very polar solutes to forms more amenable for gas chromatography. Two common derivatization reactions are esterification and silylation.

The esterification reaction involves the condensation of the carboxyl group of an acid and the hydroxyl group of an alcohol.

$$RCOOH + R'\text{-}OH \rightarrow RCOOR' + H_2O.$$

Esterification is best done in the presence of a catalyst (such as boron trichloride). The catalyst protonates an oxygen atom of the carboxyl group, making the acid much more reactive. An alcohol then combines with the protonated acid to yield an ester with the loss of water. The catalyst is removed with the water. The alcohol that is used determines the alkyl chain length of the resulting esters (the use of methanol will result in the formation of methyl esters whereas the use of ethanol will result in ethyl esters).

Silylation is the introduction of a silyl group into a molecule, usually in substitution for active hydrogen in an alcohol or carboxylic acid. Common silyl groups include such as dimethylsilyl [$-SiH(CH_3)_2$], t-butyldimethylsilyl [$-Si(CH_3)_2C(CH_3)_3$] and chloromethyldimethylsilyl [$-SiCH_2Cl(CH_3)_2$]. Replacement of active hydrogen by a silyl group reduces the polarity of the compound and reduces hydrogen bonding



Quantitative Calculations

In a GC analysis the area under the peak is proportional to the amount of analyte injected onto the column. A peak's area is determined by integration, which usually is handled by the instrument's computer or by an electronic integrating recorder. If two peak are resolved fully, the determination of their respective areas is straightforward.

> Before electronic integrating recorders and computers, two methods were used to find the area under a curve. One method used a manual planimeter; as you use the planimeter to trace an object's perimeter, it records the area. A second approach for finding a peak's area is the cut-and-weigh method. The chromatogram is recorded on a piece of paper and each peak of interest is cut

out and weighed. Assuming the paper is uniform in thickness and density of fibers, the ratio of weights for two peaks is the same as the ratio of areas. Of course, this approach destroys your chromatogram.

Overlapping peaks, however, require a choice between one of several options for dividing up the area shared by the two peaks (Figure 13.1.18). Which method we use depends on the relative size of the two peaks and their resolution. In some cases, the use of peak heights provides more accurate results [(a) Bicking, M. K. L. Chromatography Online, April 2006; (b) Bicking, M. K. L. Chromatography Online, June 2006].

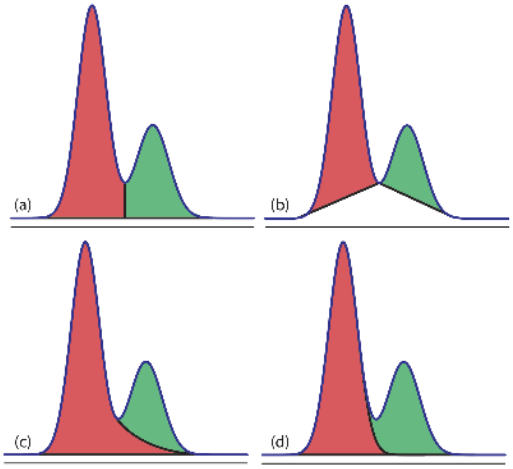


Figure 13.1.18. Four methods for determining the areas under two overlapping chromatographic peaks: (a) the drop method; (b) the valley method; (c) the exponential skim method; and (d) the Gaussian skim method. Other methods for determining areas also are available.

For quantitative work we need to establish a calibration curve that relates the detector's response to the analyte's concentration. If the injection volume is identical for every standard and sample, then an external standardization provides both accurate and precise results. Unfortunately,even under the best conditions the relative precision for replicate injections may differ by 5%; often it is substantially worse. For quantitative work that requires high accuracy and precision, the use of internal standards is recommended.

To review the method of internal standards, see Chapter 5.3.

Example 13.1.1

Marriott and Carpenter report the following data for five replicate injections of a mixture that contains 1% v/v methyl isobutyl ketone and 1% v/v *p*-xylene in dichloromethane [Marriott, P. J.; Carpenter, P. D. *J. Chem. Educ.* **1996**, *73*, 96–99].

injection	peak	peak area (arb. units)
I	1	48075
	2	78112
II	1	85829
	2	135404
III	1	84136
	2	132332
IV	1	71681
	2	112889
V	1	58054
	2	91287

Assume that *p*-xylene (peak 2) is the analyte, and that methyl isobutyl ketone (peak 1) is the internal standard. Determine the 95% confidence interval for a single-point standardization with and without using the internal standard.

Solution

For a single-point external standardization we ignore the internal standard and determine the relationship between the peak area for *p*-xylene, A_2, and the concentration, C_2, of *p*-xylene.

$$A_2 = kC_2$$

Substituting the known concentration for *p*-xylene (1% v/v) and the appropriate peak areas, gives the following values for the constant *k*.

$$78112 \quad 135404 \quad 132332 \quad 112889 \quad 91287$$

The average value for *k* is 110 000 with a standard deviation of 25 100 (a relative standard deviation of 22.8%). The 95% confidence interval is

$$\mu = \overline{X} \pm \frac{ts}{\sqrt{n}} = 111000 \pm \frac{(2.78)(25100)}{\sqrt{5}} = 111000 \pm 31200$$

For an internal standardization, the relationship between the analyte's peak area, A_2, the internal standard's peak area, A_1, and their respective concentrations, C_2 and C_1, is

$$\frac{A_2}{A_1} = k\frac{C_2}{C_1}$$

Substituting in the known concentrations and the appropriate peak areas gives the following values for the constant *k*.

$$1.5917 \quad 1.5776 \quad 1.5728 \quad 1.5749 \quad 1.5724$$

The average value for *k* is 1.5779 with a standard deviation of 0.0080 (a relative standard deviation of 0.507%). The 95% confidence interval is

$$\mu = \overline{X} \pm \frac{ts}{\sqrt{n}} = 1.5779 \pm \frac{(2.78)(0.0080)}{\sqrt{5}} = 1.5779 \pm 0.0099$$

Although there is a substantial variation in the individual peak areas for this set of replicate injections, the internal standard compensates for these variations, providing a more accurate and precise calibration.

Exercise 13.1.1

Figure **13.1.19** shows chromatograms for five standards and for one sample. Each standard and sample contains the same concentration of an internal standard, which is 2.50 mg/mL. For the five standards, the concentrations of analyte are 0.20 mg/mL, 0.40 mg/mL, 0.60 mg/mL, 0.80 mg/mL, and 1.00 mg/mL, respectively. Determine the concentration of analyte in the sample by (a) ignoring the internal standards and creating an external standards calibration curve, and by (b) creating an internal standard calibration curve. For each approach, report the analyte's concentration and the 95% confidence interval. Use peak heights instead of peak areas.

Answer

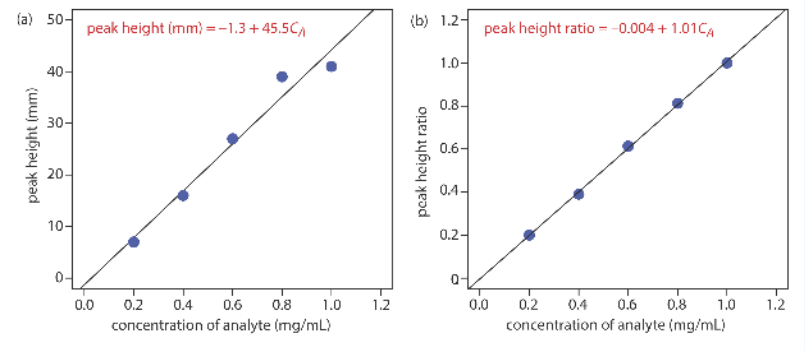
The following table summarizes my measurements of the peak heights for each standard and the sample, and their ratio (although your absolute values for peak heights will differ from mine, depending on the size of your monitor or printout, your relative peak height ratios should be similar to mine).

[standard] (mg/mL)	peak height of standard (mm)	peak height of analyte (mm)	peak height ratio
0.20	35	7	0.20
0.40	41	16	0.39
0.60	44	27	0.61
0.80	48	39	0.81
1.00	41	41	1.00
sample	39	21	0.54

Figure (a) shows the calibration curve and the calibration equation when we ignore the internal standard. Substituting the sample's peak height into the calibration equation gives the analyte's concentration in the sample as 0.49 mg/mL. The 95% confidence interval is ±0.24 mg/mL. The calibration curve shows quite a bit of scatter in the data because of uncertainty in the injection volumes.

Figure (b) shows the calibration curve and the calibration equation when we include the internal standard. Substituting the sample's peak height ratio into the calibration equation gives the analyte's concentration in the sample as 0.54 mg/mL. The 95% confidence interval is ±0.04 mg/mL.

To review the use of Excel or R for regression calculations and confidence intervals, see Chapter 5.5.



The data for this exercise were created so that the analyte's actual concentration is 0.55 mg/mL. Given the resolution of my ruler's scale, my answer is pretty reasonable. Your measurements may be slightly different, but your answers should be close to the actual values.

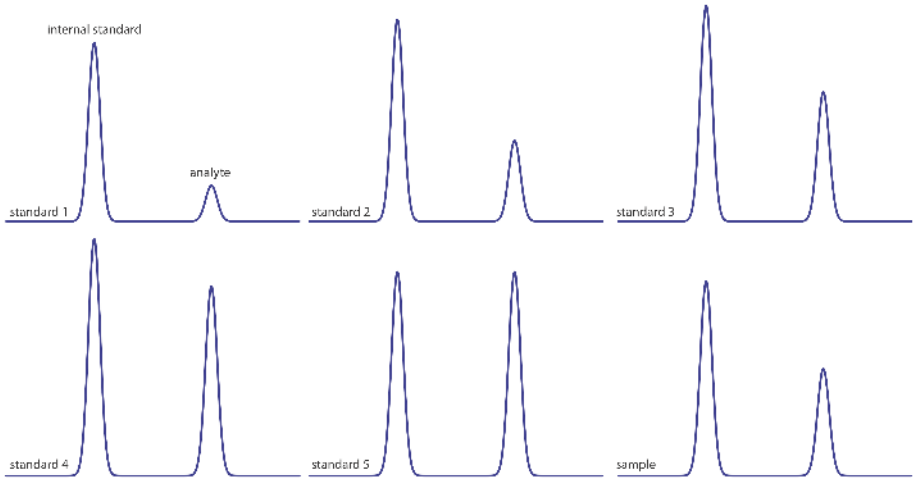


Figure 13.1.19. Chromatograms for Practice Exercise 12.5.

Qualitative Applications

In addition to a quantitative analysis, we also can use chromatography to identify the components of a mixture. As noted earlier, when using an FT–IR or a mass spectrometer as the detector we have access to the eluent's full spectrum for any retention time. By interpreting the spectrum or by searching against a library of spectra, we can identify the analyte responsible for each chromatographic peak.

> In addition to identifying the component responsible for a particular chromatographic peak, we also can use the saved spectra to evaluate peak purity. If only one component is responsible for a chromatographic peak, then the spectra should be identical throughout the peak's elution. If a spectrum at the beginning of the peak's elution is different from a spectrum taken near the end of the peak's elution, then at least two components are co-eluting.

When using a nonspectroscopic detector, such as a flame ionization detector, we must find another approach if we wish to identify the components of a mixture. One approach is to spike a sample with the suspected compound and look for an increase in peak height. We also can compare a peak's retention time to the retention time for a known compound if we use identical operating conditions.

Because a compound's retention times on two identical columns are not likely to be the same—differences in packing efficiency, for example, will affect a solute's retention time on a packed column—creating a table of standard retention times is not possible. ***Kovat's retention index*** provides one solution to the problem of matching retention times. Under isothermal conditions, the adjusted retention times for normal alkanes increase logarithmically. Kovat defined the retention index, I, for a normal alkane as 100 times the number of carbon atoms. For example, the retention index is 400 for butane, C_4H_{10}, and 500 for pentane, C_5H_{12}. To determine the a compound's retention index, I_{cpd}, we use the following formula

$$I_{cpd} = 100 \times \frac{\log t'_{r,cpd} - \log t'_{r,x}}{\log t'_{r,x+1} - \log t'_{r,x}} + I_x \tag{13.1.1}$$

where $t'_{r,cpd}$ is the compound's adjusted retention time, $t'_{r,x}$ and $t'_{r,x+1}$ are the adjusted retention times for the normal alkanes that elute immediately before the compound and immediately after the compound, respectively, and I_x is the retention index for the normal alkane that elutes immediately before the compound. A compound's retention index for a particular set of chromatographic conditions—stationary phase, mobile phase, column type, column length, temperature, etc.—is reasonably consistent from day- to-day and between different columns and instruments.

> Tables of Kovat's retention indices are available; see, for example, the NIST Chemistry Webbook. A search for toluene returns 341 values of I for over 20 different stationary phases, and for both packed columns and capillary columns.

Example 13.1.2

In a separation of a mixture of hydrocarbons the following adjusted retention times are measured: 2.23 min for propane, 5.71 min for isobutane, and 6.67 min for butane. What is the Kovat's retention index for each of these hydrocarbons?

Solution

Kovat's retention index for a normal alkane is 100 times the number of carbons; thus, for propane, I = 300 and for butane, I = 400. To find Kovat's retention index for isobutane we use equation 13.1.1.

$$I_{\text{isobutane}} = 100 \times \frac{\log(5.71) - \log(2.23)}{\log(6.67) - \log(2.23)} + 300 = 386$$

Exercise 13.1.2

When using a column with the same stationary phase as in Example **13.1.2**, you find that the retention times for propane and butane are 4.78 min and 6.86 min, respectively. What is the expected retention time for isobutane?

Answer

Because we are using the same column we can assume that isobutane's retention index of 386 remains unchanged. Using equation 13.1.1, we have

$$386 = 100 \times \frac{\log x - \log(4.78)}{\log(6.86) - \log(4.78)} + 300$$

where x is the retention time for isobutane. Solving for x, we find that

$$0.86 = \frac{\log x - \log(4.78)}{\log(6.86) - \log(4.78)}$$

$$0.135 = \log x - 0.679$$

$$0.814 = \log x$$

$$x = 6.52$$

the retention time for isobutane is 6.5 min.

The best way to appreciate the theoretical and the practical details discussed in this section is to carefully examine a typical analytical method. Although each method is unique, the following description of the determination of trihalomethanes in drinking water provides an instructive example of a typical procedure. The description here is based on a Method 6232B in *Standard Methods for the Examination of Water and Wastewater*, 20th Ed., American Public Health Association: Washing- ton, DC, 1998.

Representative Method 12.4.1: Determination of Trihalomethanes in Drinking Water

Description of Method

Trihalomethanes, such as chloroform, $CHCl_3$, and bromoform, $CHBr_3$, are found in most chlorinated waters. Because chloroform is a suspected carcinogen, the determination of trihalomethanes in public drinking water supplies is of considerable importance. In this method the trihalomethanes $CHCl_3$, $CHBrCl_2$, $CHBr_2Cl$, and $CHBr_3$ are isolated using a liquid–liquid extraction with pentane and determined using a gas chromatograph equipped with an electron capture detector.

Procedure

Collect the sample in a 40-mL glass vial equipped with a screw-cap lined with a TFE-faced septum. Fill the vial until it overflows, ensuring that there are no air bubbles. Add 25 mg of ascorbic acid as a reducing agent to quench the further production of trihalomethanes. Seal the vial and store the sample at 4°C for no longer than 14 days.

Prepare a standard stock solution for each trihalomethane by placing 9.8 mL of methanol in a 10-mL volumetric flask. Let the flask stand for 10 min, or until all surfaces wetted with methanol are dry. Weigh the flask to the nearest ±0.1 mg. Using a 100-µL syringe, add 2 or more drops of trihalomethane to the volumetric flask, allowing each drop to fall directly into the methanol. Reweigh the flask before diluting to volume and mixing. Transfer the solution to a 40-mL glass vial equipped with a TFE-lined screw-top and report the concentration in µg/mL. Store the stock solutions at –10 to –20°C and away from the light.

Prepare a multicomponent working standard from the stock standards by making appropriate dilutions of the stock solution with methanol in a volumetric flask. Choose concentrations so that calibration standards (see below) require no more than 20 µL of working standard per 100 mL of water.

Using the multicomponent working standard, prepare at least three, but preferably 5–7 calibration standards. At least one standard must be near the detection limit and the standards must bracket the expected concentration of trihalomethanes in the samples. Using an appropriate volumetric flask, prepare the standards by injecting at least 10 µL of the working standard below the surface of the water and dilute to volume. Gently mix each standard three times only. Discard the solution in the neck of the volumetric flask and then transfer the remaining solution to a 40-mL glass vial with a TFE-lined screw-top. If the standard has a headspace, it must be analyzed within 1 hr; standards without a headspace may be held for up to 24 hr.

Prepare an internal standard by dissolving 1,2-dibromopentane in hexane. Add a sufficient amount of this solution to pentane to give a final concentration of 30 µg 1,2-dibromopentane/L.

To prepare the calibration standards and samples for analysis, open the screw top vial and remove 5 mL of the solution. Recap the vial and weigh to the nearest ±0.1 mg. Add 2.00 mL of pentane (with the internal standard) to each vial and shake vigorously for 1 min. Allow the two phases to separate for 2 min and then use a glass pipet to transfer at least 1 mL of the pentane (the upper phase) to a 1.8-mL screw top sample vial equipped with a TFE septum, and store at 4°C until you are ready to inject them into the GC. After emptying, rinsing, and drying the sample's original vial, weigh it to the nearest ±0.1 mg and calculate the sample's weight to ±0.1 g. If the density is 1.0 g/mL, then the sample's weight is equivalent to its volume.

Inject a 1–5 µL aliquot of the pentane extracts into a GC equipped with a 2-mm ID, 2-m long glass column packed with a stationary phase of 10% squalane on a packing material of 80/100 mesh Chromosorb WAW. Operate the column at 67°C and a flow rate of 25 mL/min.

> A variety of other columns can be used. Another option, for example, is a 30-m fused silica column with an internal diameter of 0.32 mm and a 1 µm coating of the stationary phase DB-1. A linear flow rate of 20 cm/s is used with the following temperature program: hold for 5 min at 35°C; increase to 70°C at 10°C/min; increase to 200°C at 20°C/min.

Questions

1. A simple liquid–liquid extraction rarely extracts 100% of the analyte. How does this method account for incomplete extractions?

   Because we use the same extraction procedure for the samples and the standards, we reasonably expect that the extraction efficiency is the same for all samples and standards; thus, the relative amount of analyte in any two samples or standards is unaffected by an incomplete extraction.

2. Water samples are likely to contain trace amounts of other organic compounds, many of which will extract into pentane along with the trihalomethanes. A short, packed column, such as the one used in this method, generally does not do a particularly good job of resolving chromatographic peaks. Why do we not need to worry about these other compounds?

   An electron capture detector responds only to compounds, such as the trihalomethanes, that have electronegative functional groups. Because an electron capture detector will not respond to most of the potential interfering compounds, the chromatogram will have relatively few peaks other than those for the trihalomethanes and the internal standard.

3. Predict the order in which the four analytes elute from the GC column.

   Retention time should follow the compound's boiling points, eluting from the lowest boiling point to the highest boiling points. The expected elution order is $CHCl_3$ (61.2ºC), $CHCl_2Br$ (90ºC), $CHClBr_2$ (119ºC), and $CHBr_3$ (149.1ºC).

4. Although chloroform is an analyte, it also is an interferent because it is present at trace levels in the air. Any chloroform present in the laboratory air, for example, may enter the sample by diffusing through the sample vial's silicon septum. How can we

determine whether samples are contaminated in this manner?

> A sample blank of trihalomethane-free water is kept with the samples at all times. If the sample blank shows no evidence for chloroform, then we can safely assume that the samples also are free from contamination.

5. Why is it necessary to collect samples without a headspace (a layer of air that overlays the liquid) in the sample vial?

> Because trihalomethanes are volatile, the presence of a headspace allows for the loss of analyte from the sample to the headspace, resulting in a negative determinate error.

6. In preparing the stock solution for each trihalomethane, the procedure specifies that we add two or more drops of the pure compound by dropping them into a volumetric flask that contains methanol. When preparing the calibration standards, however, the working standard must be injected below the surface of the methanol. Explain the reason for this difference.

> When preparing a stock solution, the potential loss of the volatile trihalomethane is unimportant because we determine its concentration by weight after adding it to the methanol and diluting to volume. When we prepare the calibration standard, however, we must ensure that the addition of trihalomethane is quantitative; thus, we inject it below the surface to avoid the potential loss of analyte.

Evaluation

Scale of Operation

Gas chromatography is used to analyze analytes present at levels ranging from major to ultratrace components. Depending on the detector, samples with major and minor analytes may need to be diluted before analysis. The thermal conductivity and flame ionization detectors can handle larger amounts of analyte; other detectors, such as an electron capture detector or a mass spectrometer, require substantially smaller amounts of analyte. Although the injection volume for gas chromatography is quite small—typically about a microliter—the amount of available sample must be sufficient that the injection is a representative subsample. For a trace analyte, the actual amount of injected analyte is often in the picogram range. Using Representative Method 12.4.1 as an example, a 3.0-μL injection of 1 μg/L $CHCl_3$ is equivalent to 15 pg of $CHCl_3$, assuming a 100% extraction efficiency.

Accuracy

The accuracy of a gas chromatographic method varies substantially from sample-to-sample. For routine samples, accuracies of 1–5% are common. For analytes present at very low concentration levels, for samples with complex matrices, or for samples that require significant processing before analysis, accuracy may be substantially poorer. In the analysis for trihalomethanes described in Representative Method 12.4.1, for example, determinate errors as large as ±25% are possible.

Precision

The precision of a gas chromatographic analysis includes contributions from sampling, sample preparation, and the instrument. The relative standard deviation due to the instrument typically is 1–5%, although it can be significantly higher. The principal limitations are detector noise, which affects the determination of peak area, and the reproducibility of injection volumes. In quantitative work, the use of an internal standard compensates for any variability in injection volumes.

Sensitivity

In a gas chromatographic analysis, sensitivity is determined by the detector's characteristics. Of particular importance for quantitative work is the detector's linear range; that is, the range of concentrations over which a calibration curve is linear. Detectors with a wide linear range, such as the thermal conductivity detector and the flame ionization detector, can be used to analyze samples over a wide range of concentrations without adjusting operating conditions. Other detectors, such as the electron capture detector, have a much narrower linear range.

Selectivity

Because it combines separation with analysis, chromatographic methods provide excellent selectivity. By adjusting conditions it usually is possible to design a separation so that the analytes elute by themselves, even when the mixture is complex. Additional selectivity is obtained by using a detector, such as the electron capture detector, that does not respond to all compounds.

Time, Cost, and Equipment

Analysis time can vary from several minutes for samples that contain only a few constituents, to more than an hour for more complex samples. Preliminary sample preparation may substantially increase the analysis time. Instrumentation for gas chromatography ranges in price from inexpensive (a few thousand dollars) to expensive (>$50,000). The more expensive models are designed for capillary columns, include a variety of injection options, and use more sophisticated detectors, such as a mass spectrometer, or include multiple detectors. Packed columns typically cost <$200, and the cost of a capillary column is typically $300–$1000.

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13.2: Advances in GC

Multidimensional Gas Chromatography (GC-GC, 2D-GC)

Recently the idea of multidimensional gas chromatography separations has be proven to be very useful for closely eluting solutes in experiments with a single column. (see Liu and Phillips, *Journal of Chromatographic Science*. 29 (6), 227–231, 1991). Multidimensional GC is based upon sending groups of closely eluting solutes (on a long column of one selectivity onto a second shorter column of a different selectivity). As shown in Figure**13.2.1** a fast value is used to the red and blue co-eluting in Column 1 solutes onto Column 2 where are being further separated they are better separated.

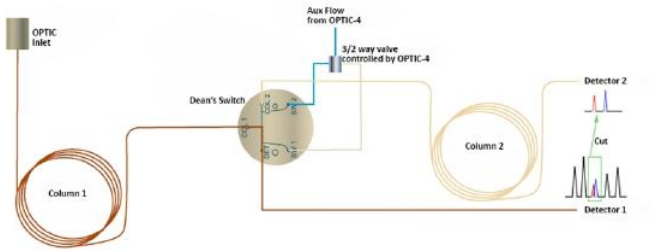


Figure**13.2.1** : A schematic diagram of a two dimension GC instrument. Image source currently unknow.

An example of the utility of multidimensional GC is shown in Figure**13.2.2** - Figure**13.2.4**. Figure**13.2.2** depicts a typical one dimensional GC separation of a large number of polycyclic aromatic compounds on an appropriate coluum. Despite the 40 m length of the capillary column, there are numerous examples of co-eluting solutes presented in the focus boxes.

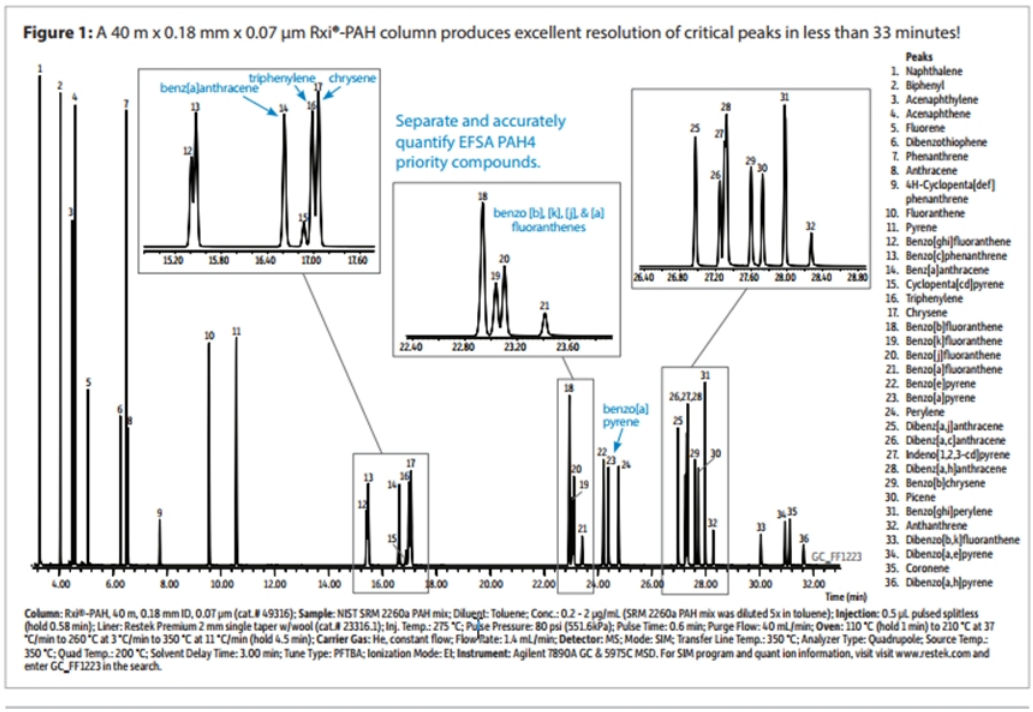


Figure**13.2.2** : The gas chromatograph for a large series of polycyclic aromoatic solutes on a 40 M RXi-PAH column. Imge source currently unknown

Figure**13.2.3** reveals the 2D chromatogram when the solutes eluting through the RXi-PAH column arriving between approximately 15 and 24 min in Figure**13.2.1** are directed onto a short, 1 M, RXi-1HT column for separation on a column with a different

selectivity. (Note an even longer RXi-PAH column, 60 M, was used in the the 2D GC instrument)

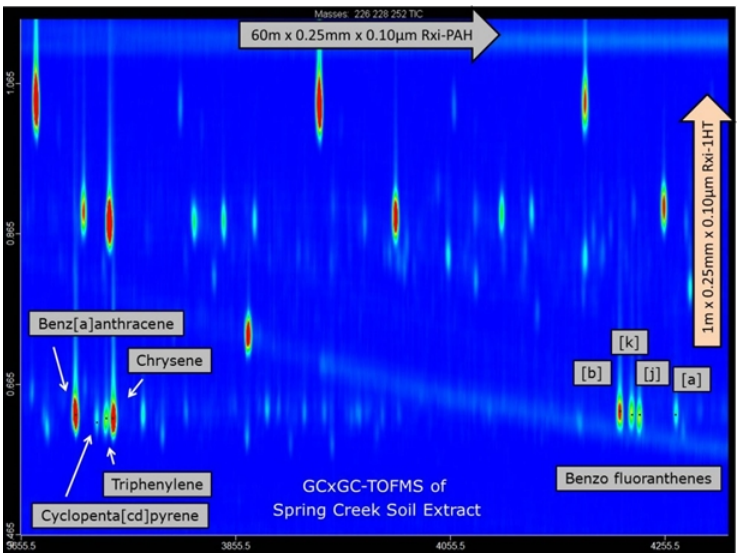
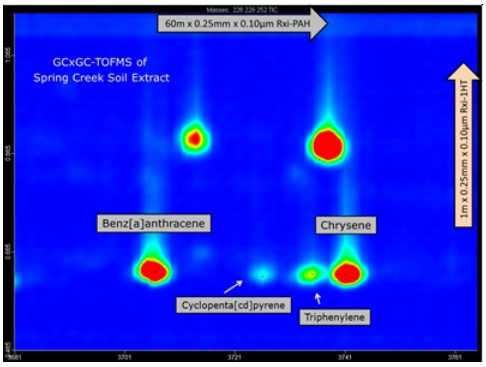


Figure**13.2.3**: A focused region in the 2D - GC experiment of the solutes eluting from approximately 15 min to 24 min in Figure 12.2.2. Image source currently unknown.

Figure**13.2.4** is a zoom of the region in Figure 12.2.3 corresponding to the box centered at 16 min in Figure**13.2.2** and containing the signals for benz[a]anthracene, chrysene, triphenylene and cyclopenta[cd]pyrene revealing the full resolution for the separation of these solutes.



ht

Figure**13.2.4** : A focused region in the 2D - GC experiment revealing the full resolution of the following solutes: benz[a]anthracene, chrysene, triphenylene and cyclopenta[cd]pyrene. Image source currently unknown.

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13.3: Problems

1. The following data were obtained for four compounds separated on a 20-m capillary column.

compound	t_r (min)	w (min)
A	8.04	0.15
B	8.26	0.15
C	8.43	0.16

(a) Calculate the number of theoretical plates for each compound and the average number of theoretical plates for the column, in mm.

(b) Calculate the average height of a theoretical plate.

(c) Explain why it is possible for each compound to have a different number of theoretical plates.

2. Using the data from Problem 1, calculate the resolution and the selectivity factors for each pair of adjacent compounds. For resolution, use both equation 12.2.1 and equation 12.3.3, and compare your results. Discuss how you might improve the resolution between compounds B and C. The retention time for an nonretained solute is 1.19 min.

3. Use the chromatogram in Figure **13.3.1**, obtained using a 2-m column, to determine values for t_r, w, t'_r, k, N, and H.

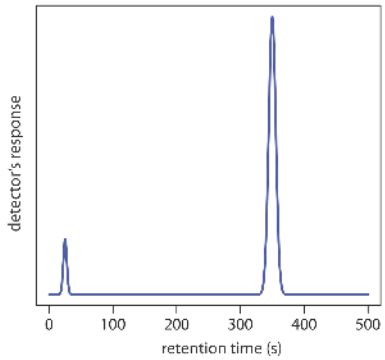


Figure 13.3.1. Chromatogram for Problem 3.

4. Use the partial chromatogram in Figure **13.3.2** to determine the resolution between the two solute bands.

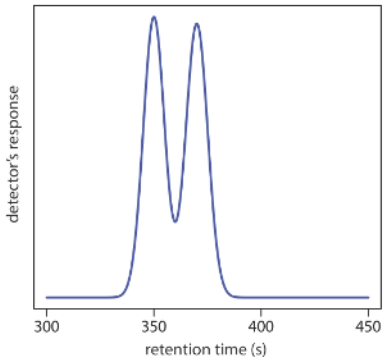


Figure 13.3.2. Chromatogram for Problem 4.

5. The chromatogram in Problem 4 was obtained on a 2-m column with a column dead time of 50 s. Suppose you want to increase the resolution between the two components to 1.5. Without changing the height of a theoretical plate, what length column do you need? What height of a theoretical plate do you need to achieve a resolution of 1.5 without increasing the column's length?

6. Complete the following table.

N_B	α	k_B	R
100000	1.05	0.50	
10000	1.10		1.50
10000		4.0	1.00
	1.05	3.0	1.75

7. Moody studied the efficiency of a GC separation of 2-butanone on a dinonyl phthalate packed column [Moody, H. W. *J. Chem. Educ.* **1982**, *59*, 218–219]. Evaluating plate height as a function of flow rate gave a van Deemter equation for which *A* is 1.65 mm, *B* is 25.8 mm•mL min^{-1}, and *C* is 0.0236 mm•min mL^{-1}.

(a) Prepare a graph of *H* versus *u* for flow rates between 5 –120 mL/min.

(b) For what range of flow rates does each term in the Van Deemter equation have the greatest effect?

(c) What is the optimum flow rate and the corresponding height of a theoretical plate?

(d) For open-tubular columns the *A* term no longer is needed. If the *B* and *C* terms remain unchanged, what is the optimum flow rate and the corresponding height of a theoretical plate?

(e) Compared to the packed column, how many more theoretical plates are in the open-tubular column?

8. Hsieh and Jorgenson prepared 12–33 μm inner diameter HPLC columns packed with 5.44-μm spherical stationary phase particles [Hsieh, S.; Jorgenson, J. W. *Anal. Chem.* **1996**, *68*, 1212–1217]. To evaluate these columns they measured reduced plate height, *h*, as a function of reduced flow rate, *v*,

$$h = \frac{H}{d_p} \quad v = \frac{ud_p}{D_m}$$

where d_p is the particle diameter and D_m is the solute's diffusion coefficient in the mobile phase. The data were analyzed using van Deemter plots. The following table contains a portion of their results for norepinephrine.

internal diameter (μm)	A	B	C
33	0.63	1.32	0.10
33	0.67	1.30	0.08
23	0.40	1.34	0.09
23	0.58	1.11	0.09
17	0.31	1.47	0.11
17	0.40	1.41	0.11
12	0.22	1.53	0.11
12	0.19	1.27	0.12

(a) Construct separate van Deemter plots using the data in the first row and in the last row for reduced flow rates in the range 0.7–15. Determine the optimum flow rate and plate height for each case given d_p = 5.44 μm and D_m = $\mathbf{6.23 \times 10^{-6}}$ cm^2 s^{-1}.

(b) The *A* term in the van Deemter equation is strongly correlated with the column's inner diameter, with smaller diameter columns providing smaller values of *A*. Offer an explanation for this observation. *Hint: consider how many particles can fit across a capillary of each diameter.*

> When comparing columns, chromatographers often use dimensionless, reduced parameters. By including particle size and the solute's diffusion coefficient, the reduced plate height and reduced flow rate correct for differences between the packing

material, the solute, and the mobile phase.

9. A mixture of *n*-heptane, tetrahydrofuran, 2-butanone, and *n*-propanol elutes in this order when using a polar stationary phase such as Carbowax. The elution order is exactly the opposite when using a nonpolar stationary phase such as polydimethyl siloxane. Explain the order of elution in each case.

10. The analysis of trihalomethanes in drinking water is described in Representative Method 12.4.1. A single standard that contains all four trihalomethanes gives the following results.

compound	concentration (ppb)	peak area
$CHCl_3$	1.30	1.35×10^4
$CHCl_2Br$	0.90	6.12×10^4
$CHClBr_2$	4.00	1.71×10^4
$CHBr_3$	1.20	1.52×10^4

Analysis of water collected from a drinking fountain gives areas of 1.56×10^4, 5.13×10^4, 1.49×10^4, and 1.76×10^4 for, respectively, $CHCl_3$, $CHCl_2Br$, $CHClBr_2$, and $CHBr_3$. All peak areas were corrected for variations in injection volumes using an internal standard of 1,2-dibromopentane. Determine the concentration of each of the trihalomethanes in the sample of water.

11. Zhou and colleagues determined the %w/w H_2O in methanol by capillary column GC using a nonpolar stationary phase and a thermal conductivity detector [Zhou, X.; Hines, P. A.; White, K. C.; Borer, M. W. *Anal. Chem.* **1998**, *70*, 390–394]. A series of calibration standards gave the following results.

%w/w H_2O	peak height (arb. units)
0.00	1.15
0.0145	2.74
0.0472	6.33
0.0951	11.58
0.1757	20.43
0.2901	32.97

(a) What is the %w/w H_2O in a sample that has a peak height of 8.63?

(b) The %w/w H_2O in a freeze-dried antibiotic is determined in the following manner. A 0.175-g sample is placed in a vial along with 4.489 g of methanol. Water in the vial extracts into the methanol. Analysis of the sample gave a peak height of 13.66. What is the %w/w H_2O in the antibiotic?

12. Loconto and co-workers describe a method for determining trace levels of water in soil [Loconto, P. R.; Pan, Y. L.; Voice, T. C. *LC•GC* **1996**, *14*, 128–132]. The method takes advantage of the reaction of water with calcium carbide, CaC_2, to produce acetylene gas, C_2H_2. By carrying out the reaction in a sealed vial, the amount of acetylene produced is determined by sampling the headspace. In a typical analysis a sample of soil is placed in a sealed vial with CaC_2. Analysis of the headspace gives a blank corrected signal of 2.70×10^5. A second sample is prepared in the same manner except that a standard addition of 5.0 mg H_2O/g soil is added, giving a blank-corrected signal of 1.06×10^6. Determine the milligrams H_2O/g soil in the soil sample.

13. Van Atta and Van Atta used gas chromatography to determine the %v/v methyl salicylate in rubbing alcohol [Van Atta, R. E.; Van Atta, R. L. *J. Chem. Educ.* **1980**, *57*, 230–231]. A set of standard additions was prepared by transferring 20.00 mL of rubbing alcohol to separate 25-mL volumetric flasks and pipeting 0.00 mL, 0.20 mL, and 0.50 mL of methyl salicylate to the flasks. All three flasks were diluted to volume using isopropanol. Analysis of the three samples gave peak heights for methyl salicylate of 57.00 mm, 88.5 mm, and 132.5 mm, respectively. Determine the %v/v methyl salicylate in the rubbing alcohol.

14. The amount of camphor in an analgesic ointment is determined by GC using the method of internal standards [Pant, S. K.; Gupta, P. N.; Thomas, K. M.; Maitin, B. K.; Jain, C. L. *LC•GC* **1990**, *8*, 322–325]. A standard sample is prepared by placing 45.2 mg of camphor and 2.00 mL of a 6.00 mg/mL internal standard solution of terpene hydrate in a 25-mL volumetric flask and diluting to volume with CCl_4. When an approximately 2-µL sample of the standard is injected, the FID signals for the two components are measured (in arbitrary units) as 67.3 for camphor and 19.8 for terpene hydrate. A 53.6-mg sample of an analgesic ointment is prepared for analysis by placing it in a 50-mL Erlenmeyer flask along with 10 mL of CCl_4. After heating to 50°C in a water bath, the sample is cooled to below room temperature and filtered. The residue is washed with two 5-mL portions of CCl_4 and the combined filtrates are collected in a 25-mL volumetric flask. After adding 2.00 mL of the internal standard solution, the contents of the flask are diluted to volume with CCl_4. Analysis of an approximately 2-µL sample gives FID signals of 13.5 for the terpene hydrate and 24.9 for the camphor. Report the %w/w camphor in the analgesic ointment.

15. The concentration of pesticide residues on agricultural products, such as oranges, is determined by GC-MS [Feigel, C. *Varian GC/MS Application Note*, Number 52]. Pesticide residues are extracted from the sample using methylene chloride and concentrated by evaporating the methylene chloride to a smaller volume. Calibration is accomplished using anthracene-d_{10} as an internal standard. In a study to determine the parts per billion heptachlor epoxide on oranges, a 50.0-g sample of orange rinds is chopped and extracted with 50.00 mL of methylene chloride. After removing any insoluble material by filtration, the methylene chloride is reduced in volume, spiked with a known amount of the internal standard and diluted to 10 mL in a volumetric flask. Analysis of the sample gives a peak–area ratio ($A_{analyte}/A_{intstd}$) of 0.108. A series of calibration standards, each containing the same amount of anthracene-d_{10} as the sample, gives the following results.

ppb heptachlor epoxide	$A_{analyte}/A_{intstd}$
20.0	0.065
60.0	0.153
200.0	0.637
500.0	1.554
1000.0	3.198

Report the nanograms per gram of heptachlor epoxide residue on the oranges. Report the uncertainty in your result at the 95% confidence level.

16. The adjusted retention times for octane, toluene, and nonane on a particular GC column are 15.98 min, 17.73 min, and 20.42 min, respectively. What is the retention index for each compound?

17. The following data were collected for a series of normal alkanes using a stationary phase of Carbowax 20M.

alkane	t_r' (min)
pentane	0.79
hexane	1.99
heptane	4.47
octane	14.12
nonane	33.11

What is the retention index for a compound whose adjusted retention time is 9.36 min?

18. The following data were reported for the gas chromatographic analysis of *p*-xylene and methylisobutylketone (MIBK) on a capillary column [Marriott, P. J.; Carpenter, P. D. *J. Chem. Educ.* **1996**, *73*, 96–99].

injection mode	compound	t_r (min)	peak area (arb. units)	peak width (min)
split	MIBK	1.878	54285	0.028
	p-xylene	5.234	123483	0.044
splitless	MIBK	3.420	2493005	1.057
	p-xylene	5.795	3396656	1.051

Explain the difference in the retention times, the peak areas, and the peak widths when switching from a split injection to a splitless injection.

19. Otto and Wegscheider report the following retention factors for the reversed-phase separation of 2-aminobenzoic acid on a C_{18} column when using 10% v/v methanol as a mobile phase [Otto, M.; Wegscheider, W. *J. Chromatog.* **1983**, *258*, 11–22].

pH	*k*
2.0	10.5
3.0	16.7
4.0	15.8
5.0	8.0
6.0	2.2
7.0	1.8

Explain the effect of pH on the retention factor for 2-aminobenzene.

20. Haddad and associates report the following retention factors for the reversed-phase separation of salicylamide and caffeine [Haddad, P.; Hutchins, S.; Tuffy, M. *J. Chem. Educ.* **1983**, *60*, 166-168].

%v/v methanol	30%	35%	40%	45%	50%	55%
k_{sal}	2.4	1.6	1.6	1.0	0.7	0.7
k_{caff}	4.3	2.8	2.3	1.4	1.1	0.9

(a) Explain the trends in the retention factors for these compounds.

(b) What is the advantage of using a mobile phase with a smaller %v/v methanol? Are there any disadvantages?

21. Suppose you need to separate a mixture of benzoic acid, aspartame, and caffeine in a diet soda. The following information is available.

	t_r in aqueous mobile phase of pH			
compound	3.0	3.5	4.0	4.5
benzoic acid	7.4	7.0	6.9	4.4
aspartame	5.9	6.0	7.1	8.1
caffeine	3.6	3.7	4.1	4.4

(a) Explain the change in each compound's retention time.

(b) Prepare a single graph that shows retention time versus pH for each compound. Using your plot, identify a pH level that will yield an acceptable separation.

22. The composition of a multivitamin tablet is determined using an HPLC with a diode array UV/Vis detector. A 5-μL standard sample that contains 170 ppm vitamin C, 130 ppm niacin, 120 ppm niacinamide, 150 ppm pyridoxine, 60 ppm thiamine, 15 ppm folic acid, and 10 ppm riboflavin is injected into the HPLC, giving signals (in arbitrary units) of, respectively, 0.22, 1.35, 0.90, 1.37, 0.82, 0.36, and 0.29. The multivitamin tablet is prepared for analysis by grinding into a powder and transferring to a 125-mL Erlenmeyer flask that contains 10 mL of 1% v/v NH_3 in dimethyl sulfoxide. After sonicating in an ultrasonic bath for 2 min, 90 mL of 2% acetic acid is added and the mixture is stirred for 1 min and sonicated at 40°C for 5 min. The extract is then filtered through a 0.45-μm membrane filter. Injection of a 5-μL sample into the HPLC gives signals of 0.87 for vitamin C, 0.00 for niacin, 1.40 for niacinamide, 0.22 for pyridoxine, 0.19 for thiamine, 0.11 for folic acid, and 0.44 for riboflavin. Report the milligrams of each vitamin present in the tablet.

23. The amount of caffeine in an analgesic tablet was determined by HPLC using a normal calibration curve. Standard solutions of caffeine were prepared and analyzed using a 10-μL fixed-volume injection loop. Results for the standards are summarized in the following table.

concentration (ppm)	signal (arb. units)
50.0	8.354
100.0	16925
150.0	25218
200.0	33584
250.0	42002

The sample is prepared by placing a single analgesic tablet in a small beaker and adding 10 mL of methanol. After allowing the sample to dissolve, the contents of the beaker, including the insoluble binder, are quantitatively transferred to a 25-mL volumetric flask and diluted to volume with methanol. The sample is then filtered, and a 1.00-mL aliquot transferred to a 10-mL volumetric flask and diluted to volume with methanol. When analyzed by HPLC, the signal for caffeine is found to be 21 469. Report the milligrams of caffeine in the analgesic tablet.

24. Kagel and Farwell report a reversed-phase HPLC method for determining the concentration of acetylsalicylic acid (ASA) and caffeine (CAF) in analgesic tablets using salicylic acid (SA) as an internal standard [Kagel, R. A.; Farwell, S. O. *J. Chem. Educ.* **1983**, *60*, 163–166]. A series of standards was prepared by adding known amounts of ace- tylsalicylic acid and caffeine to 250-mL Erlenmeyer flasks and adding 100 mL of methanol. A 10.00-mL aliquot of a standard solution of salicylic acid was then added to each. The following results were obtained for a typical set of standard solutions.

	milligrams of		peak height ratios for	
standard	ASA	CAF	ASA/SA	CAF/SA
1	200.0	20.0	20.5	10.6
2	250.0	40.0	25.1	23.0
3	300.0	60.0	30.9	36.8

A sample of an analgesic tablet was placed in a 250-mL Erlenmeyer flask and dissolved in 100 mL of methanol. After adding a 10.00-mL portion of the internal standard, the solution was filtered. Analysis of the sample gave a peak height ratio of 23.2 for ASA and of 17.9 for CAF.

(a) Determine the milligrams of ASA and CAF in the tablet.

(b) Why is it necessary to filter the sample?

(c) The directions indicate that approximately 100 mL of methanol is used to dissolve the standards and samples. Why is it not necessary to measure this volume more precisely?

(d) In the presence of moisture, ASA decomposes to SA and acetic acid. What complication might this present for this analysis? How might you evaluate whether this is a problem?

25. Bohman and colleagues described a reversed-phase HPLC method for the quantitative analysis of vitamin A in food using the method of standard additions Bohman, O.; Engdahl, K. A.; Johnsson, H. *J. Chem. Educ.* **1982**, *59*, 251–252]. In a typical example, a 10.067-g sample of cereal is placed in a 250-mL Erlenmeyer flask along with 1 g of sodium ascorbate, 40 mL of ethanol, and 10 mL of 50% w/v KOH. After refluxing for 30 min, 60 mL of ethanol is added and the solution cooled to room temperature. Vitamin A is extracted using three 100-mL portions of hexane. The combined portions of hexane are evaporated and the residue containing vitamin A transferred to a 5-mL volumetric flask and diluted to volume with methanol. A standard addition is prepared in a similar manner using a 10.093-g sample of the cereal and spiking with 0.0200 mg of vitamin A. Injecting the sample and standard addition into the HPLC gives peak areas of, respectively, $\mathbf{6.77 \times 10^3}$ and $\mathbf{1.32 \times 10^4}$. Report the vitamin A content of the sample in milligrams/100 g cereal.

26. Ohta and Tanaka reported on an ion-exchange chromatographic method for the simultaneous analysis of several inorganic anions and the cations Mg^{2+} and Ca^{2+} in water [Ohta, K.; Tanaka, K. *Anal. Chim. Acta* **1998**, *373*, 189–195]. The mobile phase includes the ligand 1,2,4-benzenetricarboxylate, which absorbs strongly at 270 nm. Indirect detection of the analytes is possible because its absorbance decreases when complexed with an anion.

(a) The procedure also calls for adding the ligand EDTA to the mobile phase. What role does the EDTA play in this analysis?

(b) A standard solution of 1.0 mM $NaHCO_3$, 0.20 mM $NaNO_2$, 0.20 mM $MgSO_4$, 0.10 mM $CaCl_2$, and 0.10 mM $Ca(NO_3)_2$ gives the following peak areas (arbitrary units).

ion	$\mathbf{HCO_3^-}$	Cl^-	$\mathbf{NO_2^-}$	$\mathbf{NO_3^-}$
peak area	373.5	322.5	264.8	262.7
ion	Ca^{2+}	Mg^{2+}	$\mathbf{SO_4^{2-}}$	
peak area	458.9	352.0	341.3	

Analysis of a river water sample (pH of 7.49) gives the following results.

ion	$\mathbf{HCO_3^-}$	Cl^-	$\mathbf{NO_2^-}$	$\mathbf{NO_3^-}$
peak area	310.0	403.1	3.97	157.6
ion	Ca^{2+}	Mg^{2+}	$\mathbf{SO_4^{2-}}$	
peak area	734.3	193.6	324.3	

Determine the concentration of each ion in the sample.

(c) The detection of $\mathbf{HCO_3^-}$ actually gives the total concentration of carbonate in solution ($[\mathbf{CO_3^{2-}}]+[\mathbf{HCO_3^-}]+[H_2CO_3]$). Given that the pH of the water is 7.49, what is the actual concentration of $\mathbf{HCO_3^-}$?

(d) An independent analysis gives the following additional concentrations for ions in the sample: $[Na^+]$ = 0.60 mM; $[\mathbf{NH_4^+}]$ = 0.014 mM; and $[K^+]$ = 0.046 mM. A solution's ion balance is defined as the ratio of the total cation charge to the total anion charge. Determine the charge balance for this sample of water and comment on whether the result is reasonable.

27. The concentrations of Cl^-, $\mathbf{NO_2^-}$, and $\mathbf{SO_4^{2-}}$ are determined by ion chromatography. A 50-µL standard sample of 10.0 ppm Cl^-, 2.00 ppm $\mathbf{NO_2^-}$, and 5.00 ppm $\mathbf{SO_4^{2-}}$ gave signals (in arbitrary units) of 59.3, 16.1, and 6.08 respectively. A sample of effluent from a wastewater treatment plant is diluted tenfold and a 50-µL portion gives signals of 44.2 for Cl^-, 2.73 for $\mathbf{NO_2^-}$, and 5.04 for $\mathbf{SO_4^{2-}}$. Report the parts per million for each anion in the effluent sample.

28. A series of polyvinylpyridine standards of different molecular weight was analyzed by size-exclusion chromatography, yielding the following results.

formula weight	retention volume (mL)
600000	6.42
100000	7.98
30000	9.30
3000	10.94

When a preparation of polyvinylpyridine of unknown formula weight is analyzed, the retention volume is 8.45 mL. Report the average formula weight for the preparation.

29. Diet soft drinks contain appreciable quantities of aspartame, benzoic acid, and caffeine. What is the expected order of elution for these compounds in a capillary zone electrophoresis separation using a pH 9.4 buffer given that aspartame has pK_a values of 2.964 and 7.37, benzoic acid has a pK_a of 4.2, and the pK_a for caffeine is less than 0. Figure **13.3.3** provides the structures of these compounds.

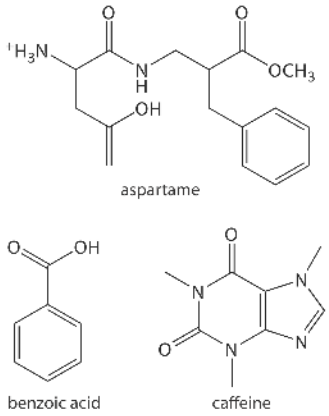


Figure 13.3.3
. Structures for the compounds in Problem 29.

30. Janusa and coworkers describe the determination of chloride by CZE [Janusa, M. A.; Andermann, L. J.; Kliebert, N. M.; Nannie, M. H. *J. Chem. Educ.* **1998**, *75*, 1463–1465]. Analysis of a series of external standards gives the following calibration curve.

$$\text{area} = -883 + 5590 \times \text{ppm } Cl^-$$

A standard sample of 57.22% w/w Cl^- is analyzed by placing 0.1011-g portions in separate 100-mL volumetric flasks and diluting to volume. Three unknowns are prepared by pipeting 0.250 mL, 0.500 mL, an 0.750 mL of the bulk unknown in separate 50-mL volumetric flasks and diluting to volume. Analysis of the three unknowns gives areas of 15 310, 31 546, and 47 582, respectively. Evaluate the accuracy of this analysis.

31. The analysis of NO_3^- in aquarium water is carried out by CZE using IO_4^- as an internal standard. A standard solution of 15.0 ppm NO_3^- and 10.0 ppm IO_4^- gives peak heights (arbitrary units) of 95.0 and 100.1, respectively. A sample of water from an aquarium is diluted 1:100 and sufficient internal standard added to make its concentration 10.0 ppm in IO_4^-. Analysis gives signals of 29.2 and 105.8 for NO_3^- and IO_4^-, respectively. Report the ppm NO_3^- in the sample of aquarium water.

32. Suggest conditions to separate a mixture of 2-aminobenzoic acid (pK_{a1} = 2.08, pK_{a2} = 4.96), benzylamine (pK_a = 9.35), and 4-methylphenol (pK_{a2} = 10.26) by capillary zone electrophoresis. Figure ***PageIndex4*** provides the structures of these compounds.

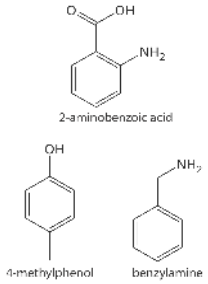


Figure 13.3.4. Structures for the compounds in Problem 32.

33. McKillop and associates examined the electrophoretic separation of some alkylpyridines by CZE [McKillop, A. G.; Smith, R. M.; Rowe, R. C.; Wren, S. A. C. *Anal. Chem.* **1999**, *71*, 497–503]. Separations were carried out using either 50-μm or 75-μm inner diameter capillaries, with a total length of 57 cm and a length of 50 cm from the point of injection to the detector. The run buffer was a pH 2.5 lithium phosphate buffer. Separations were achieved using an applied voltage of 15 kV. The electroosmotic mobility, μ_{eof}, as measured using a neutral marker, was found to be $\mathbf{6.398 \times 10^{-5}}$ $cm^2\ V^{-1}\ s^{-1}$. The diffusion coefficient for alkylpyridines is $\mathbf{1.0 \times 10^{-5}}$ $cm^2\ s^{-1}$.

(a) Calculate the electrophoretic mobility for 2-ethylpyridine given that its elution time is 8.20 min.

(b) How many theoretical plates are there for 2-ethylpyridine?

(c) The electrophoretic mobilities for 3-ethylpyridine and 4-ethylpyridine are $\mathbf{3.366 \times 10^{-4}}$ $cm^2\ V^{-1}\ s^{-1}$ and $\mathbf{3.397 \times 10^{-4}}$ $\mathbf{cm^2\ V^{-1}\ s^{-1}}$, respectively. What is the expected resolution between these two alkylpyridines?

(d) Explain the trends in electrophoretic mobility shown in the following table.

alkylpyridine	μ_{ep} ($cm^2\ V^{-1}\ s^{-1}$)
2-methylpyridine	$\mathbf{3.581 \times 10^{-4}}$
2-ethylpyridine	$\mathbf{3.222 \times 10^{-4}}$
2-propylpyridine	$\mathbf{2.923 \times 10^{-4}}$
2-pentylpyridine	$\mathbf{2.534 \times 10^{-4}}$
2-hexylpyridine	$\mathbf{2.391 \times 10^{-4}}$

(e) Explain the trends in electrophoretic mobility shown in the following table.

alkylpyridine	μ_{ep} ($cm^2\ V^{-1}\ s^{-1}$)
2-ethylpyridine	$\mathbf{3.222 \times 10^{-4}}$
3-ethylpyridine	$\mathbf{3.366 \times 10^{-4}}$
4-ethylpyridine	$\mathbf{3.397 \times 10^{-4}}$

(f) The pK_a for pyridine is 5.229. At a pH of 2.5 the electrophoretic mobility of pyridine is $\mathbf{4.176 \times 10^{-4}}$ $cm^2\ V^{-1}\ s^{-1}$. What is the expected electrophoretic mobility if the run buffer's pH is 7.5?

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CHAPTER OVERVIEW

14: Liquid Chromatography

High performance liquid chromatography has proven itself to very useful in many scientific fields, yet forces scientists to consistently choose between speed and resolution. Ultra High Performance Liquid Chromatography (UHPLC) eliminates the need to choose and creates a highly efficient method that is primarily based on small particle separations.

14.1: Scope of Liquid Chromatography
14.2: High-Performance Liquid Chromatography
14.3: Chiral Chromatography
14.4: Ion Chromatography
14.5: Size-Exclusion Chromatography
14.6: Thin-Layer Chromatography
14.7: Problems

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14.1: Scope of Liquid Chromatography

Liquid Chromatography is a term describing a wide variety of different separation methods with the common characteristic that the mobile phase is a liquid. The scope of liquid chromatography is vast and separations can be performed for complex mixtures of solutes ranging from small ions and molecules to large macromolecules. Figure 13.1.1 is a graphic illustrating the scope of liquid chromatograph in terms of solute type, solubility characteristics, and molecular mass.

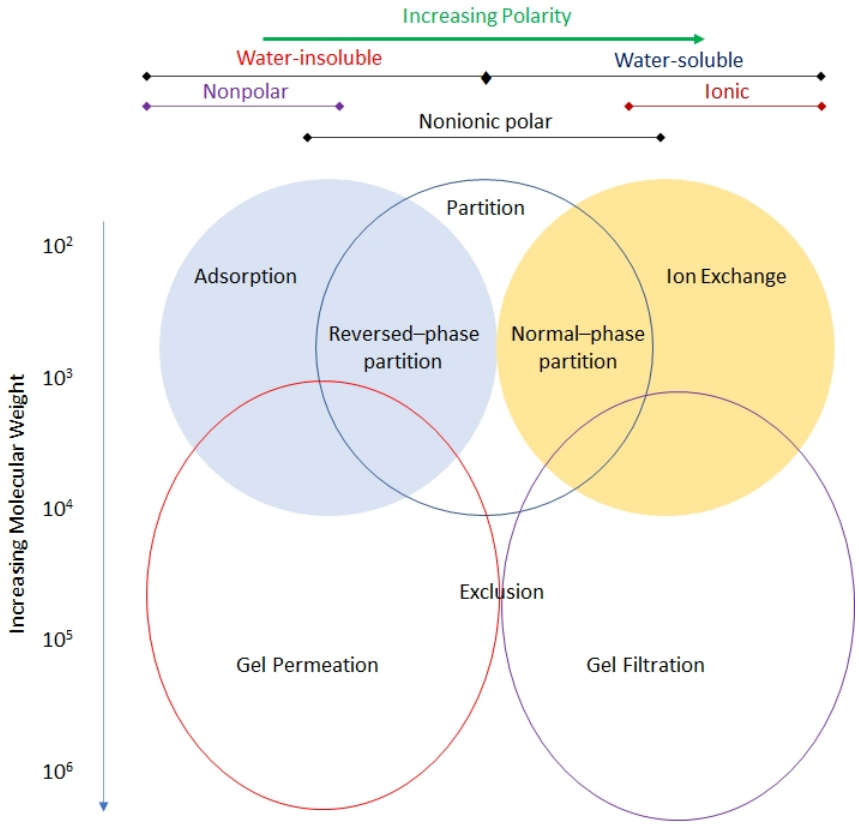


Figure 13.1.1 A illustration of the many types and applications of liquid chromatography.

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14.2: High-Performance Liquid Chromatography

In ***high-performance liquid chromatography*** (HPLC) we inject the sample, which is in solution form, into a liquid mobile phase. The mobile phase carries the sample through a packed or capillary column that separates the sample's components based on their ability to partition between the mobile phase and the stationary phase. Figure **14.2.1** shows an example of a typical HPLC instrument, which has several key components: reservoirs that store the mobile phase; a pump for pushing the mobile phase through the system; an injector for introducing the sample; a column for separating the sample into its component parts; and a detector for monitoring the eluent as it comes off the column. Let's consider each of these components.

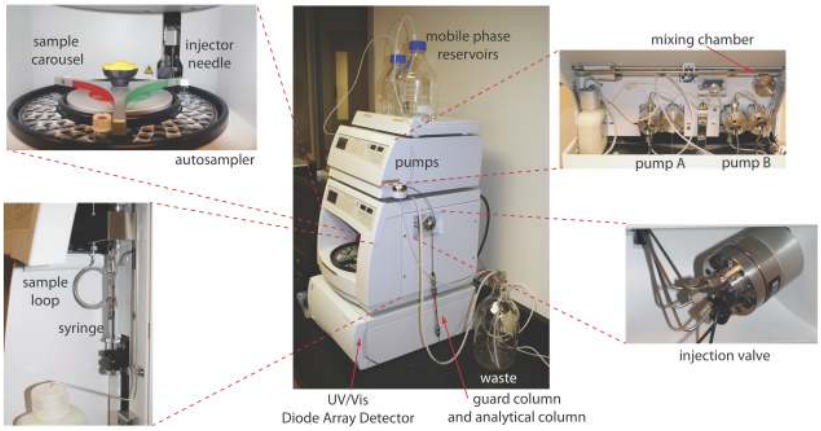


Figure 14.2.1. Example of a typical high-performance liquid chromatograph with insets showing the pumps that move the mobile phase through the system and the plumbing used to inject the sample into the mobile phase. This particular instrument includes an autosampler. An instrument in which samples are injected manually does not include the features shown in the two left-most insets, and has a different style of loop injection valve.

A solute's retention time in HPLC is determined by its interaction with the stationary phase and the mobile phase. There are several different types of solute/stationary phase interactions, including liquid–solid adsorption, liquid–liquid partitioning, ion-exchange, and size-exclusion. This chapter deals exclusively with HPLC separations based on liquid–liquid partitioning. Other forms of liquid chromatography receive consideration in Chapter 12.6.

HPLC Columns

An HPLC typically includes two columns: an analytical column, which is responsible for the separation, and a guard column that is placed before the analytical column to protect it from contamination.

Analytical Columns

The most common type of HPLC column is a stainless steel tube with an internal diameter between 2.1 mm and 4.6 mm and a length between 30 mm and 300 mm (Figure **14.2.2**). The column is packed with 3–10 nm porous silica particles with either an irregular or a spherical shape. Typical column efficiencies are 40,000–60,000 theoretical plates/m. Assuming a V_{max}/V_{min} of approximately 50, a 25-cm column with 50,000 plates/m has 12,500 theoretical plates and a peak capacity of 110.



Figure 14.2.2. Typical packed column for HPLC. This particular column has an internal diameter of 4.6 mm and a length of 150 mm, and is packed with 5 μm particles coated with stationary phase.

Over time expertise in the manufacture of silica particles leading to smaller particles and more uniform particle size distributions. As shown in the van Deemter sketch depicted in Figure (\PageIndex{3}\)) separation efficiency increases greatly with decreasing particle size. None of these lines depict the minima predicted for a van Deemter plot as these are only observable at very low flow rates that are too low for practical applications.

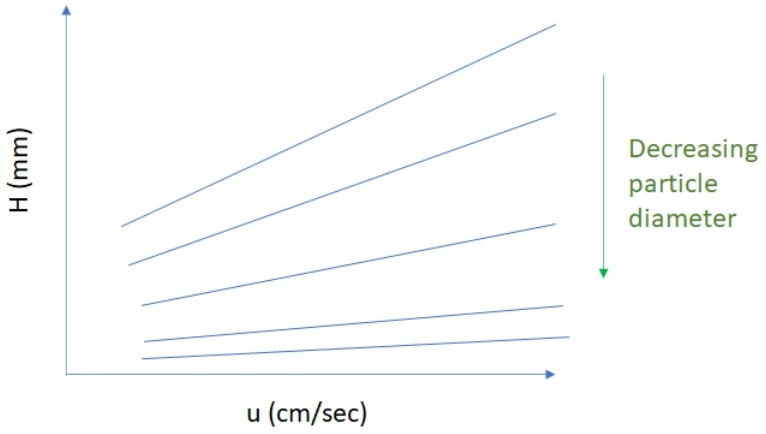


Figure (\PageIndex{3}\)): A sketch of the effect of packing particle diameter on separation efficiency where u is the linear velocity of the mobile phase flow rate and H is the hight equivalent of a theoretical plate.

Capillary columns use less solvent and, because the sample is diluted to a lesser extent, produce larger signals at the detector. These columns are made from fused silica capillaries with internal diameters from 44–200 μm and lengths of 50–250 mm. Capillary columns packed with 3–5 μm particles have been prepared with column efficiencies of up to 250,000 theoretical plates [Novotony, M. *Science*, **1989**, *246*, 51–57].

One limitation to a packed capillary column is the back pressure that develops when pumping the mobile phase through the small interstitial spaces between the particulate micron-sized packing material (Figure **14.2.4**). Because the tubing and fittings that carry the mobile phase have pressure limits, a higher back pressure requires a lower flow rate and a longer analysis time. Monolithic columns, in which the solid support is a single, porous rod, offer column efficiencies equivalent to a packed capillary column while allowing for faster flow rates. A monolithic column—which usually is similar in size to a conventional packed column, although smaller, capillary columns also are available—is prepared by forming the monolithic rod in a mold and covering it with PTFE tubing or a polymer resin. Monolithic rods made of a silica-gel polymer typically have macropores with diameters of approximately 2 μm and mesopores—pores within the macropores—with diameters of approximately 13 nm [Cabrera, K. Chromatography Online, April 1, 2008].

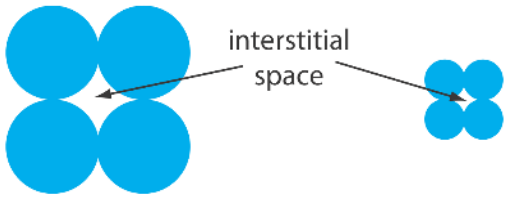


Figure 14.2.4. The packing of smaller particles creates smaller interstitial spaces than the packing of larger particles. Although reducing particle size by **2×** increases efficiency by a factor of 1.4, it also produces a 4-fold increase in back pressure.

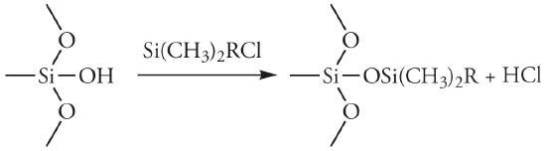
Guard Columns

Two problems tend to shorten the lifetime of an analytical column. First, solutes that bind irreversibly to the stationary phase degrade the column's performance by decreasing the amount of stationary phase available for effecting a separation. Second, particulate material injected with the sample may clog the analytical column. To minimize these problems we place a guard column before the analytical column. A Guard column usually contains the same particulate packing material and stationary phase as the analytical column, but is significantly shorter and less expensive—a length of 7.5 mm and a cost one-tenth of that for the corresponding analytical column is typical. Because they are intended to be sacrificial, guard columns are replaced regularly.

> If you look closely at Figure 14.2.1, you will see the small guard column just above the analytical column.

Stationary Phases for Liquid-Liquid Chromatography

In liquid–liquid chromatography the stationary phase is a liquid film coated on a packing material, typically 3–10 μm porous silica particles. Because the stationary phase may be partially soluble in the mobile phase, it may elute, or bleed from the column over time. To prevent the loss of stationary phase, which shortens the column's lifetime, it is bound covalently to the silica particles. ***Bonded stationary phases*** are created by reacting the silica particles with an organochlorosilane of the general form $Si(CH_3)_2RCl$, where R is an alkyl or substituted alkyl group.



To prevent unwanted interactions between the solutes and any remaining –SiOH groups, $Si(CH_3)_3Cl$ is used to convert unreacted sites to $\mathbf{-SiOSi(CH_3)_3}$; such columns are designated as end-capped.

The properties of a stationary phase depend on the organosilane's alkyl group. If R is a polar functional group, then the stationary phase is polar. Examples of polar stationary phases include those where R contains a cyano ($-C_2H_4CN$), a diol ($-C_3H_6OCH_2CHOHCH_2OH$), or an amino ($-C_3H_6NH_2$) functional group. Because the stationary phase is polar, the mobile phase is a nonpolar or a moderately polar solvent. The combination of a polar stationary phase and a nonpolar mobile phase is called ***normal-phase chromatography***.

In ***reversed-phase chromatography***, which is the more common form of HPLC, the stationary phase is nonpolar and the mobile phase is polar. The most common nonpolar stationary phases use an organochlorosilane where the R group is an *n*-octyl (C_8) or *n*-octyldecyl (C_{18}) hydrocarbon chain. Most reversed-phase separations are carried out using a buffered aqueous solution as a polar mobile phase, or using other polar solvents, such as methanol and acetonitrile. Because the silica substrate may undergo hydrolysis in basic solutions, the pH of the mobile phase must be less than 7.5.

> It seems odd that the more common form of liquid chromatography is identified as reverse-phase instead of normal phase. You might recall that one of the earliest examples of chromatography was Mikhail Tswett's separation of plant pigments using a polar column of calcium carbonate and a nonpolar mobile phase of petroleum ether. The assignment of normal and reversed, therefore, is all about precedence.

Mobile Phases

The elution order of solutes in HPLC is governed by polarity. For a normal-phase separation, a solute of lower polarity spends proportionally less time in the polar stationary phase and elutes before a solute that is more polar. Given a particular stationary phase, retention times in normal-phase HPLC are controlled by adjusting the mobile phase's properties. For example, if the resolution between two solutes is poor, switching to a less polar mobile phase keeps the solutes on the column for a longer time and provides more opportunity for their separation. In reversed-phase HPLC the order of elution is the opposite that in a normal-phase separation, with more polar solutes eluting first. Increasing the polarity of the mobile phase leads to longer retention times. Shorter retention times require a mobile phase of lower polarity.

Choosing a Mobile Phase: Using the Polarity Index

There are several indices that help in selecting a mobile phase, one of which is the polarity index [Snyder, L. R.; Glajch, J. L.; Kirkland, J. J. *Practical HPLC Method Development*, Wiley-Inter- science: New York, 1988]. Table **14.2.1** provides values of the polarity index, P', for several common mobile phases, where larger values of P' correspond to more polar solvents. Mixing together two or more mobile phases—assuming they are miscible—creates a mobile phase of intermediate polarity. For example, a binary mobile phase made by combining solvent *A* and solvent *B* has a polarity index, P'_{AB}, of

$$P'_{AB} = \Phi_A P'_A + \Phi_B P'_B \tag{14.2.1}$$

where P'_A and P'_B are the polarity indices for solvents *A* and *B*, and Φ_A and Φ_B are the volume fractions for the two solvents.

Table **14.2.1**. Properties of HPLC Mobile Phases

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mobile phase	polarity index (P')	UV cutoff (nm)
cyclohexane	0.04	210
n-hexane	0.1	210
carbon tetrachloride	1.6	265
i-propyl ether	2.4	220
toluene	2.4	286
diethyl ether	2.8	218
tetrahydrofuran	4.0	220
ethanol	4.3	210
ethyl acetate	4.4	255
dioxane	4.8	215
methanol	5.1	210
acetonitrile	5.8	190
water	10.2	—

Example 14.2.1

A reversed-phase HPLC separation is carried out using a mobile phase of 60% v/v water and 40% v/v methanol. What is the mobile phase's polarity index?

Solution

Using equation 14.2.1 and the values in Table 14.2.1, the polarity index for a 60:40 water–methanol mixture is

$$P'_{AB} = \Phi_{\text{water}} P'_{\text{water}} + \Phi_{\text{methanol}} P'_{\text{methanol}}$$

$$P'_{AB} = 0.60 \times 10.2 + 0.40 \times 5.1 = 8.2$$

Exercise 14.2.1

Suppose you need a mobile phase with a polarity index of 7.5. Explain how you can prepare this mobile phase using methanol and water.

Answer

If we let *x* be the fraction of water in the mobile phase, then 1 – *x* is the fraction of methanol. Substituting these values into equation 14.2.1 and solving for *x*

$$7.5 = 10.2x + 5.1(1 - x)$$

$$7.5 = 10.2x + 5.1 - 5.1x$$

$$2.4 = 5.1x$$

gives *x* as 0.47. The mobile phase is 47% v/v water and 53% v/v methanol.

As a general rule, a two unit change in the polarity index corresponds to an approximately 10-fold change in a solute's retention factor. Here is a simple example. If a solute's retention factor, *k*, is 22 when using water as a mobile phase (P' = 10.2), then switching to a mobile phase of 60:40 water–methanol (P' = 8.2) decreases *k* to approximately 2.2. Note that the retention factor

becomes smaller because we are switching from a more polar mobile phase to a less polar mobile phase in a reversed-phase separation.

Choosing a Mobile Phase: Adjusting Selectivity

Changing the mobile phase's polarity index changes a solute's retention factor. As we learned in Chapter 12.3, however, a change in k is not an effective way to improve resolution when the initial value of k is greater than 10. To effect a better separation between two solutes we must improve the selectivity factor, α. There are two common methods for increasing α: adding a reagent to the mobile phase that reacts with the solutes in a secondary equilibrium reaction or switching to a different mobile phase.

Taking advantage of a secondary equilibrium reaction is a useful strategy for improving a separation [(a) Foley, J. P. *Chromatography*, **1987**, *7*, 118–128; (b) Foley, J. P.; May, W. E. Anal. Chem. 1987, 59, 102–109; (c) Foley, J. P.; May, W. E. Anal. Chem. 1987, 59, 110–115]. Figure 12.3.3, which we considered earlier in this chapter, shows the reversed-phase separation of four weak acids—benzoic acid, terephthalic acid, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid—on a nonpolar C_{18} column using an aqueous buffer of acetic acid and sodium acetate as the mobile phase. The retention times for these weak acids are shorter when using a less acidic mobile phase because each solute is present in an anionic, weak base form that is less soluble in the nonpolar stationary phase. If the mobile phase's pH is sufficiently acidic, the solutes are present as neutral weak acids that are more soluble in the stationary phase and take longer to elute. Because the weak acid solutes do not have identical pK_a values, the pH of the mobile phase has a different effect on each solute's retention time, allowing us to find the optimum pH for effecting a complete separation of the four solutes.

> Acid–base chemistry is not the only example of a secondary equilibrium reaction. Other examples include ion-pairing, complexation, and the interaction of solutes with micelles. We will consider the last of these in Chapter 12.7 when we discuss micellar electrokinetic capillary chromatography.

In Example 14.2.1 we learned how to adjust the mobile phase's polarity by blending together two solvents. A polarity index, however, is just a guide, and binary mobile phase mixtures with identical polarity indices may not resolve equally a pair of solutes. Table **14.2.2**, for example, shows retention times for four weak acids in two mobile phases with nearly identical values for P'. Although the order of elution is the same for both mobile phases, each solute's retention time is affected differently by the choice of organic solvent. If we switch from using acetonitrile to tetrahydrofuran, for example, we find that benzoic acid elutes more quickly and that *p*-hydroxybenzoic acid elutes more slowly. Although we can resolve fully these two solutes using mobile phase that is 16% v/v acetonitrile, we cannot resolve them if the mobile phase is 10% tetrahydrofuran.

Table **14.2.2**. Retention Times for Four Weak Acids in Mobile Phases With Similar Polarity Indexes

retention time (min)	16% acetonitrile (CH_3CN) 84% pH 4.11 aqueous buffer (P' = 9.5)	10% tetrahydrofuran (THF) 90% pH 4.11 aqueous buffer (P' = 9.6)
$t_{r,\,BA}$	5.18	4.01
$t_{r,\,PH}$	1.67	2.91
$t_{r,\,PA}$	1.21	1.05
$t_{r,\,TP}$	0.23	0.54

Key: BA is benzoic acid; PH is *p*-hydroxybenzoic acid; PA is *p*-aminobenzoic acid; TP is terephthalic acid
Source: Harvey, D. T.; Byerly, S.; Bowman, A.; Tomlin, J. "Optimization of HPLC and GC Separations Using Re- sponse Surfaces," *J. Chem. Educ.* **1991**, *68*, 162–168.

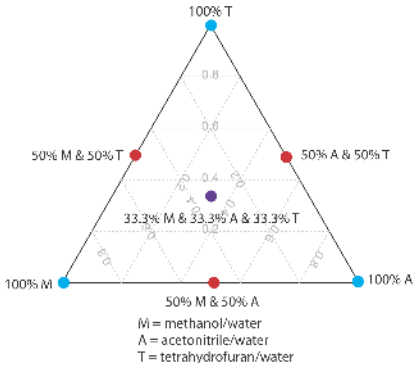


Figure 14.2.5. Solvent triangle for optimizing a reversed-phase HPLC separation. The three blue circles show mobile phases consisting of an organic solvent and water. The three red circles are binary mobile phases created by combining equal volumes of the pure mobile phases. The ternary mobile phase shown by the purple circle contains all three of the pure mobile phases.

One strategy for finding the best mobile phase is to use the solvent triangle shown in Figure **14.2.5**, which allows us to explore a broad range of mobile phases with only seven experiments. We begin by adjusting the amount of acetonitrile in the mobile phase to produce the best possible separation within the desired analysis time. Next, we use Table **14.2.3** to estimate the composition of methanol/H_2O and tetrahydrofuran/H_2O mobile phases that will produce similar analysis times. Four additional mobile phases are prepared using the binary and ternary mobile phases shown in Figure **14.2.4**. When we examine the chromatograms from these seven mobile phases we may find that one or more provides an adequate separation, or we may identify a region within the solvent triangle where a separation is feasible. Figure **14.2.6** shows a resolution map for the reversed-phase separation of benzoic acid, terephthalic acid, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid on a nonpolar C_{18} column in which the maximum desired analysis time is set to 6 min [Harvey, D. T.; Byerly, S.; Bowman, A.; Tomlin, J. *J. Chem. Educ.* **1991**, *68*, 162–168]. The areas in blue, green, and red show mobile phase compositions that do not provide baseline resolution. The unshaded area represents mobile phase compositions where a separation is possible.

> The choice to start with acetonitrile is arbitrary—we can just as easily choose to begin with methanol or with tetrahydrofuran.

Table **14.2.3**. Composition of Mobile Phases With Approximately Equal Solvent Strengths

%v/v CH_3OH	% v/v CH_3CN	%v/v THF
0	0	0
10	6	4
20	14	10
30	22	16
40	32	24
50	40	30
6	50	36
70	60	44
80	72	52
90	87	62
100	99	71

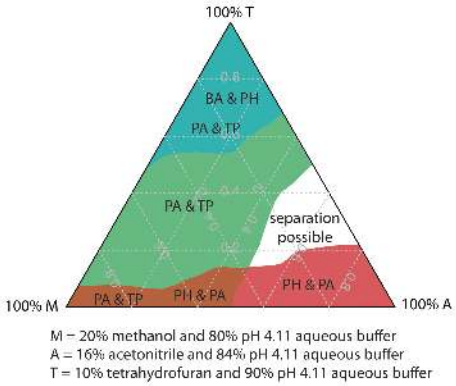


Figure 14.2.6. Resolution map for the separation of benzoic acid (BA), terephthalic acid (TP), *p*-aminobenzoic acid (PA), and *p*-hydroxybenzoic acid (PH) on a nonpolar C_{18} column subject to a maximum analysis time of 6 min. The shaded areas represent regions where a separation is not possible, with the unresolved solutes identified. A separation is possible in the unshaded area. See Harvey, D. T.; Byerly, S.; Bowman, A.; Tomlin, J. "Optimization of HPLC and GC Separations Using Response Surfaces," *J. Chem. Educ.* **1991**, *68*, 162–168 for details on the mathematical model used to generate the resolution map.

Choosing a Mobile Phase: Isocratic and Gradient Elutions

A separation using a mobile phase that has a fixed composition is an ***isocratic elution***. One difficulty with an isocratic elution is that an appropriate mobile phase strength for resolving early-eluting solutes may lead to unacceptably long retention times for late-eluting solutes. Optimizing the mobile phase for late-eluting solutes, on the other hand, may provide an inadequate separation of early-eluting solutes. Changing the mobile phase's composition as the separation progresses is one solution to this problem. For a reversed-phase separation we use an initial mobile phase that is more polar. As the separation progresses, we adjust the composition of mobile phase so that it becomes less polar (see Figure **14.2.7**). Such separations are called ***gradient elutions***.

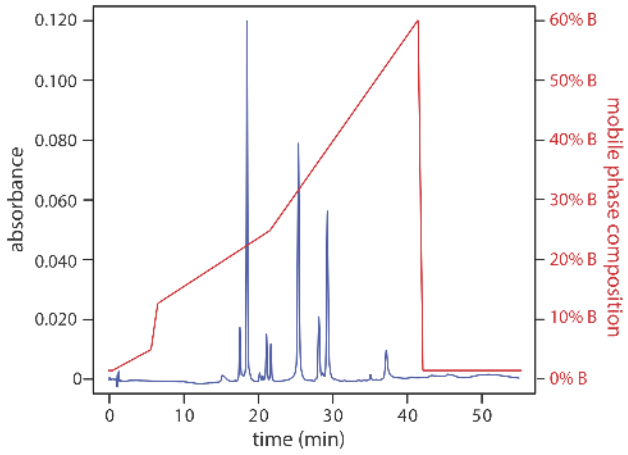


Figure 14.2.7. Gradient elution separation of a mixture of flavonoids. Mobile phase A is an aqueous solution of 0.1% formic acid and mobile phase B is 0.1% formic acid in acetonitrile. The initial mobile phase is 98% A and 2% B. The percentage of mobile phase B increases in four steps: from 2% to 5% over 5 min, beginning at 0.5 min; from 5% to 12% over 1 min, beginning at 5.5 min; from 12% to 25% over 15 min, beginning at 6.5 min; and from 25% to 60% over 20 min, beginning at 21.5 min. Data provided by Christopher Schardon, Kyle Meinhardt, and Michelle Bushey, Department of Chemistry, Trinty University.

HPLC Plumbing

In a gas chromatograph the pressure from a compressed gas cylinder is sufficient to push the mobile phase through the column. Pushing a liquid mobile phase through a column, however, takes a great deal more effort, generating pressures in excess of several hundred atmospheres. In this section we consider the basic plumbing needed to move the mobile phase through the column and to inject the sample into the mobile phase.

Moving the Mobile Phase

A typical HPLC includes between 1–4 reservoirs for storing mobile phase solvents. The instrument in Figure 14.2.1, for example, has two mobile phase reservoirs that are used for an isocratic elution or a gradient elution by drawing solvents from one or both reservoirs.

Before using a mobile phase solvent we must remove dissolved gases, such as N_2 and O_2, and small particulate matter, such as dust. Because there is a large drop in pressure across the column—the pressure at the column's entrance is as much as several hundred atmospheres, but it is atmospheric pressure at the column's exit—gases dissolved in the mobile phase are released as gas bubbles that may interfere with the detector's response. Degassing is accomplished in several ways, but the most common are the use of a vacuum pump or sparging with an inert gas, such as He, which has a low solubility in the mobile phase. Particulate materials, which may clog the HPLC tubing or column, are removed by filtering the solvents.

> Bubbling an inert gas through the mobile phase releases volatile dissolved gases. This process is called sparging.

The mobile phase solvents are pulled from their reservoirs by the action of one or more pumps. Figure **14.2.8** shows a close-up view of the pumps for the instrument in Figure 14.2.1. The working pump and the equilibrating pump each have a piston whose back and forth movement maintains a constant flow rate of up to several mL/min and provides the high output pressure needed to push the mobile phase through the chromatographic column. In this particular instrument, each pump sends its mobile phase to a mixing chamber where they combine to form the final mobile phase. The relative speed of the two pumps determines the mobile phase's final composition.

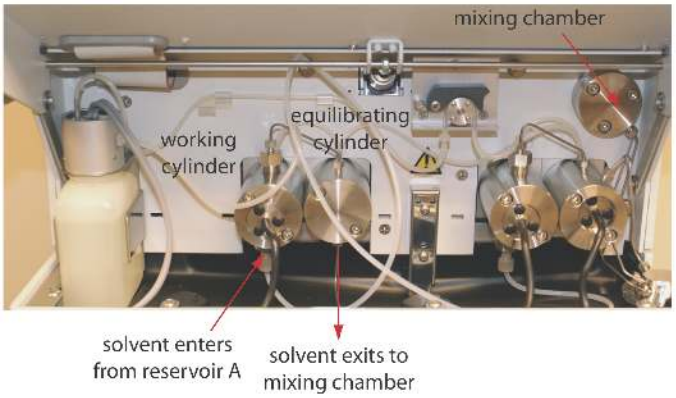


Figure 14.2.8. Close-up view of the pumps for the instrument shown in Figure 14.2.1. The working cylinder and the equilibrating cylinder for the pump on the left take solvent from reservoir A and send it to the mixing chamber. The pump on the right moves solvent from reservoir B to the mixing chamber. The mobile phase's flow rate is determined by the combined speeds of the two pumps. By changing the relative speeds of the two pumps, different binary mobile phases can be prepared.

The back and forth movement of a reciprocating pump creates a pulsed flow that contributes noise to the chromatogram. To minimize these pulses, each pump in Figure **14.2.8** has two cylinders. During the working cylinder's forward stoke it fills the equilibrating cylinder and establishes flow through the column. When the working cylinder is on its reverse stroke, the flow is maintained by the piston in the equilibrating cylinder. The result is a pulse-free flow.

> There are other possible ways to control the mobile phase's composition and flow rate. For example, instead of the two pumps in Figure **14.2.7**, we can place a solvent proportioning valve before a single pump. The solvent proportioning value connects two or more solvent reservoirs to the pump and determines how much of each solvent is pulled during each of the pump's cycles. Another approach for eliminating a pulsed flow is to include a pulse damper between the pump and the column. A pulse damper is a chamber filled with an easily compressed fluid and a flexible diaphragm. During the piston's forward stroke the fluid in the pulse damper is compressed. When the piston withdraws to refill the pump, pressure from the expanding fluid in the pulse damper maintains the flow rate.

Injecting the Sample

The operating pressure within an HPLC is sufficiently high that we cannot inject the sample into the mobile phase by inserting a syringe through a septum, as is possible in gas chromatography. Instead, we inject the sample using a ***loop injector***, a diagram of which is shown in Figure **14.2.9**. In the load position a sample loop—which is available in a variety of sizes ranging from 0.5 μL to 5 mL—is isolated from the mobile phase and open to the atmosphere. The sample loop is filled using a syringe with a capacity several times that of the sample loop, with excess sample exiting through the waste line. After loading the sample, the injector is turned to the inject position, which redirects the mobile phase through the sample loop and onto the column.

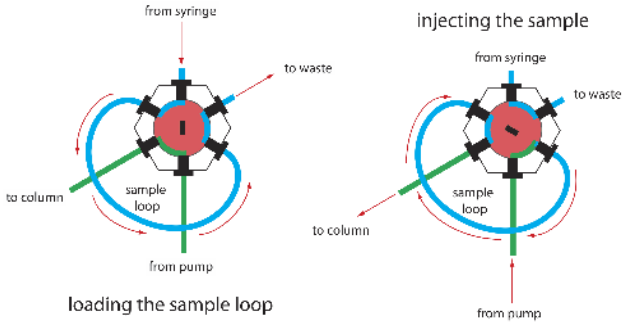


Figure 14.2.9. Schematic diagram of a manual loop injector. In the load position the flow of mobile phase from the pump to the column (shown in green) is isolated from the sample loop, which is filled using a syringe (shown in blue). Rotating the inner valve (shown in red) to the inject position directs the mobile phase through the sample loop and onto the column.

> The instrument in Figure 14.2.1 uses an autosampler to inject samples. Instead of using a syringe to push the sample into the sample loop, the syringe draws sample into the sample loop.

Detectors for HPLC

Many different types of detectors have been use to monitor HPLC separations, the most common encountered detectors are based on absorption or fluorescence. Other important detector types include refractive index detectors, light scattering detectors and amperometric electrochemical detectors. In addition, coupling a HPLC with a mass spectrometer through an electrospray, thermospray, or atmospheric pressure ionization interface leads to the creation of a very powerful instrument widely used in analytical and bioanalytical chemistry.

Spectroscopic Detectors

The most popular HPLC detectors take advantage of an analyte's UV/Vis absorption spectrum. These detectors range from simple designs, in which the analytical wavelength is selected using appropriate filters, to a modified spectrophotometer in which the sample compartment includes a flow cell. Figure **14.2.9** shows the design of a typical flow cell when using a diode array spectrometer as the detector. The flow cell has a volume of 1–10 μL and a path length of 0.2–1 cm.

> To review the details of how we measure absorbance, see Chapter 10.2. More information about different types of instruments, including the diode array spectrometer, is in Chapter 10.3.

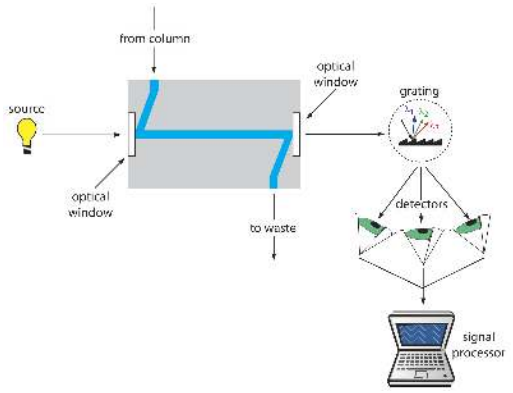


Figure 14.2.9. Schematic diagram of a flow cell for a detector equipped with a diode array spectrometer.

When using a UV/Vis detector the resulting chromatogram is a plot of absorbance as a function of elution time (see Figure **14.2.10**). If the detector is a diode array spectrometer, then we also can display the result as a three-dimensional chromatogram that shows absorbance as a function of wavelength and elution time. One limitation to using absorbance is that the mobile phase cannot absorb at the wavelengths we wish to monitor. Table 14.2.1 lists the minimum useful UV wavelength for several common HPLC solvents. Absorbance detectors provide detection limits of as little as 100 pg–1 ng of injected analyte.

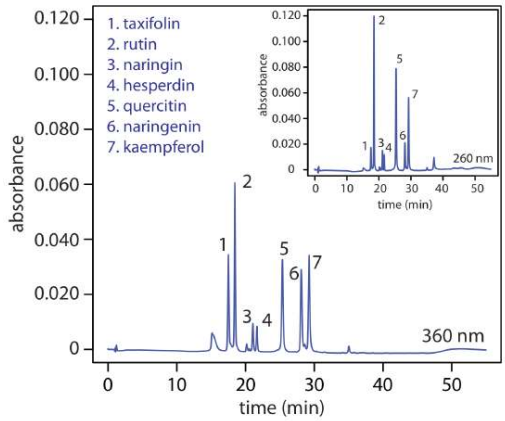


Figure 14.2.10. HPLC separation of a mixture of flavonoids with UV/Vis detection at 360 nm and, in the inset, at 260 nm. The choice of wavelength affects each analyte's signal. By carefully choosing the wavelength, we can enhance the signal for the analytes of greatest interest. Data provided by Christopher Schardon, Kyle Meinhardt, and Michelle Bushey, Department of Chemistry, Trinty University.

If an analyte is fluorescent, we can place the flow cell in a spectrofluorimeter. As shown in Figure **14.2.11**, a fluorescence detector provides additional selectivity because only a few of a sample's components are fluorescent. Detection limits are as little as 1–10 pg of injected analyte.

> See Chapter 10.6 for a review of fluorescence spectroscopy and spectrofluorimeters.

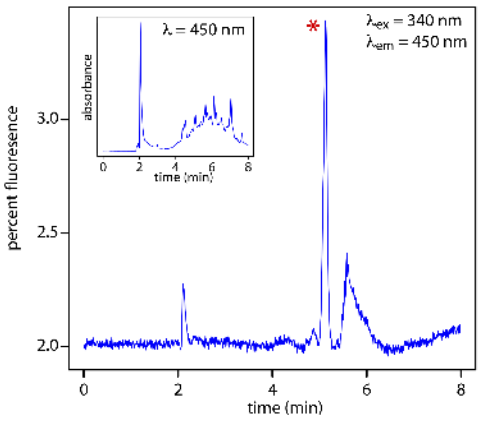


Figure 14.2.11. HPLC chromatogram for the determination of riboflavin in urine using fluorescence detection with exci-tation at a wavelength of 340 nm and detection at 450 nm. The peak corresponding to riboflavin is marked with a red asterisk (*). The inset shows the same chromatogram when using a less-selective UV/Vis detector at a wavelength of 450 nm. Data provided by Jason Schultz, Jonna Berry, Kaelene Lundstrom, and Dwight Stoll, Department of Chemistry, Gustavus Adolphus College.

Electrochemical Detectors

Another common group of HPLC detectors are those based on electrochemical measurements such as amperometry, voltammetry, coulometry, and conductivity. Figure **14.2.12**, for example, shows an amperometric flow cell. Effluent from the column passes over the working electrode—held at a constant potential relative to a downstream reference electrode—that completely oxidizes or reduces the analytes. The current flowing between the working electrode and the auxiliary electrode serves as the analytical signal. Detection limits for amperometric electrochemical detection are from 10 pg–1 ng of injected analyte.

> See Chapter 11.4 for a review of amperometry.

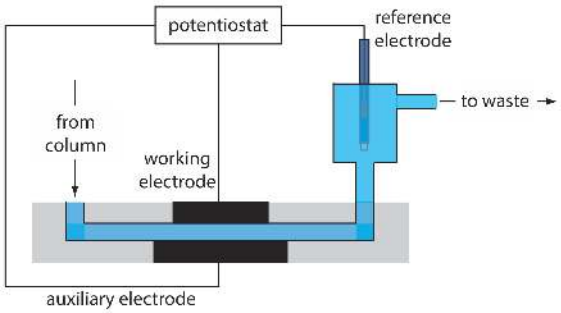


Figure 14.2.12. Schematic diagram showing a flow cell for an amperometric electrochemical detector.

Other Detectors

Several other detectors have been used in HPLC including the refractive index detector and the light scattering detector.

The refractive index detector

Measuring a change in the mobile phase's refractive index is analogous to monitoring the mobile phase's thermal conductivity in gas chromatography. A refractive index detector is nearly universal, responding to almost all compounds, but has a relatively poor detection limit of 0.1–1 μg of injected analyte. As in the thermal conductivity detector for GC, the refractive index of a small volume of mobile phase containing the eluting analyte is compared to the refractive index of the mobile phase alone (a static volume) . Consequently, an additional limitation of a refractive index detector is that it cannot be used for a gradient elution unless the mobile phase components have identical refractive indexes.

The light scattering detector

In accord with the Tyndall effect, all particles with diameters approaching the wavelength of light will scatter some of the light. Consequently a light scattering detector is a universal detector for macromolecular solutes such as synthetic polymers, proteins, DNA oligomers etc.. As shown in Figure **14.2.13**, solutes eluting off the column are converted into a fine mist of solvated particles

by a nebulizer and mixed with a drying gas, typically nitrogen. After traveling down the the detector, during which time solvent molecules are evaporating from the solute particles, the dry solute particles interact with a laser light beam, most commonly from a HeNe laser. Light scattered from the solute particles is detected with a photomultipler tube and the amount of scatterd light detected depends on the number of solute particles and their size. The light scattering detector has excellent sensitivity (0.2 ng/ml) but does requires the use of volatile mobile phase components.

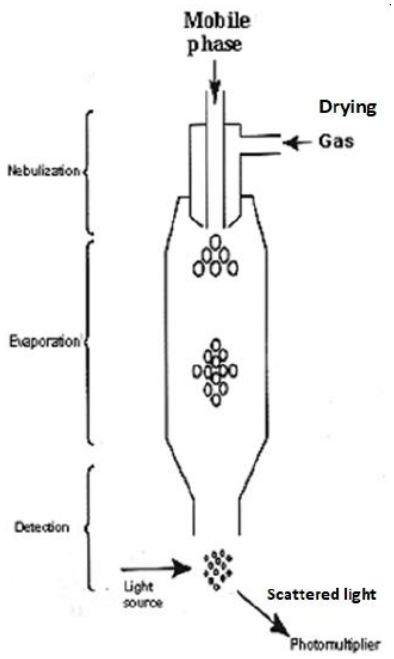


Figure **14.2.13**: A sketch of a light scattering detector. Image source currently unknown.

Another useful detector is a mass spectrometer. Figure **14.2.14** shows a block diagram of a typical HPLC–MS instrument. The effluent from the column enters the mass spectrometer's ion source using an interface the removes most of the mobile phase, an essential need because of the incompatibility between the liquid mobile phase and the mass spectrometer's high vacuum environment. In the ionization chamber the remaining molecules—a mixture of the mobile phase components and solutes—undergo ionization and fragmentation. The mass spectrometer's mass analyzer separates the ions by their mass-to-charge ratio (m/z). A detector counts the ions and displays the mass spectrum.

Interfacing a HPLC to a mass spectrometer proved to be a challenging problem because of the requirement of mass spectrometers to operate under high vacuum and solutions to this problem have been available only since the 1970's. The three most important interfaces developed to couple HPLC to a mass spectrometer are (1) the **atmospheric pressure ionization** interface developed by Horning, Carroll, and their co-works in the 1970's at the Baylor College of Medicine (Houston, TX) (2) the **thermospray** interface developed by Vestal and R. Blakley in the 1980's at the University of Houston (Houston, TX) and the electrospray ionization interface developed by Fenn and coworkers at Yale University (New Haven, CT) also in the 1980's. Each of these wil lbe presented in detail in the chapter on Molecula Mass Spectrometry.

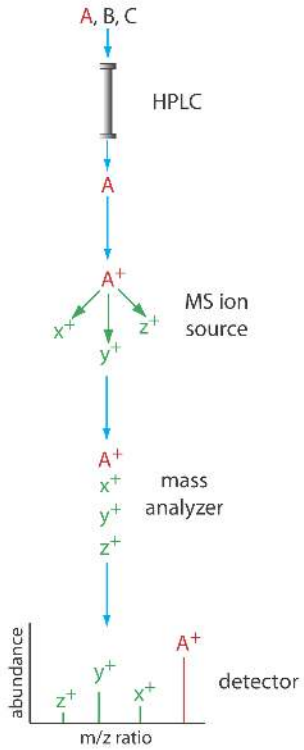


Figure 14.2.14. Block diagram of an HPLC–MS. A three component mixture enters the HPLC. When component A elutes from the column, it enters the MS ion source and ionizes to form the parent ion and several fragment ions. The ions enter the mass analyzer, which separates them by their mass-to-charge ratio, providing the mass spectrum shown at the detector.

There are several options for monitoring the chromatogram when using a mass spectrometer as the detector. The most common method is to continuously scan the entire mass spectrum and report the total signal for all ions reaching the detector during each scan. This total ion scan provides universal detection for all analytes. As seen in Figure **14.2.15**, we can achieve some degree of selectivity by monitoring only specific mass-to-charge ratios, a process called selective-ion monitoring.

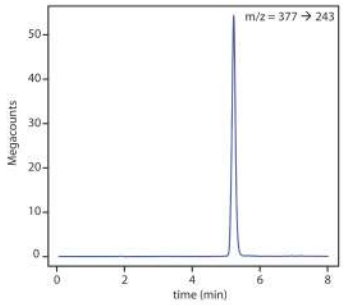


Figure 14.2.15. HPLC–MS/MS chromatogram for the determination of riboflavin in urine. An initial parent ion with an m/z ratio of 377 enters a second mass spectrometer where it undergoes additional 20 ionization; the fragment ion with an m/z ratio of 243 provides the signal. The selectivity of this detector is evident when you compare this chromatogram to the one in Figure 14.2.11, which uses fluoresence deterction. Data provided by Jason Schultz, Jonna Berry, Kaelene Lundstrom, and Dwight Stoll, Department of Chemistry, Gustavus Adolphus College.

The advantages of using a mass spectrometer in HPLC are the same as for gas chromatography. Detection limits are very good, typically 0.1–1 ng of injected analyte, with values as low as 1–10 pg for some samples. In addition, a mass spectrometer provides qualitative, structural information that can help to identify the analytes. The interface between the HPLC and the mass spectrometer is technically more difficult than that in a GC–MS because of the incompatibility of a liquid mobile phase with the mass spectrometer's high vacuum requirement.

> For more details on mass spectrometry see Introduction to Mass Spectrometry by Michael Samide and Olujide Akinbo, a resource that is part of the Analytical Sciences Digital Library.

Quantitative Applications

High-performance liquid chromatography is used routinely for both qualitative and quantitative analyses of environmental, pharmaceutical, industrial, forensic, clinical, and consumer product samples.

Preparing Samples for Analysis

Samples in liquid form are injected into the HPLC after a suitable clean-up to remove any particulate materials, or after a suitable extraction to remove matrix interferents. In determining polyaromatic hydrocarbons (PAH) in wastewater, for example, an extraction with CH_2Cl_2 serves the dual purpose of concentrating the analytes and isolating them from matrix interferents. Solid samples are first dissolved in a suitable solvent or the analytes of interest brought into solution by extraction. For example, an HPLC analysis for the active ingredients and the degradation products in a pharmaceutical tablet often begins by extracting the powdered tablet with a portion of mobile phase. Gas samples are collected by bubbling them through a trap that contains a suitable solvent. Organic isocyanates in industrial atmospheres are collected by bubbling the air through a solution of 1-(2-methoxyphenyl)piperazine in toluene. The reaction between the isocyanates and 1-(2-methoxyphenyl)piperazine both stabilizes them against degradation before the HPLC analysis and converts them to a chemical form that can be monitored by UV absorption.

Quantitative Calculations

A quantitative HPLC analysis is often easier than a quantitative GC analysis because a fixed volume sample loop provides a more precise and accurate injection. As a result, most quantitative HPLC methods do not need an internal standard and, instead, use external standards and a normal calibration curve.

> An internal standard is necessary when using HPLC–MS because the interface between the HPLC and the mass spectrometer does not allow for a reproducible transfer of the column's eluent into the MS's ionization chamber.

Example 14.2.2

The concentration of polynuclear aromatic hydrocarbons (PAH) in soil is determined by first extracting the PAHs with methylene chloride. The extract is diluted, if necessary, and the PAHs separated by HPLC using a UV/Vis or fluorescence detector. Calibration is achieved using one or more external standards. In a typical analysis a 2.013-g sample of dried soil is extracted with 20.00 mL of methylene chloride. After filtering to remove the soil, a 1.00-mL portion of the extract is removed and diluted to 10.00 mL with acetonitrile. Injecting 5 µL of the diluted extract into an HPLC gives a signal of 0.217 (arbitrary units) for the PAH fluoranthene. When 5 µL of a 20.0-ppm fluoranthene standard is analyzed using the same conditions, a signal of 0.258 is measured. Report the parts per million of fluoranthene in the soil.

Solution

For a single-point external standard, the relationship between the signal, S, and the concentration, C, of fluoranthene is

$$S = kC$$

Substituting in values for the standard's signal and concentration gives the value of k as

$$k = \frac{S}{C} = \frac{0.258}{20.0 \text{ ppm}} = 0.0129 \text{ ppm}^{-1}$$

Using this value for k and the sample's HPLC signal gives a fluoranthene concentration of

$$C = \frac{S}{k} = \frac{0.217}{0.0129 \text{ ppm}^{-1}} = 16.8 \text{ ppm}$$

for the extracted and diluted soil sample. The concentration of fluoranthene in the soil is

$$\frac{16.8 \text{ g/mL} \times \frac{10.00 \text{ mL}}{1.00 \text{ mL}} \times 20.00 \text{ mL}}{2.013 \text{ g sample}} = 1670 \text{ ppm fluoranthene}$$

Exercise 14.2.2

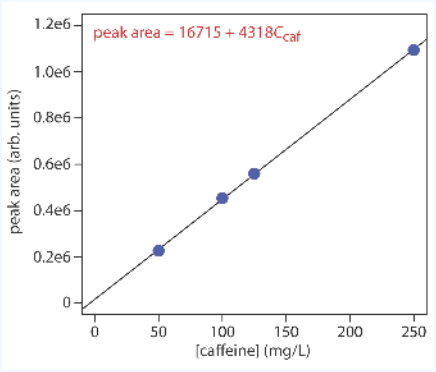
The concentration of caffeine in beverages is determined by a reversed-phase HPLC separation using a mobile phase of 20% acetonitrile and 80% water, and using a nonpolar C_8 column. Results for a series of 10-µL injections of caffeine standards are in the following table.

[caffeine] (mg/L)	peak area (arb. units)
50.0	226724
100.0	453762
125.0	559443
250.0	1093637

What is the concentration of caffeine in a sample if a 10-µL injection gives a peak area of 424195? The data in this problem comes from Kusch, P.; Knupp, G. "Simultaneous Determination of Caffeine in Cola Drinks and Other Beverages by Reversed-Phase HPTLC and Reversed-Phase HPLC," *Chem. Educator*, **2003**, *8*, 201–205.

Answer

The figure below shows the calibration curve and calibration equation for the set of external standards. Substituting the sample's peak area into the calibration equation gives the concentration of caffeine in the sample as 94.4 mg/L.



The best way to appreciate the theoretical and the practical details discussed in this section is to carefully examine a typical analytical method. Although each method is unique, the following description of the determination of fluoxetine in serum provides an instructive example of a typical procedure. The description here is based on Smyth, W. F. *Analytical Chemistry of Complex Matricies*, Wiley Teubner: Chichester, England, 1996, pp. 187–189.

Representative Method 12.5.1: Determination of Fluoxetine in Serum

Description of Method

Fluoxetine is another name for the antidepressant drug Prozac. The determination of fluoxetine in serum is an important part of monitoring its therapeutic use. The analysis is complicated by the complex matrix of serum samples. A solid-phase extraction followed by an HPLC analysis using a fluorescence detector provides the necessary selectivity and detection limits.

Procedure

Add a known amount of the antidepressant protriptyline, which serves as an internal standard, to each serum sample and to each external standard. To remove matrix interferents, pass a 0.5-mL aliquot of each serum sample or standard through a C_{18} solid-phase extraction cartridge. After washing the cartridge to remove the interferents, elute the remaining constituents, including the analyte and the internal standard, by washing the cartridge with 0.25 mL of a 25:75 v/v mixture of 0.1 M $HClO_4$ and acetonitrile. Inject a 20-µL aliquot onto a 15-cm × 4.6-mm column packed with a 5 µm C_8-bonded stationary phase. The isocratic mobile phase is

37.5:62.5 v/v acetonitrile and water (that contains 1.5 g of tetramethylammonium perchlorate and 0.1 mL of 70% v/v $HClO_4$). Monitor the chromatogram using a fluorescence detector set to an excitation wave- length of 235 nm and an emission wavelength of 310 nm.

Questions

1. The solid-phase extraction is important because it removes constitutions in the serum that might interfere with the analysis. What types of interferences are possible?

> Blood serum, which is a complex mixture of compounds, is approximately 92% water, 6–8% soluble proteins, and less than 1% each of various salts, lipids, and glucose. A direct injection of serum is not advisable for three reasons. First, any particulate materials in the serum will clog the column and restrict the flow of mobile phase. Second, some of the compounds in the serum may absorb too strongly to the stationary phase, degrading the column's performance. Finally, although an HPLC can separate and analyze complex mixtures, an analysis is difficult if the number of constituents exceeds the column's peak capacity.

2. One advantage of an HPLC analysis is that a loop injector often eliminates the need for an internal standard. Why is an internal standard used in this analysis? What assumption(s) must we make when using the internal standard?

> An internal standard is necessary because of uncertainties introduced during the solid-phase extraction. For example, the volume of serum transferred to the solid-phase extraction cartridge, 0.5 mL, and the volume of solvent used to remove the analyte and internal standard, 0.25 mL, are very small. The precision and accuracy with which we can measure these volumes is not as good as when we use larger volumes. For example, if we extract the analyte into a volume of 0.24 mL instead of a volume of 0.25 mL, then the analyte's concentration increases by slightly more than 4%. In addition, the concentration of eluted analytes may vary from trial-to-trial due to variations in the amount of solution held up by the cartridge. Using an internal standard compensates for these variation. To be useful we must assume that the analyte and the internal standard are retained completely during the initial loading, that they are not lost when the cartridge is washed, and that they are extracted completely during the final elution.

3. Why does the procedure monitor fluorescence instead of monitoring UV absorption?

> Fluorescence is a more selective technique for detecting analytes. Many other commonly prescribed antidepressants (and their metabolites) elute with retention times similar to that of fluoxetine. These compounds, however, either do not fluoresce or are only weakly fluorescent.

4. If the peaks for fluoxetine and protriptyline are resolved insufficiently, how might you alter the mobile phase to improve their separation?

> Decreasing the amount of acetonitrile and increasing the amount of water in the mobile will increase retention times, providing more time to effect a separation.

Evaluation

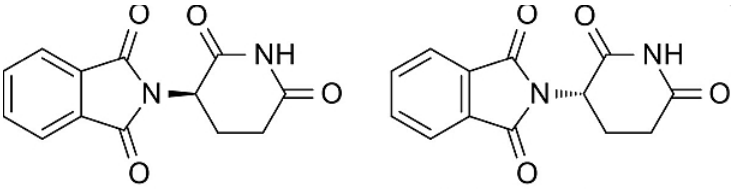
With a few exceptions, the scale of operation, accuracy, precision, sensitivity, selectivity, analysis time, and cost for an HPLC method are similar to GC methods. Injection volumes for an HPLC method usually are larger than for a GC method because HPLC columns have a greater capacity. Because it uses a loop injection, the precision of an HPLC method often is better than a GC method. HPLC is not limited to volatile analytes, which means we can analyze a broader range of compounds. Capillary GC columns, on the other hand, have more theoretical plates, and can separate more complex mixtures.

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14.3: Chiral Chromatography

The separation of enantiomers of chiral compounds by chromatographic methods and related techniques is one of the most important tasks in modern analytical chemistry, especially in the analysis of compounds of biological and pharmaceutical interest. While chiral separations can be accomplished using gas chromatography they are more often accomplished with liquid chromatography and capillary electrophoresis due to the focus on molecules of importance to the pharmeceutical industry.

When the enantiomers of a drug are administered into a chirally selective living system, these enantiomers often exhibit differences in bioavailability, distribution, metabolic and excretion behavior, and action. One of the enantiomers is often the more active stereoisomer for a given action (eutomer), while the other, less active one (distomer) may either contribute side-effects, display toxicity or act as an antagonist. The differences in biological properties of enantiomers arise from the differences in protein transport and binding, the kinetics of their metabolism and their stability in the environment. A tragic case in point is the racemic drug n-Thalidomide which was marketed in the the United Kingdom late 1950's as a sedative and with tragic effect. Exclusively the R-(+) enantiomer possesses therapeutic activity while the S-(+) enantiomer is teratogenic.



(R)-thalidomide **(S)-thalidomide**

As a result, the pharmaceutical industry has raised its emphasis on the generation of enantiomerically pure compounds in the search for safer and more effective drugs. Single enantiomers can be obtained via (a) the selective synthesis of one enantiomer or (b) the separation of racemic mixtures Stereoselective syntheses are rarely selected for largescale separations, particularly at the early stages of development of new drugs in the pharmaceutical industry because they are both expensive and time-consuming. Yet, These methods yield the enantiomerically pure substances and are generally the most advantageous and are a major focus of modern synthetic chemistry. The enantiomers of a racemic mixture can be separated indirectly when diastereomer pairs are formed covalently; their separation can be achieved by taking advantage of their different chemical or physical properties on crystallization, nonstereoselective chromatography or distillation. Alternatively, direct processes are based on the formation of noncovalent diastereomeric pairs of molecules. The direct resolution of enantiomers can be achieved by interaction of a racemic mixture with a chiral selector, either a part of a chiral stationary phase (CSP) or as a chiral mobile phase additive (CMA).

Indirect chromatographic methods

The application of chiral derivatizing agents (CDAs) to produce diastereomer pairs with appropriate separation and detection possibilities was the first method widely used for the enantioseparation of optically active molecules in liquid chromatography. Labeling with CDAs is carried out by reaction with a functional group in the analyte, e.g. amine, carboxy, carbonyl, hydroxy or thiol.

Among various functional groups, the tagging reactions of primary and secondary amines are unquestionably of major significance. The derivatization reactions for chiral amines are mainly based on the formation of amides, carbamates, ureas and thioureas. Only the most important members of the CDA types are shown in Figure **14.3.1**.

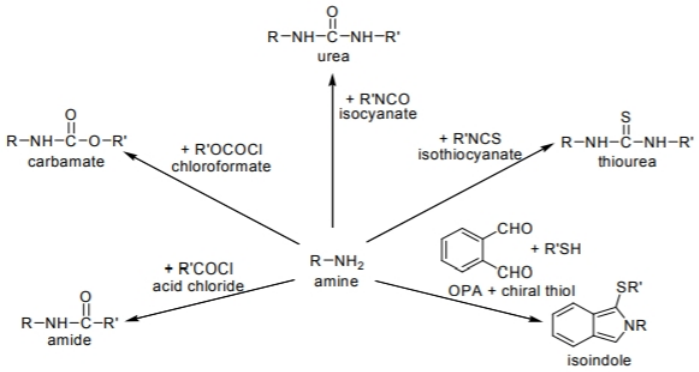


Figure **14.3.1**: Some examples of chiral derivatization reactions for amino groups (both R and R' contain a chiral center) leading to the formation of diastereomer pairs for each solute enantiomer.

Advantages of indirect methods for chiral separations include: 1. Good chromatographic properties of derivatives 2. Elution sequence predictable 3. Good chromophoric or fluorophoric properties of the reagent (enhanced sensitivity can be achieved) 4. Low cost of achiral columns 5. Method development is simple and 6. Selectivity can be increased (better separation is often achieved than with a direct method). Disadvantages include: 1. The purity of the CDA is critical and 2. The excess of reagent and side-products may interfere with the separation.

Direct Chromatographic Methods

Chiral Mobile Phase Additives (CMAs)

Enantiomers can be resolved on conventional achiral stationary phases by adding an appropriate chiral selector to the mobile phase. These additives can be cyclodextrins, chiral crown ethers, chiral counter-ions or chiral ligands which are capable of forming ternary complexes with the solute enantiomers in the presence of a transition metal ion.

Chiral Stationary Phases

The 1980s proved to be a major turning-point in the field of enantiomer separation in HPLC. A tremendous number of new and improved CSPs were introduced and accompanied by a corresponding increase in the number of publications in this area. CSPs can be grouped in several ways. Depending on their separation principles, the main classes are as follows: Pirkle CSPs, helical polymers, mainly cellulose and amylose, cavity phases, macrocyclic antibiotic phases and protein- and ligand-exchange phases.

Pirkle Phases

The first commercial chiral stationary phase was developed in W. Pirkle's laboratory at the University of Illinois in the 19080's. This chiral stationary phase was based on immobilized (R)-2,2,2-trifluoro-1-(9-anthryl)ethanol (previously used in magnetic resonance studies) on a silica support. This CSP was shown to be useful for the separation of enantiomers of several π-acidic racemates, e.g. 3,5-dinitrobenzoyl (DNB) derivatives of amines, amino acids, amino alcohols, etc. *pi*-Basic phases such as 1-aryl-1-aminoalkanes, N-arylamino esters, phthalides, phospine oxides, etc. contain a *pi*-electrondonor group and are expected to interact and resolve compounds bearing a π-electronacceptor group. Typically, DNB and 3,5-dinitrophenylurea derivatives of amino acids and amines were resolved on these CSPs. Among the DNB derivatives, phenylglycine exhibited large separation factors. This observation led to the investigation of DNB phenylglycine as a *pi*-acid-based CSP. Finally, the Pirkle laboraotry designed CSPs containing both *pi*-acidic and *pi*-basic sites [63]. These phases were expected to allow the efficient resolution of compounds containing appropriately located *pi*-acidic and *pi*-basic moieties. Pirkle and his coworkers commercialized many of their CSP sold through Regis Technologies (registech.com) where CSPs such as the very versatile Welk-O 1, shown in Figure **14.3.2** are named after the co-worker that developed the CSP.

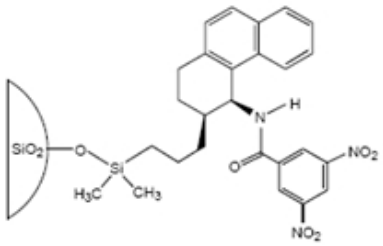


Figure 14.3.2: The Whelk-O 1 chrial stationary phase sold by Regis Technolgies. Image from registech.com.

CSPs based on polysaccharides

Polysaccharides (especially cellulose) are naturally-occurring polymers; their derivatives were found to exhibit the ability of chiral recognition as CSPs. Cellulose is a crystalline polymer composed of linear poly-β-D-1,4-glucoside residues which form a helical structure. Although crystalline cellulose displays chiral recognition, it does not yield practical CSPs. The reason for this is the poor resolution, and broad peaks are obtained due to slow mass transfer and slow diffusion through the polymer network. The highly polar hydroxy groups of cellulose often lead to nonstereoselective binding with the enantiomers of the analyte. Additionally, cellulose is unable to withstand normal HPLC pressures.

Derivatization of cellulose brings about practically useful CSPs which can separate a wide range of racemic compounds with high selectivities. Among the derivatives, cellulose tris-4-methylbenzoate and tris-3,5-dimethylphenyl carbamate, shown in Figure 14.3.3vatives containing both an electron-donating and an electron-withdrawing group on the phenyl moiety were prepared to perfect their chiral recognition abilities.

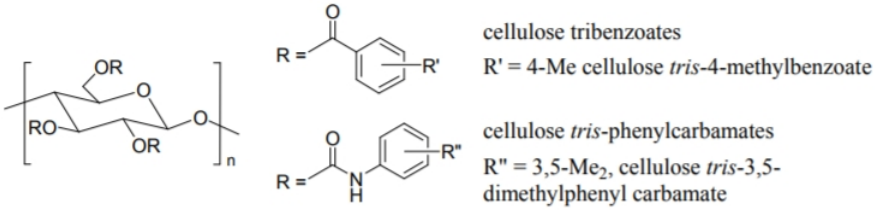


Figure 14.3.3: Two examples of derrivatized polysaccaride CSPs based on celluose.

Cavity phases

Chiral separations based on inclusion are achieved through a mechanism by which the guest molecule is accepted into the cavity in a host molecule. The exterior of the host molecule generally possesses functional groups that act as barriers or interact with the guest molecule in a fashion that includes enantioselectivity. The most often used CSP of this type is CD bound to a silica support.

CDs are cyclic oligomers with 6 (α-CD), 7 (β-CD) or 8 (γ-CD) D-glucopyranose units through an (α-1,4- linkage that adopt a tapered cylindrical or toroidal shape (see Figure 14.3.4). The toroid has a maximum diameter ranging from 5.7 Å (α-CD) to 9.5 Å (γ-CD) with a depth of about 7 Å. The exterior of the toroid is relatively hydrophilic because of the presence of the primary C-6 hydroxy groups at the smaller rim of the toroid and the secondary C-2 and C-3 hydroxy groups at the opposite end. The internal cavity is hydroxy-free and hydrophobic, which favors the enantioseparation of partially nonpolar compounds via selective inclusion. The modification of CDs and their complexation behavior involves substitution of one or more of the C-2, C-3 and C-6 hydroxy groups. The most commonly used derivatized CDs are sulfated, acetylated, permethylated, perphenylated, 2-hydroxypropylated, 3,5- dimethylcarbamoylated, and naphthylethyl-carbamoylated [73] CDs. Most of the studies involving CDs as CSPs in HPLC were accomplished in the RP mode, in the NP mode or in the polar-organic (PO) mode. Thus, CD-bonded CSPs are regarded as multi-modal phases.

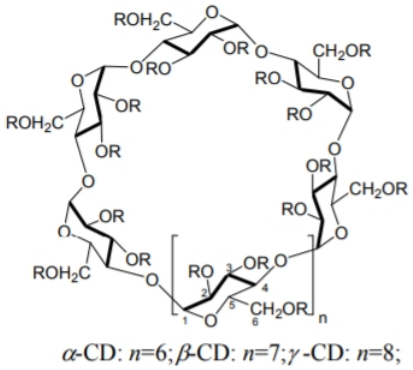


Figure 14.3.4: Structures of α-, β- and γ-CDs

A nother group of cavity based CSPs are formed using crown ethers, heteroatomic macrocycles with repeating (-X-C_2H_4-) units, where X, the heteroatom, is commonly oxygen, but may also be a sulfur or nitrogen atom. an example crown ether compound is shown in Figure 14.3.5. Unlike CDs, the host-guest interaction within the chiral cavity is hydrophilic in nature. Crown ethers, and especially 18-crown-6 ethers, can complex inorganic cations and alkylammonium compounds. This inclusion interaction is based mainly on H-bonding between the hydrogens of the ammonium group and the heteroatom of the crown ether. Additional electrostatic interaction occurs between the nitrogen and the crown ether's oxygen lone pair electrons.

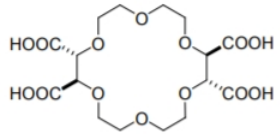


Figure 14.3.5: The structure of (+)-(18-crown-6)-2,3,11,12- tetracarboxylic acid.

Macrocyclic Antibiotic Phases

Macrocyclic antibiotics have proved to be an exceptionally useful class of chiral selectors for the separation of enantiomers of biological and pharmaceutical importance by means of HPLC, TLC and CE. The macrocyclic antibiotics are covalently bonded to silica gel via chains of different linkages, such as carboxylic acid, amine, epoxy or isocyanate-terminated organosilanes. This kind of attachment ensures the stability of the chiral selectors, while their chiral recognition properties are retained. All macrocyclic antibiotic stationary phases are multimodal CSPs, i.e. they can be used in normal phase, reverse phase, polar organic or polar-ionic separations. The antibiotics used for chiral separations in HPLC include the ansamycins (rifamycins), the glycopeptides (avoparcin, teicoplanin, ristocetin A, vancomycin and their analogs) and the polypeptide antibiotic thiostrepton. One of the most frequently used teicoplanin, Figure 14.3.6, consists of four fused macrocyclic rings, which contain seven aromatic rings, two of them bearing a chlorine substituent, and the others having a phenolic character.

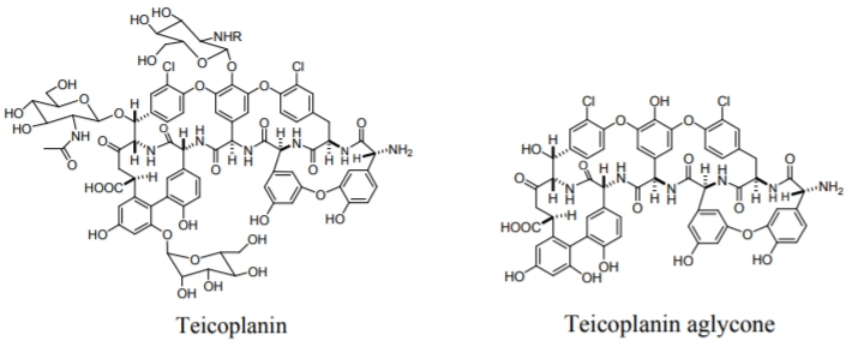


Figure 14.3.6: Structures of the macrocyclic glycopeptide teicoplanin and teicoplanin aglycone.

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14.4: Ion Chromatography

In ion-exchange chromatography (IEC) the stationary phase is a cross-linked polymer resin, usually divinylbenzene cross-linked polystyrene, with covalently attached ionic functional groups. As shown in Figure 1 for a styrene–divinylbenzene co-polymer modified for use as an ion-exchange resin, the ion-exchange sites—indicated here by R and shown in blue—are mostly in the *para* position and are not necessarily bound to all styrene units. The cross-linking is shown in red. The counterions to these fixed charges are mobile and can be displaced by ions that compete more favorably for the exchange sites.

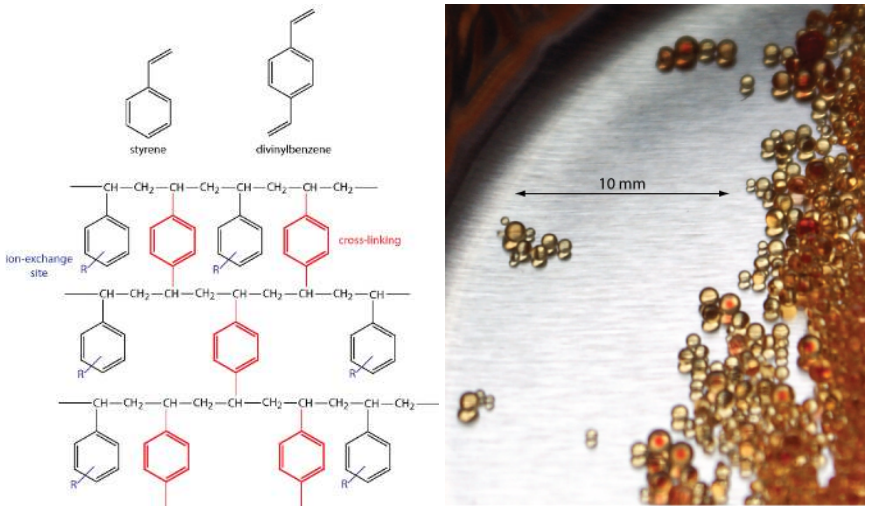


***Figure 1**: (left) styrene–divinylbenzene co-polymer modified for use as an ion-exchange resin. (right) The photo here shows an example of ion-exchange polymer beads. These beads are approximately 0.30–0.85 mm in diameter. Resins for use in ion-exchange chromatography are smaller, typically 5–11 mm in diameter.*

Imagine if we had a tube whose surfaces were coated with an immobilized cation. These would have electrostatic attraction for anions. If a solution containing a mixture of positively and negatively charged groups flows through this tube, the anions would preferentially bind, and the cations in the solution would flow through (Figure 2).

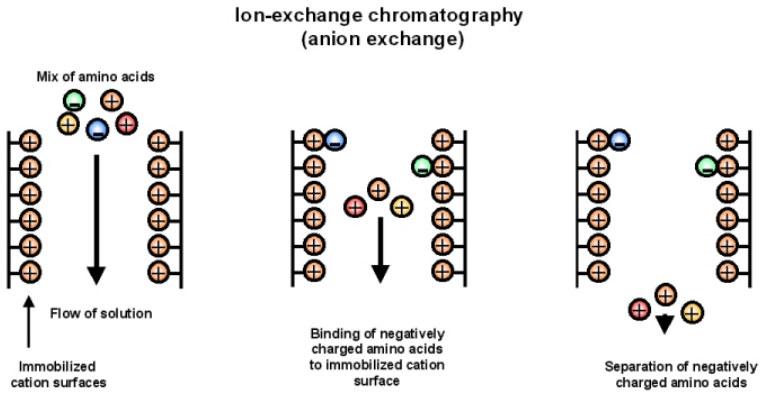


***Figure 2**: Separating action of amino acids in an anion exchange column.*

- This is the basis of ion exchange chromatography. The example above is termed "anion exchange" because the inert surface is interacting with anions
- If the immobile surface was coated with anions, then the chromatography would be termed "cation exchange" chromatography (and cations would selectively bind and be removed from the solution flowing through
- Strength of binding can be affected by pH, and salt concentration of the buffer. The ionic species "stuck" to the column can be removed (i.e. "eluted") and collected by changing one of these conditions. For example, we could lower the pH of the buffer and protonate anions. This would eliminate their electrostatic attraction to the immobilized cation surface. Or, we could increase the salt concentration of the buffer, the anions in the salt would "compete off" bound anions on the cation surface.

A common detector used exclusively in liquid chromatography for ion chromatography is the conductivity detector. In this detector two closely spaced electrodes are placed after the separation column and changes in the conductivity of the solution passing between the two electrodes is monitored. In order to improve the sensitivity of this simple detector and ion suppressor is typically placed between the separation column and the conductivity detector. Pictures in Figure 3 is a schematic of an ion suppressor used , in this example for improving the detectability of a series of anions, X-.

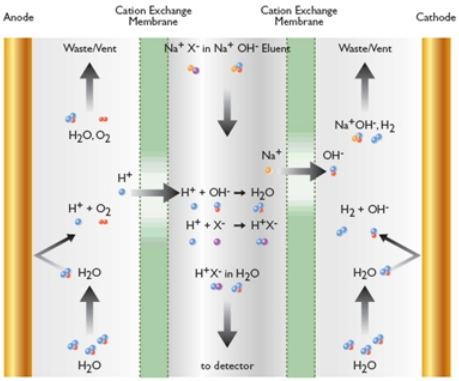


Figure 3. An ion suppressor used in ion chromatography. Image source currently unknown

As evident in Figure 3 an ion suppressor is based on simple acid base neutralization chemistry, i.e. $H^+ + OH^- \rightarrow H_2O$ or $2H^+ + CO_3^{2-} \rightarrow H_2CO_3$. In the example in Figure 3, hydronium ions generated at the anode of an electroylsis cell pass through the ion exchange membrane where sodium ions exit due to charge balance and the hydronium ions neutralize the hydroxide ions in the mobile phase, thus reducing the net ion concentration and conductivity of the solution passing to the conductivity detector.

An example of the effect of an ion suppressor is illustrated in Figure 4 for a separation of a series of anions.

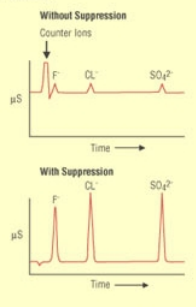
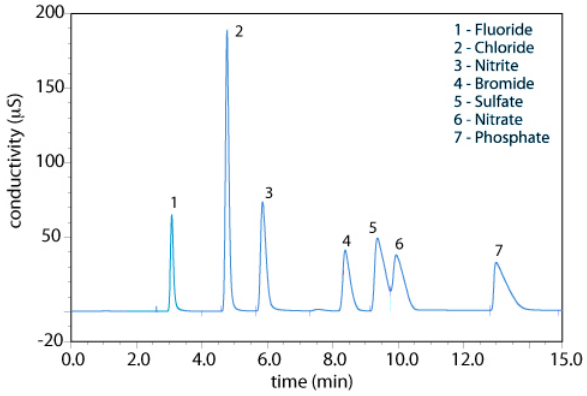


Figure 5: An example of the expected improvement in detectability in the separation of a series of anions with an ion suppressor. Image source currently unknown.

Ion-exchange chromatography is an important technique for the analysis of anions and cations in environmental samples. For example, an IEC analysis for the anions F^-, Cl^-, Br^-, NO_2^-, NO_3^-, PO_4^{3-}, and SO_4^{2-} takes approximately 15 minutes. A complete analysis of the same set of anions by a combination of potentiometry and spectrophotometry requires 1–2 days.



Contributors and Attributions

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14.5: Size-Exclusion Chromatography

Steric exclusion chromatography is a technique that separates compounds solely on the basis of size. In order for the results of steric exclusion separations to be meaningful, there can be no directed forces between the compounds being separated and the surface of the particles used as the stationary phase. Instead, the particles are prepared with well-characterized pore sizes. Figure 50 shows a particle with one pore in it. Figure 50 also shows representations for several molecules of different size (note, the pore size and molecule size are not representative of the scale that would exist in real steric exclusion phases – the pore is bigger than would actually occur and the molecules would never have sizes on the order of that of the particle).

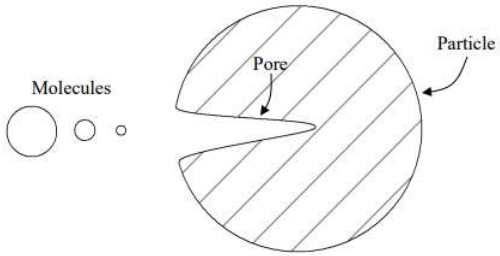
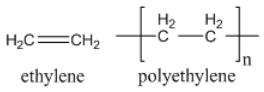


Figure 50. *Representation of a particle with a pore.*

The smallest molecule is small enough to fit entirely into all regions of the pore. The largest molecule is too big to fit into the pore. The intermediate sized one can only sample some of the pore volume. Provided these molecules have no interaction with the surface of the particle and are only separated on the basis of how much of the pore volume they can sample, the largest molecule would elute first from the column and the smallest one last.

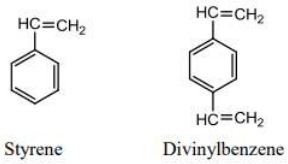
Suppose we took a molecule that was even larger than the biggest one in the picture above. Note that it would not fit into any of the pores either, and would elute with the biggest in the picture. If we wanted to separate these large species, we would need a particle with even larger pores. Similarly, if we took a smaller molecule than the smallest pictured above, it would sample all of the pore volume and elute with the smallest one pictured above. To separate these two compounds, we would need particles with smaller pores.

Steric exclusion chromatography requires large compounds, and is not generally effective on things with molecular weights of less than 1000. It is commonly used in biochemistry for the separation of proteins and nucleic acids. It is also used for separation or characterization purposes in polymer chemistry (a polymer is a large compound prepared from repeating monomeric units – polyethylene is a long, linear polymer made of repeating ethylene units).



One critical question is how to remove the possibility of an attractive force between the compounds being separated and the surface of the porous particles. The way this is done is to use a particle with very different properties than the compound being separated, and to use a solvent that the solutes are very soluble in. For example, if we want to separate proteins or nucleic acids, which are polar and very soluble in water, we would need to use porous particles made from an organic material that had a relatively non-polar surface. If we wanted to separate polyethylenes of different chain lengths, which are non-polar, we would dissolve the polyethylene in a non-polar organic solvent and use porous particles made from a material that had a highly polar surface. This might involve the use of a polydextran (carbohydrate), which has hydroxyl groups on the surface of the pores.

The classic organic polymer that has been used to prepare porous particles for the steric exclusion separation of water-soluble polymers is a co-polymer of styrene and divinylbenzene.



If we just had styrene, and conducted a polymerization, we would get a long, single-bonded chain of carbon atoms with phenyl rings attached to it. Linear polystyrenes are soluble in certain non-polar organic solvents. The divinylbenzene acts as a cross-linking agent that bridges individual styrene chains (Figure 51). A typical ratio of styrene to divinylbenzene is 12:1. The cross-linking serves to make the polymer into particles rather than linear chains. The cross-linked polymer is an insoluble material with a very high molecular weight. By controlling the reaction conditions including the amount of cross-linker and rate of reaction, it is possible to make polystyrenes with a range of pore sizes.

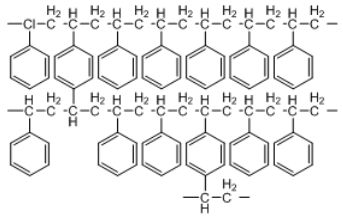


Figure 51. *Cross-linked styrene-divinylbenzene copolymer.*

If we think about what is inside the column, there are four important volume terms to consider. One is the total volume of the column (V_T). The second is the interstitial volume between the particles (V_I). The third is the volume of all of the pores (V_P). The last one is the actual volume occupied by the mass of the material that makes up the particles.

It turns out that V_I is usually about 0.3 of V_T for a column. There is simply no way to crunch the particles closer together to reduce this term appreciably.

$$V_I = 0.3V_T \tag{14.5.1}$$

It turns out that the maximum pore volume that can be achieved is about 0.4 of V_T. If you make this larger, the particles have more pores than structure, become excessively fragile, and get crushed as you try to push a liquid mobile phase through the column.

$$V_P = 0.4V_T \tag{14.5.2}$$

Totaling this up, we can write that:

$$V_I + VP = 0.7V_T \tag{14.5.3}$$

Since there are no directed forces in a steric exclusion column, this means that all of the separation must happen in one column volume, or in 0.7 V_T. If we then showed a chromatogram for a series of compounds being separated on a steric exclusion, it might look like that shown in Figure 52.

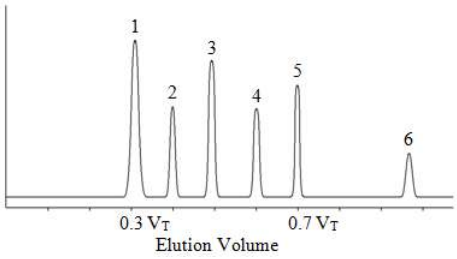


Figure 52. *Representation of a chromatogram on a steric exclusion column.*

The peak labeled 1, which has the shortest retention time (0.3 V_T), corresponds to all molecules in the sample that had a size that was too large to fit into the pores. It cannot be known whether this is one compound or a mixture of large compounds. The peak labeled 5, which has the longest retention time (0.7 V_T), corresponds to all molecules in the sample that had a size small enough to sample all of the pore volume. It cannot be known whether it is one compound or a mixture of two or more small compounds. The peaks labeled 2 through 4 sample some of the pore volume depending on their size. The peak labeled 6 elutes with a retention volume that is greater than 0.7 V_T. How would we explain the elution volume of this peak? The only way we can do so is if there are attractive forces between this molecule and the surface of the particles. This is a serious problem and we would need to examine this system and eliminate the cause of this attractive force.

Note that this method separates things on the basis of their size. What people try to do when using steric exclusion chromatography is equate size with molecular weight. The two representations for the shape of a molecule shown in Figure 53 point out a potential problem with equating size with molecular weight. One molecule is spherical. The other is rod shaped. The spherical molecule probably has a larger molecular weight, because it does occupy more volume. However, the size of a molecule is determined by what is known as its hydrodynamic radius. This is the volume that the molecule sweeps out in space as it tumbles. If you took the rod shaped molecule below and allowed it to tumble, you would see that its size is essentially comparable to that of the spherical molecule.

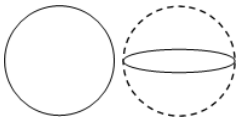


Figure 53. *Representation of the molecular size of spherical (left) and rod-shaped (right) molecules.*

When performing steric exclusion chromatography, you need to use a series of molecular weight standards. It is essential that these standards approximate the molecular features and shapes of the molecules you are analyzing. If you are analyzing proteins, you need to use proteins as standards. The same would apply to nucleic acids, or to particular organic polymers. Figure 54 shows the typical outcome of the calibration of elution time with molecular weight (always plotted on a log scale) for a steric exclusion column.

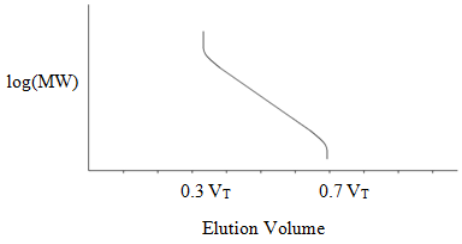


Figure 54. *Generalized calibration curve for a steric exclusion column.*

Note that excessively large molecules never enter the pores and elute together at 0.3 V_T. Excessively small molecules sample all of the pore volume and elute at 0.7 V_T. There is then some range of size (or molecular weight) where the compounds sample some of the pore volume and elute at volumes between 0.3 and 0.7 V_T.

The plots that would occur for particles with the next larger (dashed line) and smaller pore sizes (dotted line) are shown in Figure 55.

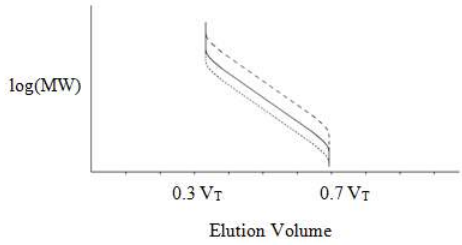


Figure 55. *Calibration curves for a steric exclusion column. The dashed line is the column with the largest pore sizes. The dotted line is the column with the smallest pore sizes.*

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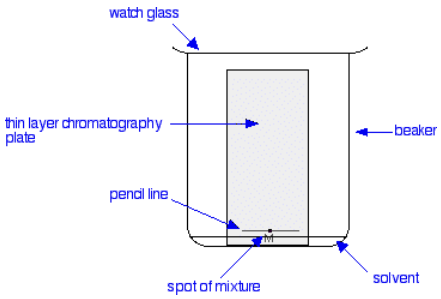
- **Steric Exclusion Chromatography** by Thomas Wenzel is licensed CC BY-NC-SA 4.0. Original source: https://asdlib.org.

14.6: Thin-Layer Chromatography

Chromatography is used to separate mixtures of substances into their components. All forms of chromatography work on the same principle. They all have a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rates.

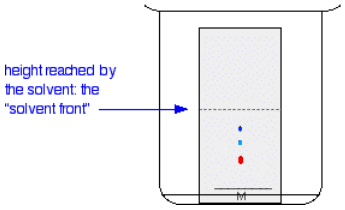
Thin layer chromatography is done exactly as it says - using a thin, uniform layer of silica gel or alumina coated onto a piece of glass, metal or rigid plastic. The silica gel (or the alumina) is the stationary phase. The stationary phase for thin layer chromatography also often contains a substance which fluoresces in UV light - for reasons you will see later. The mobile phase is a suitable liquid solvent or mixture of solvents.

We'll start with a very simple case - just trying to show that a particular dye is in fact a mixture of simpler dyes.



A pencil line is drawn near the bottom of the plate and a small drop of a solution of the dye mixture is placed on it. Any labelling on the plate to show the original position of the drop must also be in pencil. If any of this was done in ink, dyes from the ink would also move as the chromatogram developed. When the spot of mixture is dry, the plate is stood in a shallow layer of solvent in a covered beaker. It is important that the solvent level is below the line with the spot on it.

The reason for covering the beaker is to make sure that the atmosphere in the beaker is saturated with solvent vapor. To help this, the beaker is often lined with some filter paper soaked in solvent. Saturating the atmosphere in the beaker with vapor stops the solvent from evaporating as it rises up the plate. As the solvent slowly travels up the plate, the different components of the dye mixture travel at different rates and the mixture is separated into different coloured spots.

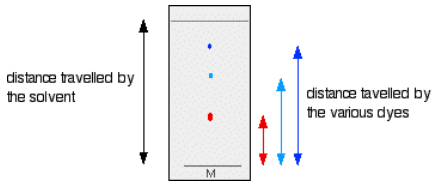


The diagram shows the plate after the solvent has moved about half way up it. The solvent is allowed to rise until it almost reaches the top of the plate. That will give the maximum separation of the dye components for this particular combination of solvent and stationary phase.

Measuring R_f values

If all you wanted to know is how many different dyes made up the mixture, you could just stop there. However, measurements are often taken from the plate in order to help identify the compounds present. These measurements are the distance traveled by the solvent, and the distance traveled by individual spots. When the solvent front gets close to the top of the plate, the plate is removed from the beaker and the position of the solvent is marked with another line before it has a chance to evaporate.

These measurements are then taken:



The R_f value for each dye is then worked out using the formula:

$$R_f = \frac{\text{distance traveled by sample}}{\text{distance traveled by solvent}}$$

For example, if the red component traveled 1.7 cm from the base line while the solvent had traveled 5.0 cm, then the R_f value for the red dye is:

$$R_f = \frac{1.7}{5.0}$$
$$= 0.34$$

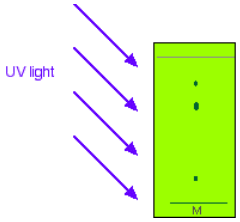
If you could repeat this experiment under exactly the same conditions, then the R_f values for each dye would always be the same. For example, the R_f value for the red dye would always be 0.34. However, if anything changes (the temperature, the exact composition of the solvent, and so on), that is no longer true. You have to bear this in mind if you want to use this technique to identify a particular dye. We'll look at how you can use thin layer chromatography for analysis further down the page.

What if the substances you are interested in are colorless?

There are two simple ways of getting around this problem.

Using fluorescence

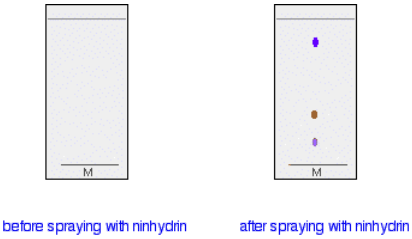
You may remember that I mentioned that the stationary phase on a thin layer plate often has a substance added to it which will fluoresce when exposed to UV light. That means that if you shine UV light on it, it will glow. That glow is masked at the position where the spots are on the final chromatogram - even if those spots are invisible to the eye. That means that if you shine UV light on the plate, it will all glow apart from where the spots are. The spots show up as darker patches.



While the UV is still shining on the plate, you obviously have to mark the positions of the spots by drawing a pencil circle around them. As soon as you switch off the UV source, the spots will disappear again.

Showing the spots up chemically

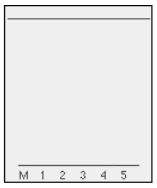
In some cases, it may be possible to make the spots visible by reacting them with something which produces a coloured product. A good example of this is in chromatograms produced from amino acid mixtures. The chromatogram is allowed to dry and is then sprayed with a solution of ninhydrin. Ninhydrin reacts with amino acids to give coloured compounds, mainly brown or purple.



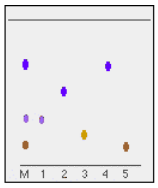
In another method, the chromatogram is again allowed to dry and then placed in an enclosed container (such as another beaker covered with a watch glass) along with a few iodine crystals. The iodine vapor in the container may either react with the spots on the chromatogram, or simply stick more to the spots than to the rest of the plate. Either way, the substances you are interested in may show up as brownish spots.

Using thin layer chromatography to identify compounds

Suppose you had a mixture of amino acids and wanted to find out which particular amino acids the mixture contained. For simplicity we'll assume that you know the mixture can only possibly contain five of the common amino acids. A small drop of the mixture is placed on the base line of the thin layer plate, and similar small spots of the known amino acids are placed alongside it. The plate is then stood in a suitable solvent and left to develop as before. In the diagram, the mixture is M, and the known amino acids are labelled 1 to 5.



before spraying with ninhydrin



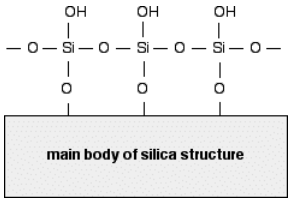
after spraying with ninhydrin

The left-hand diagram shows the plate after the solvent front has almost reached the top. The spots are still invisible. The second diagram shows what it might look like after spraying with ninhydrin. There is no need to measure the R_f values because you can easily compare the spots in the mixture with those of the known amino acids - both from their positions and their colours. In this example, the mixture contains the amino acids labelled as 1, 4 and 5. And what if the mixture contained amino acids other than the ones we have used for comparison? There would be spots in the mixture which didn't match those from the known amino acids. You would have to re-run the experiment using other amino acids for comparison.

How does thin layer chromatography work?

The stationary phase - silica gel

Silica gel is a form of silicon dioxide (silica). The silicon atoms are joined via oxygen atoms in a giant covalent structure. However, at the surface of the silica gel, the silicon atoms are attached to -OH groups. So, at the surface of the silica gel you have Si-O-H bonds instead of Si-O-Si bonds. The diagram shows a small part of the silica surface.



The surface of the silica gel is very polar and, because of the -OH groups, can form hydrogen bonds with suitable compounds around it as well as van der Waals dispersion forces and dipole-dipole attractions.

The other commonly used stationary phase is alumina - aluminium oxide. The aluminium atoms on the surface of this also have -OH groups attached. Anything we say about silica gel therefore applies equally to alumina.

What separates the compounds as a chromatogram develops?

As the solvent begins to soak up the plate, it first dissolves the compounds in the spot that you have put on the base line. The compounds present will then tend to get carried up the chromatography plate as the solvent continues to move upwards.How fast the compounds get carried up the plate depends on two things:

- How soluble the compound is in the solvent. This will depend on how much attraction there is between the molecules of the compound and those of the solvent.

- How much the compound sticks to the stationary phase - the silica gel, for example. This will depend on how much attraction there is between the molecules of the compound and the silica gel.

Suppose the original spot contained two compounds - one of which can form hydrogen bonds, and one of which can only take part in weaker van der Waals interactions. The one which can hydrogen bond will stick to the surface of the silica gel more firmly than the other one. We say that one is adsorbed more strongly than the other. Adsorption is the name given to one substance forming some sort of bonds to the surface of another one.

Adsorption isn't permanent - there is a constant movement of a molecule between being adsorbed onto the silica gel surface and going back into solution in the solvent. Obviously the compound can only travel up the plate during the time that it is dissolved in the solvent. While it is adsorbed on the silica gel, it is temporarily stopped - the solvent is moving on without it. That means that the more strongly a compound is adsorbed, the less distance it can travel up the plate.

In the example we started with, the compound which can hydrogen bond will adsorb more strongly than the one dependent on van der Waals interactions, and so won't travel so far up the plate.

What if both components of the mixture can hydrogen bond?

It is very unlikely that both will hydrogen bond to exactly the same extent, and be soluble in the solvent to exactly the same extent. It isn't just the attraction of the compound for the silica gel which matters. Attractions between the compound and the solvent are also important - they will affect how easily the compound is pulled back into solution away from the surface of the silica. However, it may be that the compounds don't separate out very well when you make the chromatogram. In that case, changing the solvent may well help - including perhaps changing the pH of the solvent. This is to some extent just a matter of trial and error - if one solvent or solvent mixture doesn't work very well, you try another one. (Or, more likely, given the level you are probably working at, someone else has already done all the hard work for you, and you just use the solvent mixture you are given and everything will work perfectly!)

Contributors and Attributions

- Jim Clark (Chemguide.co.uk)

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14.7: Problems

1. The following data were obtained for four compounds separated on a 20-m capillary column.

compound	t_r (min)	w (min)
A	8.04	0.15
B	8.26	0.15
C	8.43	0.16

(a) Calculate the number of theoretical plates for each compound and the average number of theoretical plates for the column, in mm.

(b) Calculate the average height of a theoretical plate.

(c) Explain why it is possible for each compound to have a different number of theoretical plates.

2. Using the data from Problem 1, calculate the resolution and the selectivity factors for each pair of adjacent compounds. For resolution, use both equation 12.2.1 and equation 12.3.3, and compare your results. Discuss how you might improve the resolution between compounds B and C. The retention time for an nonretained solute is 1.19 min.

3. Use the chromatogram in Figure 12.8.1 , obtained using a 2-m column, to determine values for t_r, w, t'_r, k, N, and H.

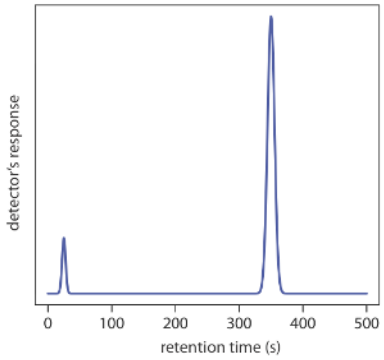


Figure 12.8.1 . Chromatogram for Problem 3.

4. Use the partial chromatogram in Figure 12.8.2 to determine the resolution between the two solute bands.

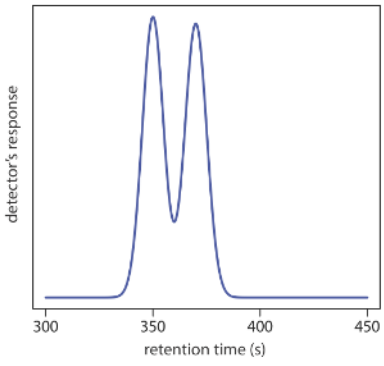


Figure 12.8.2 . Chromatogram for Problem 4.

5. The chromatogram in Problem 4 was obtained on a 2-m column with a column dead time of 50 s. Suppose you want to increase the resolution between the two components to 1.5. Without changing the height of a theoretical plate, what length column do you need? What height of a theoretical plate do you need to achieve a resolution of 1.5 without increasing the column's length?

6. Complete the following table.

N_B	α	k_B	R
100000	1.05	0.50	
10000	1.10		1.50
10000		4.0	1.00
	1.05	3.0	1.75

7. Moody studied the efficiency of a GC separation of 2-butanone on a dinonyl phthalate packed column [Moody, H. W. *J. Chem. Educ.* **1982**, *59*, 218–219]. Evaluating plate height as a function of flow rate gave a van Deemter equation for which *A* is 1.65 mm, *B* is 25.8 mm•mL min^{-1}, and *C* is 0.0236 mm•min mL^{-1}.

(a) Prepare a graph of *H* versus *u* for flow rates between 5 –120 mL/min.

(b) For what range of flow rates does each term in the Van Deemter equation have the greatest effect?

(c) What is the optimum flow rate and the corresponding height of a theoretical plate?

(d) For open-tubular columns the *A* term no longer is needed. If the *B* and *C* terms remain unchanged, what is the optimum flow rate and the corresponding height of a theoretical plate?

(e) Compared to the packed column, how many more theoretical plates are in the open-tubular column?

8. Hsieh and Jorgenson prepared 12–33 μm inner diameter HPLC columns packed with 5.44-μm spherical stationary phase particles [Hsieh, S.; Jorgenson, J. W. *Anal. Chem.* **1996**, *68*, 1212–1217]. To evaluate these columns they measured reduced plate height, *h*, as a function of reduced flow rate, *v*,

$$h = \frac{H}{d_p} \quad v = \frac{ud_p}{D_m}$$

where d_p is the particle diameter and D_m is the solute's diffusion coefficient in the mobile phase. The data were analyzed using van Deemter plots. The following table contains a portion of their results for norepinephrine.

internal diameter (μm)	A	B	C
33	0.63	1.32	0.10
33	0.67	1.30	0.08
23	0.40	1.34	0.09
23	0.58	1.11	0.09
17	0.31	1.47	0.11
17	0.40	1.41	0.11
12	0.22	1.53	0.11
12	0.19	1.27	0.12

(a) Construct separate van Deemter plots using the data in the first row and in the last row for reduced flow rates in the range 0.7–15. Determine the optimum flow rate and plate height for each case given d_p = 5.44 μm and D_m = $\mathbf{6.23 \times 10^{-6}}$ cm^2 s^{-1}.

(b) The *A* term in the van Deemter equation is strongly correlated with the column's inner diameter, with smaller diameter columns providing smaller values of *A*. Offer an explanation for this observation. *Hint: consider how many particles can fit across a capillary of each diameter.*

> When comparing columns, chromatographers often use dimensionless, reduced parameters. By including particle size and the solute's diffusion coefficient, the reduced plate height and reduced flow rate correct for differences between the packing material, the solute, and the mobile phase.

9. A mixture of *n*-heptane, tetrahydrofuran, 2-butanone, and *n*-propanol elutes in this order when using a polar stationary phase such as Carbowax. The elution order is exactly the opposite when using a nonpolar stationary phase such as polydimethyl siloxane. Explain the order of elution in each case.

10. The analysis of trihalomethanes in drinking water is described in Representative Method 12.4.1. A single standard that contains all four trihalomethanes gives the following results.

compound	concentration (ppb)	peak area
$CHCl_3$	1.30	1.35×10^4
$CHCl_2Br$	0.90	6.12×10^4
$CHClBr_2$	4.00	1.71×10^4
$CHBr_3$	1.20	1.52×10^4

Analysis of water collected from a drinking fountain gives areas of 1.56×10^4, 5.13×10^4, 1.49×10^4, and 1.76×10^4 for, respectively, $CHCl_3$, $CHCl_2Br$, $CHClBr_2$, and $CHBr_3$. All peak areas were corrected for variations in injection volumes using an internal standard of 1,2-dibromopentane. Determine the concentration of each of the trihalomethanes in the sample of water.

11. Zhou and colleagues determined the %w/w H_2O in methanol by capillary column GC using a polar stationary phase and a thermal conductivity detector [Zhou, X.; Hines, P. A.; White, K. C.; Borer, M. W. *Anal. Chem.* **1998**, *70*, 390–394]. A series of calibration standards gave the following results.

%w/w H_2O	peak height (arb. units)
0.00	1.15
0.0145	2.74
0.0472	6.33
0.0951	11.58
0.1757	20.43
0.2901	32.97

(a) What is the %w/w H_2O in a sample that has a peak height of 8.63?

(b) The %w/w H_2O in a freeze-dried antibiotic is determined in the following manner. A 0.175-g sample is placed in a vial along with 4.489 g of methanol. Water in the vial extracts into the methanol. Analysis of the sample gave a peak height of 13.66. What is the %w/w H_2O in the antibiotic?

12. Loconto and co-workers describe a method for determining trace levels of water in soil [Loconto, P. R.; Pan, Y. L.; Voice, T. C. *LC•GC* **1996**, *14*, 128–132]. The method takes advantage of the reaction of water with calcium carbide, CaC_2, to produce acetylene gas, C_2H_2. By carrying out the reaction in a sealed vial, the amount of acetylene produced is determined by sampling the headspace. In a typical analysis a sample of soil is placed in a sealed vial with CaC_2. Analysis of the headspace gives a blank corrected signal of 2.70×10^5. A second sample is prepared in the same manner except that a standard addition of 5.0 mg H_2O/g soil is added, giving a blank-corrected signal of 1.06×10^6. Determine the milligrams H_2O/g soil in the soil sample.

13. Van Atta and Van Atta used gas chromatography to determine the %v/v methyl salicylate in rubbing alcohol [Van Atta, R. E.; Van Atta, R. L. *J. Chem. Educ.* **1980**, *57*, 230–231]. A set of standard additions was prepared by transferring 20.00 mL of rubbing

alcohol to separate 25-mL volumetric flasks and pipeting 0.00 mL, 0.20 mL, and 0.50 mL of methyl salicylate to the flasks. All three flasks were diluted to volume using isopropanol. Analysis of the three samples gave peak heights for methyl salicylate of 57.00 mm, 88.5 mm, and 132.5 mm, respectively. Determine the %v/v methyl salicylate in the rubbing alcohol.

14. The amount of camphor in an analgesic ointment is determined by GC using the method of internal standards [Pant, S. K.; Gupta, P. N.; Thomas, K. M.; Maitin, B. K.; Jain, C. L. *LC•GC* **1990**, *8*, 322–325]. A standard sample is prepared by placing 45.2 mg of camphor and 2.00 mL of a 6.00 mg/mL internal standard solution of terpene hydrate in a 25-mL volumetric flask and diluting to volume with CCl_4. When an approximately 2-µL sample of the standard is injected, the FID signals for the two components are measured (in arbitrary units) as 67.3 for camphor and 19.8 for terpene hydrate. A 53.6-mg sample of an analgesic ointment is prepared for analysis by placing it in a 50-mL Erlenmeyer flask along with 10 mL of CCl_4. After heating to 50°C in a water bath, the sample is cooled to below room temperature and filtered. The residue is washed with two 5-mL portions of CCl_4 and the combined filtrates are collected in a 25-mL volumetric flask. After adding 2.00 mL of the internal standard solution, the contents of the flask are diluted to volume with CCl_4. Analysis of an approximately 2-µL sample gives FID signals of 13.5 for the terpene hydrate and 24.9 for the camphor. Report the %w/w camphor in the analgesic ointment.

15. The concentration of pesticide residues on agricultural products, such as oranges, is determined by GC-MS [Feigel, C. *Varian GC/MS Application Note*, Number 52]. Pesticide residues are extracted from the sample using methylene chloride and concentrated by evaporating the methylene chloride to a smaller volume. Calibration is accomplished using anthracene-d_{10} as an internal standard. In a study to determine the parts per billion heptachlor epoxide on oranges, a 50.0-g sample of orange rinds is chopped and extracted with 50.00 mL of methylene chloride. After removing any insoluble material by filtration, the methylene chloride is reduced in volume, spiked with a known amount of the internal standard and diluted to 10 mL in a volumetric flask. Analysis of the sample gives a peak–area ratio ($A_{analyte}/A_{intstd}$) of 0.108. A series of calibration standards, each containing the same amount of anthracene-d_{10} as the sample, gives the following results.

ppb heptachlor epoxide	$A_{analyte}/A_{intstd}$
20.0	0.065
60.0	0.153
200.0	0.637
500.0	1.554
1000.0	3.198

Report the nanograms per gram of heptachlor epoxide residue on the oranges.

16. The adjusted retention times for octane, toluene, and nonane on a particular GC column are 15.98 min, 17.73 min, and 20.42 min, respectively. What is the retention index for each compound?

17. The following data were collected for a series of normal alkanes using a stationary phase of Carbowax 20M.

alkane	t_r' (min)
pentane	0.79
hexane	1.99
heptane	4.47
octane	14.12
nonane	33.11

What is the retention index for a compound whose adjusted retention time is 9.36 min?

18. The following data were reported for the gas chromatographic analysis of *p*-xylene and methylisobutylketone (MIBK) on a capillary column [Marriott, P. J.; Carpenter, P. D. *J. Chem. Educ.* **1996**, *73*, 96–99].

injection mode	compound	t_r (min)	peak area (arb. units)	peak width (min)
split	MIBK	1.878	54285	0.028
	p-xylene	5.234	123483	0.044
splitless	MIBK	3.420	2493005	1.057
	p-xylene	5.795	3396656	1.051

Explain the difference in the retention times, the peak areas, and the peak widths when switching from a split injection to a splitless injection.

19. Otto and Wegscheider report the following retention factors for the reversed-phase separation of 2-aminobenzoic acid on a C_{18} column when using 10% v/v methanol as a mobile phase [Otto, M.; Wegscheider, W. *J. Chromatog.* **1983**, *258*, 11–22].

pH	*k*
2.0	10.5
3.0	16.7
4.0	15.8
5.0	8.0
6.0	2.2
7.0	1.8

Explain the effect of pH on the retention factor for 2-aminobenzene.

20. Haddad and associates report the following retention factors for the reversed-phase separation of salicylamide and caffeine [Haddad, P.; Hutchins, S.; Tuffy, M. *J. Chem. Educ.* **1983**, *60*, 166-168].

%v/v methanol	30%	35%	40%	45%	50%	55%
k_{sal}	2.4	1.6	1.6	1.0	0.7	0.7
k_{caff}	4.3	2.8	2.3	1.4	1.1	0.9

(a) Explain the trends in the retention factors for these compounds.

(b) What is the advantage of using a mobile phase with a smaller %v/v methanol? Are there any disadvantages?

21. Suppose you need to separate a mixture of benzoic acid, aspartame, and caffeine in a diet soda. The following information is available.

	t_r in aqueous mobile phase of pH			
compound	3.0	3.5	4.0	4.5
benzoic acid	7.4	7.0	6.9	4.4
aspartame	5.9	6.0	7.1	8.1
caffeine	3.6	3.7	4.1	4.4

(a) Explain the change in each compound's retention time.

(b) Prepare a single graph that shows retention time versus pH for each compound. Using your plot, identify a pH level that will yield an acceptable separation.

22. The composition of a multivitamin tablet is determined using an HPLC with a diode array UV/Vis detector. A 5-µL standard sample that contains 170 ppm vitamin C, 130 ppm niacin, 120 ppm niacinamide, 150 ppm pyridoxine, 60 ppm thiamine, 15 ppm folic acid, and 10 ppm riboflavin is injected into the HPLC, giving signals (in arbitrary units) of, respectively, 0.22, 1.35, 0.90, 1.37, 0.82, 0.36, and 0.29. The multivitamin tablet is prepared for analysis by grinding into a powder and transferring to a 125-mL Erlenmeyer flask that contains 10 mL of 1% v/v NH_3 in dimethyl sulfoxide. After sonicating in an ultrasonic bath for 2 min, 90 mL of 2% acetic acid is added and the mixture is stirred for 1 min and sonicated at 40°C for 5 min. The extract is then filtered through a 0.45-µm membrane filter. Injection of a 5-µL sample into the HPLC gives signals of 0.87 for vitamin C, 0.00 for niacin, 1.40 for niacinamide, 0.22 for pyridoxine, 0.19 for thiamine, 0.11 for folic acid, and 0.44 for riboflavin. Report the milligrams of each vitamin present in the tablet.

23. The amount of caffeine in an analgesic tablet was determined by HPLC using a normal calibration curve. Standard solutions of caffeine were prepared and analyzed using a 10-µL fixed-volume injection loop. Results for the standards are summarized in the following table.

concentration (ppm)	signal (arb. units)
50.0	8.354
100.0	16925
150.0	25218
200.0	33584
250.0	42002

The sample is prepared by placing a single analgesic tablet in a small beaker and adding 10 mL of methanol. After allowing the sample to dissolve, the contents of the beaker, including the insoluble binder, are quantitatively transferred to a 25-mL volumetric flask and diluted to volume with methanol. The sample is then filtered, and a 1.00-mL aliquot transferred to a 10-mL volumetric flask and diluted to volume with methanol. When analyzed by HPLC, the signal for caffeine is found to be 21 469. Report the milligrams of caffeine in the analgesic tablet.

24. Kagel and Farwell report a reversed-phase HPLC method for determining the concentration of acetylsalicylic acid (ASA) and caffeine (CAF) in analgesic tablets using salicylic acid (SA) as an internal standard [Kagel, R. A.; Farwell, S. O. *J. Chem. Educ.* **1983**, *60*, 163–166]. A series of standards was prepared by adding known amounts of ace- tylsalicylic acid and caffeine to 250-mL Erlenmeyer flasks and adding 100 mL of methanol. A 10.00-mL aliquot of a standard solution of salicylic acid was then added to each. The following results were obtained for a typical set of standard solutions.

	milligrams of		peak height ratios for	
standard	ASA	CAF	ASA/SA	CAF/SA
1	200.0	20.0	20.5	10.6
2	250.0	40.0	25.1	23.0
3	300.0	60.0	30.9	36.8

A sample of an analgesic tablet was placed in a 250-mL Erlenmeyer flask and dissolved in 100 mL of methanol. After adding a 10.00-mL portion of the internal standard, the solution was filtered. Analysis of the sample gave a peak height ratio of 23.2 for ASA and of 17.9 for CAF.

(a) Determine the milligrams of ASA and CAF in the tablet.

(b) Why is it necessary to filter the sample?

(c) The directions indicate that approximately 100 mL of methanol is used to dissolve the standards and samples. Why is it not necessary to measure this volume more precisely?

(d) In the presence of moisture, ASA decomposes to SA and acetic acid. What complication might this present for this analysis? How might you evaluate whether this is a problem?

25. Bohman and colleagues described a reversed-phase HPLC method for the quantitative analysis of vitamin A in food using the method of standard additions Bohman, O.; Engdahl, K. A.; Johnsson, H. *J. Chem. Educ.* **1982**, *59*, 251–252]. In a typical example, a 10.067-g sample of cereal is placed in a 250-mL Erlenmeyer flask along with 1 g of sodium ascorbate, 40 mL of ethanol, and 10 mL of 50% w/v KOH. After refluxing for 30 min, 60 mL of ethanol is added and the solution cooled to room temperature. Vitamin A is extracted using three 100-mL portions of hexane. The combined portions of hexane are evaporated and the residue containing vitamin A transferred to a 5-mL volumetric flask and diluted to volume with methanol. A standard addition is prepared in a similar manner using a 10.093-g sample of the cereal and spiking with 0.0200 mg of vitamin A. Injecting the sample and standard addition into the HPLC gives peak areas of, respectively, $\mathbf{6.77 \times 10^3}$ and $\mathbf{1.32 \times 10^4}$. Report the vitamin A content of the sample in milligrams/100 g cereal.

26. Ohta and Tanaka reported on an ion-exchange chromatographic method for the simultaneous analysis of several inorganic anions and the cations Mg^{2+} and Ca^{2+} in water [Ohta, K.; Tanaka, K. *Anal. Chim. Acta* **1998**, *373*, 189–195]. The mobile phase includes the ligand 1,2,4-benzenetricarboxylate, which absorbs strongly at 270 nm. Indirect detection of the analytes is possible because its absorbance decreases when complexed with an anion.

(a) The procedure also calls for adding the ligand EDTA to the mobile phase. What role does the EDTA play in this analysis?

(b) A standard solution of 1.0 mM $NaHCO_3$, 0.20 mM $NaNO_2$, 0.20 mM $MgSO_4$, 0.10 mM $CaCl_2$, and 0.10 mM $Ca(NO_3)_2$ gives the following peak areas (arbitrary units).

ion	$\mathbf{HCO_3^-}$	Cl^-	$\mathbf{NO_2^-}$	$\mathbf{NO_3^-}$
peak area	373.5	322.5	264.8	262.7
ion	Ca^{2+}	Mg^{2+}	$\mathbf{SO_4^{2-}}$	
peak area	458.9	352.0	341.3	

Analysis of a river water sample (pH of 7.49) gives the following results.

ion	$\mathbf{HCO_3^-}$	Cl^-	$\mathbf{NO_2^-}$	$\mathbf{NO_3^-}$
peak area	310.0	403.1	3.97	157.6
ion	Ca^{2+}	Mg^{2+}	$\mathbf{SO_4^{2-}}$	
peak area	734.3	193.6	324.3	

Determine the concentration of each ion in the sample.

(c) The detection of $\mathbf{HCO_3^-}$ actually gives the total concentration of carbonate in solution ($[\mathbf{CO_3^{2-}}]+[\mathbf{HCO_3^-}]+[H_2CO_3]$). Given that the pH of the water is 7.49, what is the actual concentration of $\mathbf{HCO_3^-}$?

(d) An independent analysis gives the following additional concentrations for ions in the sample: $[Na^+]$ = 0.60 mM; $[\mathbf{NH_4^+}]$ = 0.014 mM; and $[K^+]$ = 0.046 mM. A solution's ion balance is defined as the ratio of the total cation charge to the total anion charge. Determine the charge balance for this sample of water and comment on whether the result is reasonable.

27. The concentrations of Cl^-, $\mathbf{NO_2^-}$, and $\mathbf{SO_4^{2-}}$ are determined by ion chromatography. A 50-µL standard sample of 10.0 ppm Cl^-, 2.00 ppm $\mathbf{NO_2^-}$, and 5.00 ppm $\mathbf{SO_4^{2-}}$ gave signals (in arbitrary units) of 59.3, 16.1, and 6.08 respectively. A sample of effluent from a wastewater treatment plant is diluted tenfold and a 50-µL portion gives signals of 44.2 for Cl^-, 2.73 for $\mathbf{NO_2^-}$, and 5.04 for $\mathbf{SO_4^{2-}}$. Report the parts per million for each anion in the effluent sample.

28. A series of polyvinylpyridine standards of different molecular weight was analyzed by size-exclusion chromatography, yielding the following results.

formula weight	retention volume (mL)
600000	6.42
100000	7.98
30000	9.30
3000	10.94

When a preparation of polyvinylpyridine of unknown formula weight is analyzed, the retention volume is 8.45 mL. Report the average formula weight for the preparation.

29. Diet soft drinks contain appreciable quantities of aspartame, benzoic acid, and caffeine. What is the expected order of elution for these compounds in a capillary zone electrophoresis separation using a pH 9.4 buffer given that aspartame has pK_a values of 2.964 and 7.37, benzoic acid has a pK_a of 4.2, and the pK_a for caffeine is less than 0. Figure 12.8.3 provides the structures of these compounds.

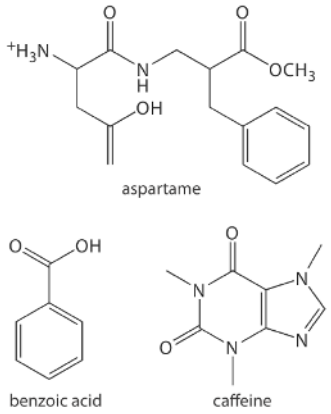


Figure 12.8.3
. Structures for the compounds in Problem 29.

30. Janusa and coworkers describe the determination of chloride by CZE [Janusa, M. A.; Andermann, L. J.; Kliebert, N. M.; Nannie, M. H. *J. Chem. Educ.* **1998**, *75*, 1463–1465]. Analysis of a series of external standards gives the following calibration curve.

$$\text{area} = -883 + 5590 \times \text{ppm } Cl^-$$

A standard sample of 57.22% w/w Cl^- is analyzed by placing 0.1011-g portions in separate 100-mL volumetric flasks and diluting to volume. Three unknowns are prepared by pipeting 0.250 mL, 0.500 mL, an 0.750 mL of the bulk unknown in separate 50-mL volumetric flasks and diluting to volume. Analysis of the three unknowns gives areas of 15 310, 31 546, and 47 582, respectively. Evaluate the accuracy of this analysis.

31. The analysis of NO_3^- in aquarium water is carried out by CZE using IO_4^- as an internal standard. A standard solution of 15.0 ppm NO_3^- and 10.0 ppm IO_4^- gives peak heights (arbitrary units) of 95.0 and 100.1, respectively. A sample of water from an aquarium is diluted 1:100 and sufficient internal standard added to make its concentration 10.0 ppm in IO_4^-. Analysis gives signals of 29.2 and 105.8 for NO_3^- and IO_4^-, respectively. Report the ppm NO_3^- in the sample of aquarium water.

32. Suggest conditions to separate a mixture of 2-aminobenzoic acid (pK_{a1} = 2.08, pK_{a2} = 4.96), benzylamine (pK_a = 9.35), and 4-methylphenol (pK_{a2} = 10.26) by capillary zone electrophoresis. Figure ***PageIndex4*** provides the structures of these compounds.

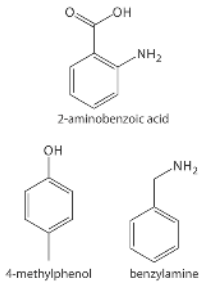


Figure 12.8.4 . Structures for the compounds in Problem 32.

33. McKillop and associates examined the electrophoretic separation of some alkylpyridines by CZE [McKillop, A. G.; Smith, R. M.; Rowe, R. C.; Wren, S. A. C. *Anal. Chem.* **1999**, *71*, 497–503]. Separations were carried out using either 50-μm or 75-μm inner diameter capillaries, with a total length of 57 cm and a length of 50 cm from the point of injection to the detector. The run buffer was a pH 2.5 lithium phosphate buffer. Separations were achieved using an applied voltage of 15 kV. The electroosmotic mobility, μ_{eof}, as measured using a neutral marker, was found to be $\mathbf{6.398 \times 10^{-5}}$ $cm^2\ V^{-1}\ s^{-1}$. The diffusion coefficient for alkylpyridines is $\mathbf{1.0 \times 10^{-5}}$ $cm^2\ s^{-1}$.

(a) Calculate the electrophoretic mobility for 2-ethylpyridine given that its elution time is 8.20 min.

(b) How many theoretical plates are there for 2-ethylpyridine?

(c) The electrophoretic mobilities for 3-ethylpyridine and 4-ethylpyridine are $\mathbf{3.366 \times 10^{-4}}$ $cm^2\ V^{-1}\ s^{-1}$ and $\mathbf{3.397 \times 10^{-4}}$ $\mathbf{cm^2\ V^{-1}\ s^{-1}}$, respectively. What is the expected resolution between these two alkylpyridines?

(d) Explain the trends in electrophoretic mobility shown in the following table.

alkylpyridine	μ_{ep} ($cm^2\ V^{-1}\ s^{-1}$)
2-methylpyridine	$\mathbf{3.581 \times 10^{-4}}$
2-ethylpyridine	$\mathbf{3.222 \times 10^{-4}}$
2-propylpyridine	$\mathbf{2.923 \times 10^{-4}}$
2-pentylpyridine	$\mathbf{2.534 \times 10^{-4}}$
2-hexylpyridine	$\mathbf{2.391 \times 10^{-4}}$

(e) Explain the trends in electrophoretic mobility shown in the following table.

alkylpyridine	μ_{ep} ($cm^2\ V^{-1}\ s^{-1}$)
2-ethylpyridine	$\mathbf{3.222 \times 10^{-4}}$
3-ethylpyridine	$\mathbf{3.366 \times 10^{-4}}$
4-ethylpyridine	$\mathbf{3.397 \times 10^{-4}}$

(f) The pK_a for pyridine is 5.229. At a pH of 2.5 the electrophoretic mobility of pyridine is $\mathbf{4.176 \times 10^{-4}}$ $cm^2\ V^{-1}\ s^{-1}$. What is the expected electrophoretic mobility if the run buffer's pH is 7.5?

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CHAPTER OVERVIEW

15: Capillary Electrophoresis and Electrochromatography

15.1: Electrophoresis
15.2: Problems

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15.1: Electrophoresis

Electrophoresis is a class of separation techniques in which we separate analytes by their ability to move through a conductive medium—usually an aqueous buffer—in response to an applied electric field. In the absence of other effects, cations migrate toward the electric field's negatively charged cathode. Cations with larger charge-to-size ratios—which favors ions of greater charge and of smaller size—migrate at a faster rate than larger cat- ions with smaller charges. Anions migrate toward the positively charged anode and neutral species do not experience the electrical field and remain stationary.

> As we will see shortly, under normal conditions even neutral species and anions migrate toward the cathode.

There are several forms of electrophoresis. In slab gel electrophoresis the conducting buffer is retained within a porous gel of agarose or polyacrylamide. Slabs are formed by pouring the gel between two glass plates separated by spacers. Typical thicknesses are 0.25–1 mm. Gel electrophoresis is an important technique in biochemistry where it frequently is used to separate DNA fragments and proteins. Although it is a powerful tool for the qualitative analysis of complex mixtures, it is less useful for quantitative work.

In ***capillary electrophoresis*** the conducting buffer is retained within a capillary tube with an inner diameter that typically is 25–75 μm. The sample is injected into one end of the capillary tube, and as it migrates through the capillary the sample's components separate and elute from the column at different times. The resulting ***electropherogram*** looks similar to a GC or an HPLC chromatogram, and provides both qualitative and quantitative information. Only capillary electrophoretic methods receive further consideration in this section.

Theory of Electrophoresis

In capillary electrophoresis we inject the sample into a buffered solution retained within a capillary tube. When an electric field is applied across the capillary tube, the sample's components migrate as the result of two types of actions: electrophoretic mobility and electroosmotic mobility. ***Electrophoretic mobility*** is the solute's response to the applied electrical field in which cations move toward the negatively charged cathode, anions move toward the positively charged anode, and neutral species remain stationary. The other contribution to a solute's migration is ***electroosmotic flow***, which occurs when the buffer moves through the capillary in response to the applied electrical field. Under normal conditions the buffer moves toward the cathode, sweeping most solutes, including the anions and neutral species, toward the negatively charged cathode.

Electrophoretic Mobility

The velocity with which a solute moves in response to the applied electric field is called its ***electrophoretic velocity***, ν_{ep}; it is defined as

$$\nu_{ep} = \mu_{ep} E \tag{15.1.1}$$

where μ_{ep} is the solute's electrophoretic mobility, and E is the magnitude of the applied electrical field. A solute's electrophoretic mobility is defined as

$$\mu_{ep} = \frac{q}{6\pi\eta r} \tag{15.1.2}$$

where q is the solute's charge, η is the buffer's viscosity, and r is the solute's radius. Using equation 15.1.1 and equation 15.1.2 we can make several important conclusions about a solute's electrophoretic velocity. Electrophoretic mobility and, therefore, electrophoretic velocity, increases for more highly charged solutes and for solutes of smaller size. Because q is positive for a cation and negative for an anion, these species migrate in opposite directions. A neutral species, for which q is zero, has an electrophoretic velocity of zero.

Electroosmotic Mobility

When an electric field is applied to a capillary filled with an aqueous buffer we expect the buffer's ions to migrate in response to their electrophoretic mobility. Because the solvent, H_2O, is neutral we might reasonably expect it to remain stationary. What we observe under normal conditions, however, is that the buffer moves toward the cathode. This phenomenon is called the electroosmotic flow.

Electroosmotic flow occurs because the walls of the capillary tubing carry a charge. The surface of a silica capillary contains large numbers of silanol groups (–SiOH). At a pH level greater than approximately 2 or 3, the silanol groups ionize to form negatively charged silanate ions ($–SiO^-$). Cations from the buffer are attracted to the silanate ions. As shown in Figure **15.1.1**, some of these cations bind tightly to the silanate ions, forming a fixed layer. Because the cations in the fixed layer only partially neutralize the negative charge on the capillary walls, the solution adjacent to the fixed layer—which is called the diffuse layer—contains more cations than anions. Together these two layers are known as the double layer. Cations in the diffuse layer migrate toward the cathode. Because these cations are solvated, the solution also is pulled along, producing the electroosmotic flow.

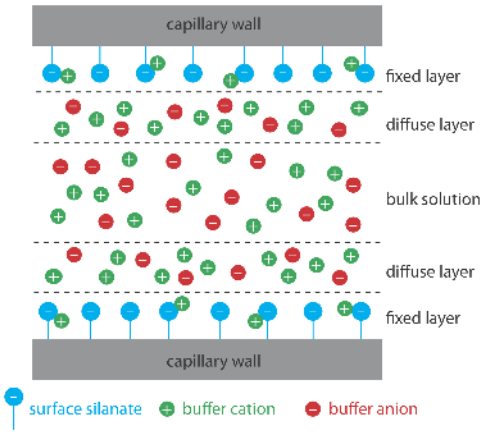


Figure 15.1.1. Schematic diagram showing the origin of the double layer within a capillary tube. Although the net charge within the capillary is zero, the distribution of charge is not. The walls of the capillary have an excess of negative charge, which decreases across the fixed layer and the diffuse layer, reaching a value of zero in bulk solution.

> The anions in the diffuse layer, which also are solvated, try to move toward the anode. Because there are more cations than anions, however, the cations win out and the electroosmotic flow moves in the direction of the cathode.

The rate at which the buffer moves through the capillary, what we call its ***electroosmotic flow velocity***, ν_{eof}, is a function of the applied electric field, E, and the buffer's electroosmotic mobility, μ_{eof}.

$$\nu_{eof} = \mu_{eof} E \tag{15.1.3}$$

Electroosmotic mobility is defined as

$$\mu_{eof} = \frac{\varepsilon \zeta}{4\pi\eta} \tag{15.1.4}$$

where ϵ is the buffer dielectric constant, ζ is the zeta potential, and η is the buffer's viscosity.

The ***zeta potential***—the potential of the diffuse layer at a finite distance from the capillary wall—plays an important role in determining the electroosmotic flow velocity. Two factors determine the zeta potential's value. First, the zeta potential is directly proportional to the charge on the capillary walls, with a greater density of silanate ions corresponding to a larger zeta potential. Below a pH of 2 there are few silanate ions and the zeta potential and the electroosmotic flow velocity approach zero. As the pH increases, both the zeta potential and the electroosmotic flow velocity increase. Second, the zeta potential is directly proportional to the thickness of the double layer. Increasing the buffer's ionic strength provides a higher concentration of cations, which decreases the thickness of the double layer and decreases the electroosmotic flow.

> The definition of zeta potential given here admittedly is a bit fuzzy. For a more detailed explanation see Delgado, A. V.; González-Caballero, F.; Hunter, R. J.; Koopal, L. K.; Lyklema, J. "Measurement and Interpretation of Electrokinetic Phenomena," *Pure. Appl. Chem.* **2005**, *77*, 1753–1805. Although this is a very technical report, Sections 1.3–1.5 provide a good introduction to the difficulty of defining the zeta potential and of measuring its value.

The electroosmotic flow profile is very different from that of a fluid moving under forced pressure. Figure **15.1.2** compares the electroosmotic flow profile with the hydrodynamic flow profile in gas chromatography and liquid chromatography. The uniform, flat profile for electroosmosis helps minimize band broadening in capillary electrophoresis, improving separation efficiency.

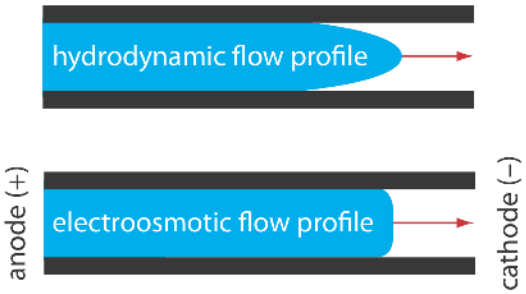


Figure 15.1.2. Comparison of hydrodynamic flow and electroosmotic flow. The nearly uniform electroosmotic flow profile means that the electroosmotic flow velocity is nearly constant across the capillary.

Total Mobility

A solute's total velocity, ν_{tot} , as it moves through the capillary is the sum of its electrophoretic velocity and the electroosmotic flow velocity.

$$\nu_{tot} = \nu_{ep} + \nu_{eof}$$

As shown in Figure **15.1.3**, under normal conditions the following general relationships hold true.

$$(\nu_{tot})_{cations} > \nu_{eof}$$

$$(\nu_{tot})_{neutrals} = \nu_{eof}$$

$$(\nu_{tot})_{anions} < \nu_{eof}$$

Cations elute first in an order that corresponds to their electrophoretic mobilities, with small, highly charged cations eluting before larger cations of lower charge. Neutral species elute as a single band with an elution rate equal to the electroosmotic flow velocity. Finally, anions are the last components to elute, with smaller, highly charged anions having the longest elution time.

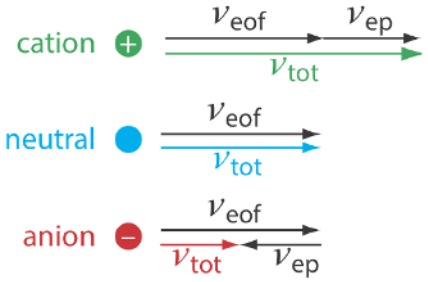


Figure 15.1.3. Visual explanation for the general elution order in capillary electrophoresis. Each species has the same electroosmotic flow, ν_{eof}. Cations elute first because they have a positive electrophoretic velocity, ν_{ep}. Anions elute last because their negative electrophoretic velocity partially offsets the electroosmotic flow velocity. Neutrals elute with a velocity equal to the electroosmotic flow.

Migration Time

Another way to express a solute's velocity is to divide the distance it travels by the elapsed time

$$\nu_{tot} = \frac{l}{t_m} \tag{15.1.5}$$

where l is the distance between the point of injection and the detector, and t_m is the solute's migration time. To understand the experimental variables that affect migration time, we begin by noting that

$$\nu_{tot} = \mu_{tot} E = (\mu_{ep} + \mu_{eof}) E \tag{15.1.6}$$

Combining equation 15.1.5 and equation 15.1.6 and solving for t_m leaves us with

$$t_m = \frac{l}{(\mu_{ep} + \mu_{eof})\, E} \tag{15.1.7}$$

The magnitude of the electrical field is

$$E = \frac{V}{L} \tag{15.1.8}$$

where V is the applied potential and L is the length of the capillary tube. Finally, substituting equation 15.1.8 into equation 15.1.7 leaves us with the following equation for a solute's migration time.

$$t_m = \frac{lL}{(\mu_{ep} + \mu_{eof})V} \tag{15.1.9}$$

To decrease a solute's migration time—which shortens the analysis time—we can apply a higher voltage or use a shorter capillary tube. We can also shorten the migration time by increasing the electroosmotic flow, although this decreases resolution.

Efficiency

As we learned in Chapter 12.2, the efficiency of a separation is given by the number of theoretical plates, N. In capillary electrophoresis the number of theoretic plates is

$$N = \frac{l^2}{2Dt_m} = \frac{(\mu_{ep} + \mu_{eof})El}{2DL} \tag{15.1.10}$$

where D is the solute's diffusion coefficient. From equation 15.1.10, the efficiency of a capillary electrophoretic separation increases with higher voltages. Increasing the electroosmotic flow velocity improves efficiency, but at the expense of resolution. Two additional observations deserve comment. First, solutes with larger electrophoretic mobilities—in the same direction as the electroosmotic flow—have greater efficiencies; thus, smaller, more highly charged cations are not only the first solutes to elute, but do so with greater efficiency. Second, efficiency in capillary electrophoresis is independent of the capillary's length. Theoretical plate counts of approximately 100 000–200 000 are not unusual.

> It is possible to design an electrophoretic experiment so that anions elute before cations—more about this later—in which smaller, more highly charged anions elute with greater efficiencies.

Selectivity

In chromatography we defined the selectivity between two solutes as the ratio of their retention factors. In capillary electrophoresis the analogous expression for selectivity is

$$\alpha = \frac{\mu_{ep,1}}{\mu_{ep,2}}$$

where $\mu_{ep,1}$ and $\mu_{ep,2}$ are the electrophoretic mobilities for the two solutes, chosen such that $\alpha \geq 1$. We can often improve selectivity by adjusting the pH of the buffer solution. For example, NH_4^+ is a weak acid with a pK_a of 9.75. At a pH of 9.75 the concentrations of NH_4^+ and NH_3 are equal. Decreasing the pH below 9.75 increases its electrophoretic mobility because a greater fraction of the solute is present as the cation NH_4^+. On the other hand, raising the pH above 9.75 increases the proportion of neutral NH_3, decreasing its electrophoretic mobility.

Resolution

The resolution between two solutes is

$$R = \frac{0.177(\mu_{ep,2} - \mu_{ep,1})\sqrt{V}}{\sqrt{D(\mu_{avg} + \mu_{eof}}} \tag{15.1.11}$$

where μ_{avg} is the average electrophoretic mobility for the two solutes. Increasing the applied voltage and decreasing the electroosmotic flow velocity improves resolution. The latter effect is particularly important. Although increasing electroosmotic flow improves analysis time and efficiency, it de- creases resolution.

Instrumentation

The basic instrumentation for capillary electrophoresis is shown in Figure 15.1.4 and includes a power supply for applying the electric field, anode and cathode compartments that contain reservoirs of the buffer solution, a sample vial that contains the sample,

the capillary tube, and a detector. Each part of the instrument receives further consideration in this section.

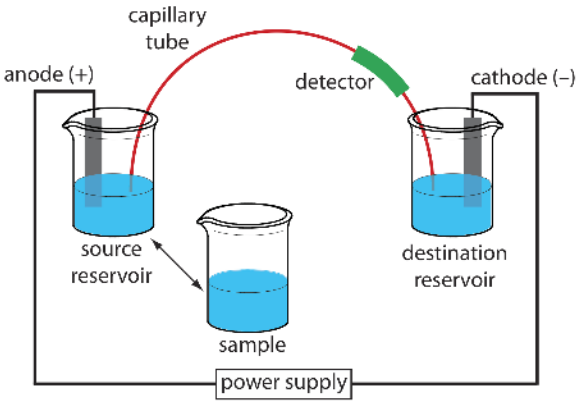


Figure 15.1.4. Schematic diagram of the basic instrumentation for capillary electrophoresis. The sample and the source reservoir are switched when making injections.

Capillary Tubes

Figure 15.1.5 shows a cross-section of a typical capillary tube. Most capillary tubes are made from fused silica coated with a 15–35 μm layer of polyimide to give it mechanical strength. The inner diameter is typically 25–75 μm, which is smaller than the internal diameter of a capillary GC column, with an outer diameter of 200–375 μm.

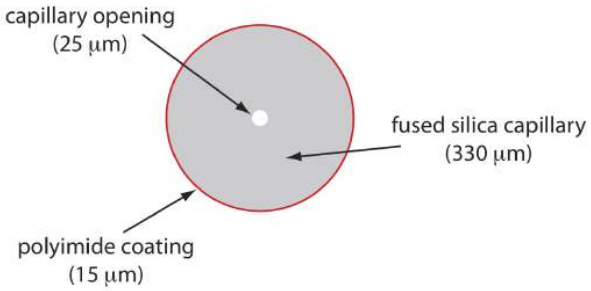


Figure 15.1.5. Cross section of a capillary column for capillary electrophoresis. The dimensions shown here are typical and are scaled proportionally in this figure.

The capillary column's narrow opening and the thickness of its walls are important. When an electric field is applied to the buffer solution, current flows through the capillary. This current leads to the release of heat, which we call ***Joule heating***. The amount of heat released is proportional to the capillary's radius and to the magnitude of the electrical field. Joule heating is a problem because it changes the buffer's viscosity, with the solution at the center of the capillary being less viscous than that near the capillary walls. Because a solute's electrophoretic mobility depends on its viscosity (see equation 15.1.2), solute species in the center of the capillary migrate at a faster rate than those near the capillary walls. The result is an additional source of band broadening that degrades the separation. Capillaries with smaller inner diameters generate less Joule heating, and capillaries with larger outer diameters are more effective at dissipating the heat. Placing the capillary tube inside a thermostated jacket is another method for minimizing the effect of Joule heating; in this case a smaller outer diameter allows for a more rapid dissipation of thermal energy.

Injecting the Sample

There are two common methods for injecting a sample into a capillary electrophoresis column: hydrodynamic injection and electrokinetic injection. In both methods the capillary tube is filled with the buffer solution. One end of the capillary tube is placed in the destination reservoir and the other end is placed in the sample vial.

Hydrodynamic injection uses pressure to force a small portion of sample into the capillary tubing. A difference in pressure is applied across the capillary either by pressurizing the sample vial or by applying a vacuum to the destination reservoir. The volume of sample injected, in liters, is given by the following equation

$$V_{\text{inj}} = \frac{\Delta P d^4 \pi t}{128 \eta L} \times 10^3 \tag{15.1.12}$$

where ΔP is the difference in pressure across the capillary in pascals, d is the capillary's inner diameter in meters, t is the amount of time the pressure is applied in seconds, η is the buffer's viscosity in kg m^{-1} s^{-1}, and L is the length of the capillary tubing in meters. The factor of 10^3 changes the units from cubic meters to liters.

For a hydrodynamic injection we move the capillary from the source reservoir to the sample. The anode remains in the source reservoir. A hydrodynamic injection also is possible if we raise the sample vial above the destination reservoir and briefly insert the filled capillary.

Example 15.1.1

In a hydrodynamic injection we apply a pressure difference of 2.5×10^3 Pa (a $\Delta P \approx 0.02$ atm) for 2 s to a 75-cm long capillary tube with an internal diameter of 50 μm. Assuming the buffer's viscosity is 10^{-3} kg m^{-1} s^{-1}, what volume and length of sample did we inject?

Solution

Making appropriate substitutions into equation 15.1.12 gives the sample's volume as

$$V_{inj} = \frac{\left(2.5 \times 10^3 \text{ kg m}^{-1} \text{ s}^{-2}\right)\left(50 \times 10^{-6} \text{ m}\right)^4 (3.14)(2 \text{ s})}{(128)\left(0.001 \text{ kg m}^{-1} \text{ s}^{-1}\right)(0.75 \text{ m})} \times 10^3 \text{L/m}^3$$

$$V_{inj} = 1 \times 10^{-9} \text{ L} = 1 \text{ nL}$$

Because the interior of the capillary is cylindrical, the length of the sample, l, is easy to calculate using the equation for the volume of a cylinder; thus

$$l = \frac{V_{\text{inj}}}{\pi r^2} = \frac{\left(1 \times 10^{-9} \text{ L}\right)\left(10^{-3} \text{ m}^3\text{/L}\right)}{(3.14)\left(25 \times 10^{-6} \text{ m}\right)^2} = 5 \times 10^{-4} \text{ m} = 0.5 \text{ mm}$$

Exercise 15.1.1

Suppose you need to limit your injection to less than 0.20% of the capillary's length. Using the information from Example 15.1.1, what is the maximum injection time for a hydrodynamic injection?

Answer

The capillary is 75 cm long, which means that 0.20% of that sample's maximum length is 0.15 cm. To convert this to the maximum volume of sample we use the equation for the volume of a cylinder.

$$V_{inj} = l \pi r^2 = (0.15 \text{ cm})(3.14)\left(25 \times 10^{-4} \text{ cm}\right)^2 = 2.94 \times 10^{-6} \text{ cm}^3$$

Given that 1 cm^3 is equivalent to 1 mL, the maximum volume is 2.94×10^{-6} mL or 2.94×10^{-9} L. To find the maximum injection time, we first solve equation 15.1.12 for t

$$t = \frac{128 V_{inj} \eta L}{P d^4 \pi} \times 10^{-3} \text{ m}^3\text{/L}$$

and then make appropriate substitutions.

$$t = \frac{(128)\left(2.94 \times 10^{-9} \text{ L}\right)\left(0.001 \text{ kg m}^{-1} \text{ s}^{-1}\right)(0.75 \text{ m})}{\left(2.5 \times 10^3 \text{ kg m}^{-1} \text{ s}^{-2}\right)\left(50 \times 10^{-6} \text{ m}\right)^4 (3.14)} \times \frac{10^{-3} \text{ m}^3}{\text{L}} = 5.8 \text{ s}$$

The maximum injection time, therefore, is 5.8 s.

In an ***electrokinetic injection*** we place both the capillary and the anode into the sample and briefly apply an potential. The volume of injected sample is the product of the capillary's cross sectional area and the length of the capillary occupied by the sample. In

turn, this length is the product of the solute's velocity (see equation 15.1.6) and time; thus

$$V_{inj} = \pi r^2 L = \pi r^2 (\mu_{ep} + \mu_{eof}) E' t \tag{15.1.13}$$

where r is the capillary's radius, L is the capillary's length, and E' is the effective electric field in the sample. An important consequence of equation 15.1.13 is that an electrokinetic injection is biased toward solutes with larger electrophoretic mobilities. If two solutes have equal concentrations in a sample, we inject a larger volume—and thus more moles—of the solute with the larger μ_{ep}.

> The electric field in the sample is different that the electric field in the rest of the capillary because the sample and the buffer have different ionic compositions. In general, the sample's ionic strength is smaller, which makes its conductivity smaller. The effective electric field is
>
> $$E' = E \times \frac{\chi_{\text{buffer}}}{\chi_{\text{sample}}}$$
>
> where χ_{buffer} and χ_{sample} are the conductivities of the buffer and the sample, respectively.

When an analyte's concentration is too small to detect reliably, it maybe possible to inject it in a manner that increases its concentration. This method of injection is called ***stacking***. Stacking is accomplished by placing the sample in a solution whose ionic strength is significantly less than that of the buffer in the capillary tube. Because the sample plug has a lower concentration of buffer ions, the effective field strength across the sample plug, E', is larger than that in the rest of the capillary.

We know from equation 15.1.1 that electrophoretic velocity is directly proportional to the electrical field. As a result, the cations in the sample plug migrate toward the cathode with a greater velocity, and the anions migrate more slowly—neutral species are unaffected and move with the electroosmotic flow. When the ions reach their respective boundaries between the sample plug and the buffer, the electrical field decreases and the electrophoretic velocity of the cations decreases and that for the anions increases. As shown in Figure **15.1.6**, the result is a stacking of cations and anions into separate, smaller sampling zones. Over time, the buffer within the capillary becomes more homogeneous and the separation proceeds without additional stacking.

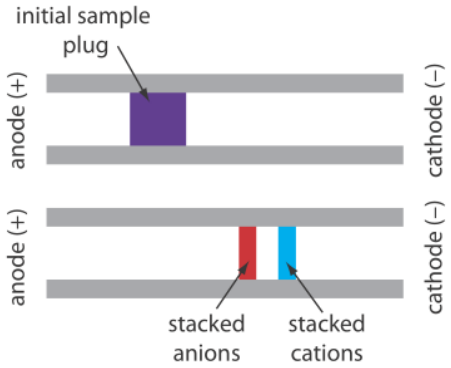


Figure 15.1.6. The stacking of cations and anions. The top diagram shows the initial sample plug and the bottom diagram shows how the cations and anions are concentrated at opposite sides of the sample plug.

Applying the Electrical Field

Migration in electrophoresis occurs in response to an applied electric field. The ability to apply a large electric field is important because higher voltages lead to shorter analysis times (equation 15.1.9), more efficient separations (equation 15.1.10), and better resolution (equation 15.1.11). Because narrow bored capillary tubes dissipate Joule heating so efficiently, voltages of up to 40 kV are possible.

> Because of the high voltages, be sure to follow your instrument's safety guidelines.

Detectors

Most of the detectors used in HPLC also find use in capillary electrophoresis. Among the more common detectors are those based on the absorption of UV/Vis radiation, fluorescence, conductivity, amperometry, and mass spectrometry. Whenever possible,

detection is done “on-column” before the solutes elute from the capillary tube and additional band broadening occurs.

UV/Vis detectors are among the most popular. Because absorbance is directly proportional to path length, the capillary tubing’s small diameter leads to signals that are smaller than those obtained in HPLC. Several approaches have been used to increase the pathlength, including a Z-shaped sample cell and multiple reflections (see Figure **15.1.7**). Detection limits are about 10^{-7} M.

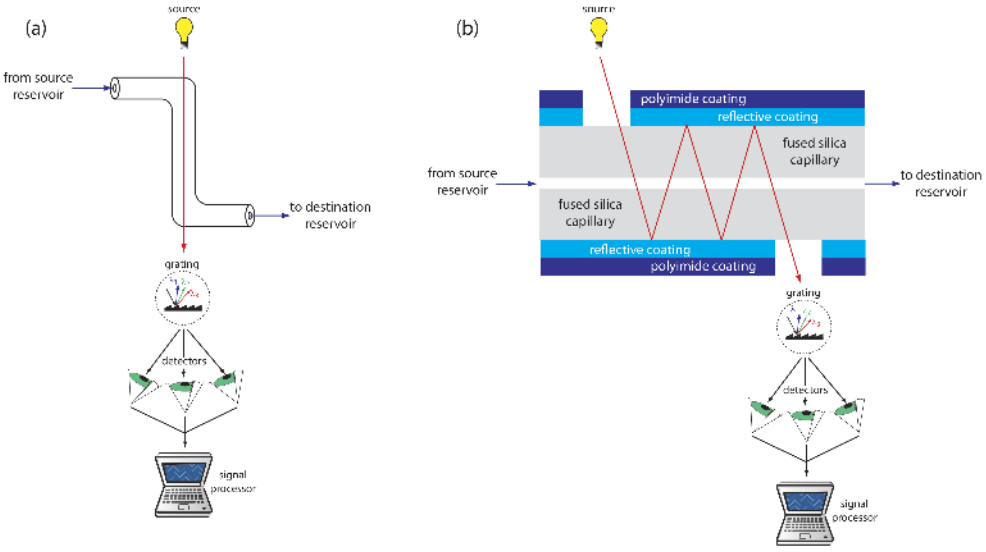


Figure 15.1.7. Two approaches to on-column detection in capillary electrophoresis using a UV/Vis diode array spectrometer: (a) Z-shaped bend in capillary, and (b) multiple reflections.

Better detection limits are obtained using fluorescence, particularly when using a laser as an excitation source. When using fluorescence detection a small portion of the capillary’s protective coating is removed and the laser beam is focused on the inner portion of the capillary tubing. Emission is measured at an angle of 90º to the laser. Because the laser provides an intense source of radiation that can be focused to a narrow spot, detection limits are as low as 10^{-16} M.

Solutes that do not absorb UV/Vis radiation or that do not undergo fluorescence can be detected by other detectors. Table **15.1.1** provides a list of detectors for capillary electrophoresis along with some of their important characteristics.

Table **15.1.1**. Characteristics of Detectors for Capillary Electrophoresis

detector	selectivity (universal or analyte must ...)	detection limited (moles injected)	detection limit (molarity)	on-column detection?
UV/Vis absorbance	have a UV/Vis chromophore	$10^{-13} - 10^{-16}$	$10^{-5} - 10^{-7}$	yes
indirect absorbancd	universal	$10^{-12} - 10^{-15}$	$10^{-4} - 10^{-6}$	yes
fluoresence	have a favorable quantum yield	$10^{-13} - 10^{-17}$	$10^{-7} - 10^{-9}$	yes
laser fluorescence	have a favorable quantum yield	$10^{-18} - 10^{-20}$	$10^{-13} - 10^{-16}$	yes
mass spectrometer	universal (total ion) selective (single ion)	$10^{-16} - 10^{-17}$	$10^{-8} - 10^{-10}$	no
amperometry	undergo oxidation or reduction	$10^{-18} - 10^{-19}$	$10^{-7} - 10^{-10}$	no
conductivity	universal	$10^{-15} - 10^{-16}$	$10^{-7} - 10^{-9}$	no
radiometric	be radioactive	$10^{-17} - 10^{-19}$	$10^{-10} - 10^{-12}$	yes

Capillary Electrophoresis Methods

There are several different forms of capillary electrophoresis, each of which has its particular advantages. Four of these methods are described briefly in this section.

Capillary Zone Electrophoresis (CZE)

The simplest form of capillary electrophoresis is ***capillary zone electrophoresis***. In CZE we fill the capillary tube with a buffer and, after loading the sample, place the ends of the capillary tube in reservoirs that contain additional buffer. Usually the end of the capillary containing the sample is the anode and solutes migrate toward the cathode at a velocity determined by their respective electrophoretic mobilities and the electroosmotic flow. Cations elute first, with smaller, more highly charged cations eluting before larger cations with smaller charges. Neutral species elute as a single band. Anions are the last species to elute, with smaller, more negatively charged anions being the last to elute.

We can reverse the direction of electroosmotic flow by adding an alkylammonium salt to the buffer solution. As shown in Figure **15.1.8**, the positively charged end of the alkyl ammonium ions bind to the negatively charged silanate ions on the capillary's walls. The tail of the alkyl ammonium ion is hydrophobic and associates with the tail of another alkyl ammonium ion. The result is a layer of positive charges that attract anions in the buffer. The migration of these solvated anions toward the anode reverses the electroosmotic flow's direction. The order of elution is exactly opposite that observed under normal conditions.

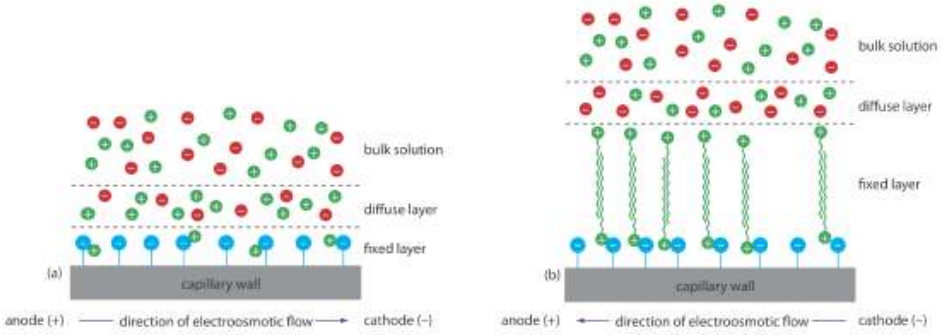


Figure 15.1.8. Two modes of capillary zone electrophoresis showing (a) normal migration with electroosmotic flow toward the cathode and (b) reversed migration in which the electroosmotic flow is toward the anode.

Coating the capillary's walls with a nonionic reagent eliminates the electroosmotic flow. In this form of CZE the cations migrate from the anode to the cathode. Anions elute into the source reservoir and neutral species remain stationary.

Capillary zone electrophoresis provides effective separations of charged species, including inorganic anions and cations, organic acids and amines, and large biomolecules such as proteins. For example, CZE was used to separate a mixture of 36 inorganic and organic ions in less than three minutes [Jones, W. R.; Jandik, P. *J. Chromatog.* **1992**, *608*, 385–393]. A mixture of neutral species, of course, can not be resolved.

Micellar Electrokinetic Capillary Chromatography (MEKC)

One limitation to CZE is its inability to separate neutral species. ***Micellar electrokinetic capillary chromatography*** overcomes this limitation by adding a surfactant, such as sodium dodecylsulfate (Figure **15.1.9**a) to the buffer solution. Sodium dodecylsulfate, or SDS, consists of a long-chain hydrophobic tail and a negatively charged ionic functional group at its head. When the concentration of SDS is sufficiently large, above the critical micelle concnetration, a micelle forms. A micelle consists of a spherical agglomeration of 40–100 surfactant molecules in which the hydrocarbon tails point inward and the negatively charged heads point outward (Figure **15.1.9**b).

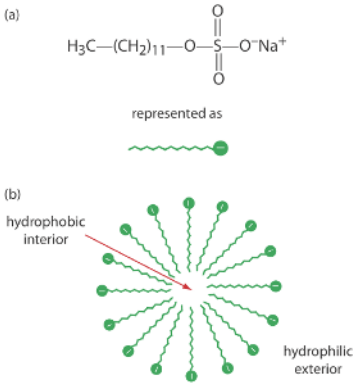


Figure 15.1.9. (a) Structure of sodium dodecylsulfate and (b) cross section through a micelle showing its hydrophobic interior and its hydrophilic exterior.

Because SDS micelles have a negative charge, they migrate toward the cathode with a velocity less than the electroosmotic flow velocity. Neutral species partition themselves between the micelles and the buffer solution in a manner similar to the partitioning of solutes between the two liquid phases in HPLC. This is illustrated in Figure **15.1.10**. Because there is a partitioning between two phases, we include the descriptive term chromatography in the techniques name. Note that in MEKC both phases are mobile and positively charged micelles can be formed using cetylammonium bromide.

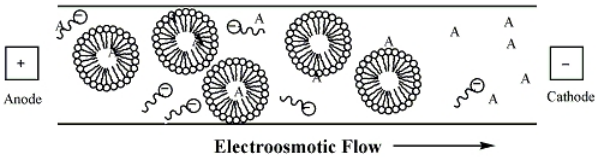


Figure **15.1.10**: A schematic illustrating the partitioning of a solute A in and out of a micelle. While the micelles have a negative charge, they are carried from the anode to the cathode due to the *eof*, albeit at a slower rate because their migration towards the anode. Image source currently unknown.

The elution order for neutral species in MEKC depends on the extent to which each species partitions into the micelles. Hydrophilic neutrals are insoluble in the micelle's hydrophobic inner environment and elute as a single band, as they would in CZE. Neutral solutes that are extremely hydrophobic are completely soluble in the micelle, eluting with the micelles as a single band. Those neutral species that exist in a partition equilibrium between the buffer and the micelles elute between the completely hydrophilic and completely hydrophobic neutral species. Those neutral species that favor the buffer elute before those favoring the micelles. Micellar electrokinetic chromatography is used to separate a wide variety of samples, including mixtures of pharmaceutical compounds, vitamins, and explosives.

The addition of micelles formed from chiral additives combined with the tremendous separation efficiency of capillary electrophoresis due to the plug flow yields the most powerful methods for chiral separations. Chiral additives of the type shown in Figure **15.1.11**

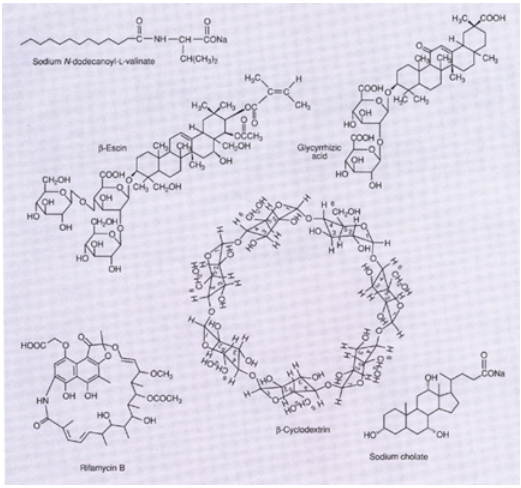


Figure 15.1.11: Examples of the micelle forming chiral additives commonly used in MEKC for recemic mixtures of isomers. Image source Analytical Chemistry, Vol. 66, No. 11, June 1, 1994 633 A

Capillary Gel Electrophoresis (CGE)

In ***capillary gel electrophoresis*** the capillary tubing is filled with a polymeric gel. Because the gel is porous, a solute migrates through the gel with a velocity determined both by its electrophoretic mobility and by its size. The ability to effect a separation using size is helpful when the solutes have similar electrophoretic mobilities. For example, fragments of DNA of varying length have similar charge-to-size ratios, making their separation by CZE difficult. Because the DNA fragments are of different size, a CGE separation is possible.

The capillary used for CGE usually is treated to eliminate electroosmotic flow to prevent the gel from extruding from the capillary tubing. Samples are injected electrokinetically because the gel provides too much resistance for hydrodynamic sampling. The primary application of CGE is the separation of large biomolecules, including DNA fragments, proteins, and oligonucleotides.

Capillary Electrochromatography (CEC)

Another approach to separating neutral species is ***capillary electrochromatography***. In CEC the capillary tubing is packed with 1.5–3 μm particles coated with a bonded stationary phase. Neutral species separate based on their ability to partition between the stationary phase and the buffer, which is moving as a result of the electroosmotic flow; Figure **15.1.12** provides a representative example for the separation of a mixture of hydrocarbons. A CEC separation is similar to the analogous HPLC separation, but without the need for high pressure pumps. Efficiency in CEC is better than in HPLC, and analysis times are shorter.

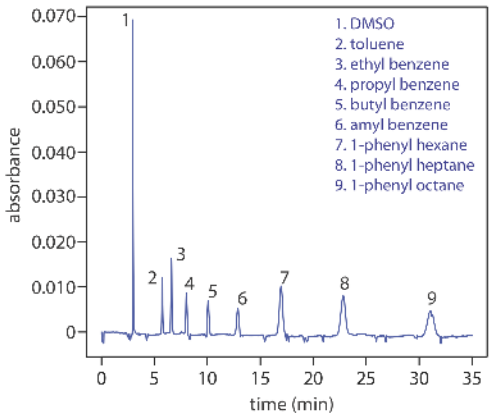


Figure 15.1.12. Capillary electrochromatographic separation of a mixture of hydrocarbons in DMSO. The column contains a porous polymer of butyl methacrylate and lauryl acrylate (25%:75% mol:mol) with butane dioldacrylate as a crosslinker. Data provided by Zoe LaPier and Michelle Bushey, Department of Chemistry, Trinity University.

The best way to appreciate the theoretical and the practical details discussed in this section is to carefully examine a typical analytical method. Although each method is unique, the following description of the determination of a vitamin B complex by capillary zone electrophoresis or by micellar electrokinetic capillary chromatography provides an instructive example of a typical procedure. The description here is based on Smyth, W. F. *Analytical Chemistry of Complex Matrices*, Wiley Teubner: Chichester, England, 1996, pp. 154–156.

Representative Method 12.7.1: Determination of Vitamin B Complex by CZE or MEKC

Description of Method

The water soluble vitamins B_1 (thiamine hydrochloride), B_2 (riboflavin), B_3 (niacinamide), and B_6 (pyridoxine hydrochloride) are determined by CZE using a pH 9 sodium tetraborate-sodium dihydrogen phosphate buffer, or by MEKC using the same buffer with the addition of sodium dodecyl sulfate. Detection is by UV absorption at 200 nm. An internal standard of *o*-ethoxybenzamide is used to standardize the method.

Procedure

Crush a vitamin B complex tablet and place it in a beaker with 20.00 mL of a 50 % v/v methanol solution that is 20 mM in sodium tetraborate and 100.0 ppm in *o*-ethoxybenzamide. After mixing for 2 min to ensure that the B vitamins are dissolved, pass a 5.00-mL portion through a 0.45-μm filter to remove insoluble binders. Load an approximately 4 nL sample into a capillary column with an inner diameter of a 50 μm. For CZE the capillary column contains a 20 mM pH 9 sodium tetraborate-sodium dihydrogen phosphate buffer. For MEKC the buffer is also 150 mM in sodium dodecyl sulfate. Apply a 40 kV/m electrical field to effect both the CZE and MEKC separations.

Questions

1. Methanol, which elutes at 4.69 min, is included as a neutral species to indicate the electroosmotic flow. When using standard solutions of each vitamin, CZE peaks are found at 3.41 min, 4.69 min, 6.31 min, and 8.31 min. Examine the structures and pK_a information in Figure **15.1.13** and identify the order in which the four B vitamins elute.

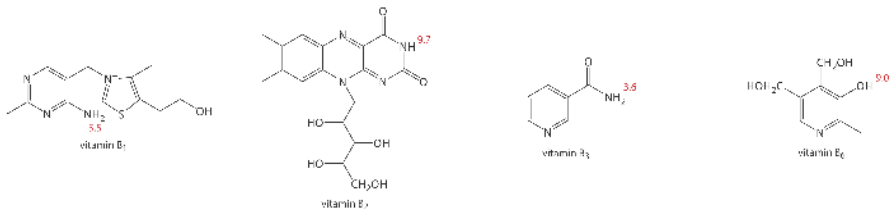


Figure 15.1.13. Structures of the four water soluble B vitamins in their predominate forms at a pH of 9; pK_a values are shown in red.

At a pH of 9, vitamin B_1 is a cation and elutes before the neutral species methanol; thus it is the compound that elutes at 3.41 min. Vitamin B_3 is a neutral species at a pH of 9 and elutes with methanol at 4.69 min. The remaining two B vitamins are weak acids that partially ionize to weak base anions at a pH of 9. Of the two, vitamin B_6 is the stronger acid (a pK_a of 9.0 versus a pK_a of 9.7) and is present to a greater extent in its anionic form. Vitamin B_6, therefore, is the last of the vitamins to elute.

2. The order of elution when using MEKC is vitamin B_3 (5.58 min), vitamin B_6 (6.59 min), vitamin B_2 (8.81 min), and vitamin B_1 (11.21 min). What conclusions can you make about the solubility of the B vitamins in the sodium dodecylsulfate micelles? The micelles elute at 17.7 min.

The elution time for vitamin B_1 shows the greatest change, increasing from 3.41 min to 11.21 minutes. Clearly vitamin B_1 has the greatest solubility in the micelles. Vitamin B_2 and vitamin B3 have a more limited solubility in the micelles, and show only slightly longer elution times in the presence of the micelles. Interestingly, the elution time for vitamin B_6 decreases in the presence of the micelles.

3. For quantitative work an internal standard of *o*-ethoxybenzamide is added to all samples and standards. Why is an internal standard necessary?

Although the method of injection is not specified, neither a hydrodynamic injection nor an electrokinetic injection is particularly reproducible. The use of an internal standard compensates for this limitation.

Evaluation

When compared to GC and HPLC, capillary electrophoresis provides similar levels of accuracy, precision, and sensitivity, and it provides a comparable degree of selectivity. The amount of material injected into a capillary electrophoretic column is significantly smaller than that for GC and HPLC—typically 1 nL versus 0.1 μL for capillary GC and 1–100 μL for HPLC. Detection limits for capillary electrophoresis, however, are 100–1000 times poorer than that for GC and HPLC. The most significant advantages of capillary electrophoresis are improvements in separation efficiency, time, and cost. Capillary electrophoretic columns contain substantially more theoretical plates ($\approx 10^6$ plates/m) than that found in HPLC ($\approx 10^5$ plates/m) and capillary GC columns ($\approx 10^3$ plates/m), providing unparalleled resolution and peak capacity. Separations in capillary electrophoresis are fast and efficient. Furthermore, the capillary column's small volume means that a capillary electrophoresis separation requires only a few microliters of buffer, compared to 20–30 mL of mobile phase for a typical HPLC separation.

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15.2: Problems

1. The following data were obtained for four compounds separated on a 20-m capillary column.

compound	t_r (min)	w (min)
A	8.04	0.15
B	8.26	0.15
C	8.43	0.16

(a) Calculate the number of theoretical plates for each compound and the average number of theoretical plates for the column, in mm.

(b) Calculate the average height of a theoretical plate.

(c) Explain why it is possible for each compound to have a different number of theoretical plates.

2. Using the data from Problem 1, calculate the resolution and the selectivity factors for each pair of adjacent compounds. For resolution, use both equation 12.2.1 and equation 12.3.3, and compare your results. Discuss how you might improve the resolution between compounds B and C. The retention time for an nonretained solute is 1.19 min.

3. Use the chromatogram in Figure **15.2.1**, obtained using a 2-m column, to determine values for t_r, w, t'_r, k, N, and H.

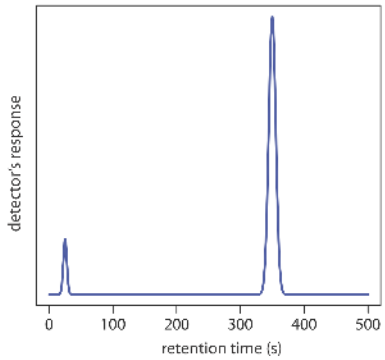


Figure 15.2.1. Chromatogram for Problem 3.

4. Use the partial chromatogram in Figure **15.2.2** to determine the resolution between the two solute bands.

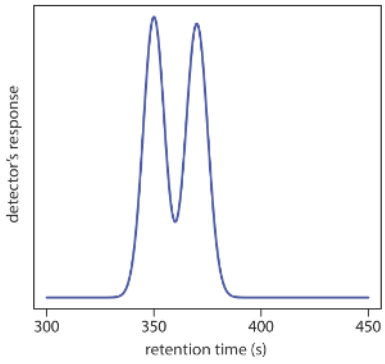


Figure 15.2.2. Chromatogram for Problem 4.

5. The chromatogram in Problem 4 was obtained on a 2-m column with a column dead time of 50 s. Suppose you want to increase the resolution between the two components to 1.5. Without changing the height of a theoretical plate, what length column do you need? What height of a theoretical plate do you need to achieve a resolution of 1.5 without increasing the column's length?

6. Complete the following table.

N_B	α	k_B	R
100000	1.05	0.50	
10000	1.10		1.50
10000		4.0	1.00
	1.05	3.0	1.75

7. Moody studied the efficiency of a GC separation of 2-butanone on a dinonyl phthalate packed column [Moody, H. W. *J. Chem. Educ.* **1982**, *59*, 218–219]. Evaluating plate height as a function of flow rate gave a van Deemter equation for which A is 1.65 mm, B is 25.8 mm•mL min^{-1}, and C is 0.0236 mm•min mL^{-1}.

(a) Prepare a graph of H versus u for flow rates between 5 –120 mL/min.

(b) For what range of flow rates does each term in the Van Deemter equation have the greatest effect?

(c) What is the optimum flow rate and the corresponding height of a theoretical plate?

(d) For open-tubular columns the A term no longer is needed. If the B and C terms remain unchanged, what is the optimum flow rate and the corresponding height of a theoretical plate?

(e) Compared to the packed column, how many more theoretical plates are in the open-tubular column?

8. Hsieh and Jorgenson prepared 12–33 μm inner diameter HPLC columns packed with 5.44-μm spherical stationary phase particles [Hsieh, S.; Jorgenson, J. W. *Anal. Chem.* **1996**, *68*, 1212–1217]. To evaluate these columns they measured reduced plate height, h, as a function of reduced flow rate, v,

$$h = \frac{H}{d_p} \quad v = \frac{ud_p}{D_m}$$

where d_p is the particle diameter and D_m is the solute's diffusion coefficient in the mobile phase. The data were analyzed using van Deemter plots. The following table contains a portion of their results for norepinephrine.

internal diameter (μm)	A	B	C
33	0.63	1.32	0.10
33	0.67	1.30	0.08
23	0.40	1.34	0.09
23	0.58	1.11	0.09
17	0.31	1.47	0.11
17	0.40	1.41	0.11
12	0.22	1.53	0.11
12	0.19	1.27	0.12

(a) Construct separate van Deemter plots using the data in the first row and in the last row for reduced flow rates in the range 0.7–15. Determine the optimum flow rate and plate height for each case given d_p = 5.44 μm and D_m = $\mathbf{6.23 \times 10^{-6}}$ cm^2 s^{-1}.

(b) The A term in the van Deemter equation is strongly correlated with the column's inner diameter, with smaller diameter columns providing smaller values of A. Offer an explanation for this observation. *Hint: consider how many particles can fit across a capillary of each diameter.*

> When comparing columns, chromatographers often use dimensionless, reduced parameters. By including particle size and the solute's diffusion coefficient, the reduced plate height and reduced flow rate correct for differences between the packing

material, the solute, and the mobile phase.

9. A mixture of *n*-heptane, tetrahydrofuran, 2-butanone, and *n*-propanol elutes in this order when using a polar stationary phase such as Carbowax. The elution order is exactly the opposite when using a nonpolar stationary phase such as polydimethyl siloxane. Explain the order of elution in each case.

10. The analysis of trihalomethanes in drinking water is described in Representative Method 12.4.1. A single standard that contains all four trihalomethanes gives the following results.

compound	concentration (ppb)	peak area
$CHCl_3$	1.30	1.35×10^4
$CHCl_2Br$	0.90	6.12×10^4
$CHClBr_2$	4.00	1.71×10^4
$CHBr_3$	1.20	1.52×10^4

Analysis of water collected from a drinking fountain gives areas of 1.56×10^4, 5.13×10^4, 1.49×10^4, and 1.76×10^4 for, respectively, $CHCl_3$, $CHCl_2Br$, $CHClBr_2$, and $CHBr_3$. All peak areas were corrected for variations in injection volumes using an internal standard of 1,2-dibromopentane. Determine the concentration of each of the trihalomethanes in the sample of water.

11. Zhou and colleagues determined the %w/w H_2O in methanol by capillary column GC using a nonpolar stationary phase and a thermal conductivity detector [Zhou, X.; Hines, P. A.; White, K. C.; Borer, M. W. *Anal. Chem.* **1998**, *70*, 390–394]. A series of calibration standards gave the following results.

%w/w H_2O	peak height (arb. units)
0.00	1.15
0.0145	2.74
0.0472	6.33
0.0951	11.58
0.1757	20.43
0.2901	32.97

(a) What is the %w/w H_2O in a sample that has a peak height of 8.63?

(b) The %w/w H_2O in a freeze-dried antibiotic is determined in the following manner. A 0.175-g sample is placed in a vial along with 4.489 g of methanol. Water in the vial extracts into the methanol. Analysis of the sample gave a peak height of 13.66. What is the %w/w H_2O in the antibiotic?

12. Loconto and co-workers describe a method for determining trace levels of water in soil [Loconto, P. R.; Pan, Y. L.; Voice, T. C. *LC•GC* **1996**, *14*, 128–132]. The method takes advantage of the reaction of water with calcium carbide, CaC_2, to produce acetylene gas, C_2H_2. By carrying out the reaction in a sealed vial, the amount of acetylene produced is determined by sampling the headspace. In a typical analysis a sample of soil is placed in a sealed vial with CaC_2. Analysis of the headspace gives a blank corrected signal of 2.70×10^5. A second sample is prepared in the same manner except that a standard addition of 5.0 mg H_2O/g soil is added, giving a blank-corrected signal of 1.06×10^6. Determine the milligrams H_2O/g soil in the soil sample.

13. Van Atta and Van Atta used gas chromatography to determine the %v/v methyl salicylate in rubbing alcohol [Van Atta, R. E.; Van Atta, R. L. *J. Chem. Educ.* **1980**, *57*, 230–231]. A set of standard additions was prepared by transferring 20.00 mL of rubbing alcohol to separate 25-mL volumetric flasks and pipeting 0.00 mL, 0.20 mL, and 0.50 mL of methyl salicylate to the flasks. All three flasks were diluted to volume using isopropanol. Analysis of the three samples gave peak heights for methyl salicylate of 57.00 mm, 88.5 mm, and 132.5 mm, respectively. Determine the %v/v methyl salicylate in the rubbing alcohol.

14. The amount of camphor in an analgesic ointment is determined by GC using the method of internal standards [Pant, S. K.; Gupta, P. N.; Thomas, K. M.; Maitin, B. K.; Jain, C. L. *LC•GC* **1990**, *8*, 322–325]. A standard sample is prepared by placing 45.2 mg of camphor and 2.00 mL of a 6.00 mg/mL internal standard solution of terpene hydrate in a 25-mL volumetric flask and diluting to volume with CCl_4. When an approximately 2-µL sample of the standard is injected, the FID signals for the two components are measured (in arbitrary units) as 67.3 for camphor and 19.8 for terpene hydrate. A 53.6-mg sample of an analgesic ointment is prepared for analysis by placing it in a 50-mL Erlenmeyer flask along with 10 mL of CCl_4. After heating to 50°C in a water bath, the sample is cooled to below room temperature and filtered. The residue is washed with two 5-mL portions of CCl_4 and the combined filtrates are collected in a 25-mL volumetric flask. After adding 2.00 mL of the internal standard solution, the contents of the flask are diluted to volume with CCl_4. Analysis of an approximately 2-µL sample gives FID signals of 13.5 for the terpene hydrate and 24.9 for the camphor. Report the %w/w camphor in the analgesic ointment.

15. The concentration of pesticide residues on agricultural products, such as oranges, is determined by GC-MS [Feigel, C. *Varian GC/MS Application Note*, Number 52]. Pesticide residues are extracted from the sample using methylene chloride and concentrated by evaporating the methylene chloride to a smaller volume. Calibration is accomplished using anthracene-d_{10} as an internal standard. In a study to determine the parts per billion heptachlor epoxide on oranges, a 50.0-g sample of orange rinds is chopped and extracted with 50.00 mL of methylene chloride. After removing any insoluble material by filtration, the methylene chloride is reduced in volume, spiked with a known amount of the internal standard and diluted to 10 mL in a volumetric flask. Analysis of the sample gives a peak–area ratio ($A_{analyte}/A_{intstd}$) of 0.108. A series of calibration standards, each containing the same amount of anthracene-d_{10} as the sample, gives the following results.

ppb heptachlor epoxide	$A_{analyte}/A_{intstd}$
20.0	0.065
60.0	0.153
200.0	0.637
500.0	1.554
1000.0	3.198

Report the nanograms per gram of heptachlor epoxide residue on the oranges.

16. The adjusted retention times for octane, toluene, and nonane on a particular GC column are 15.98 min, 17.73 min, and 20.42 min, respectively. What is the retention index for each compound?

17. The following data were collected for a series of normal alkanes using a stationary phase of Carbowax 20M.

alkane	t_r' (min)
pentane	0.79
hexane	1.99
heptane	4.47
octane	14.12
nonane	33.11

What is the retention index for a compound whose adjusted retention time is 9.36 min?

18. The following data were reported for the gas chromatographic analysis of *p*-xylene and methylisobutylketone (MIBK) on a capillary column [Marriott, P. J.; Carpenter, P. D. *J. Chem. Educ.* **1996**, *73*, 96–99].

injection mode	compound	t_r (min)	peak area (arb. units)	peak width (min)
split	MIBK	1.878	54285	0.028
	p-xylene	5.234	123483	0.044
splitless	MIBK	3.420	2493005	1.057
	p-xylene	5.795	3396656	1.051

Explain the difference in the retention times, the peak areas, and the peak widths when switching from a split injection to a splitless injection.

19. Otto and Wegscheider report the following retention factors for the reversed-phase separation of 2-aminobenzoic acid on a C_{18} column when using 10% v/v methanol as a mobile phase [Otto, M.; Wegscheider, W. *J. Chromatog.* **1983**, *258*, 11–22].

pH	*k*
2.0	10.5
3.0	16.7
4.0	15.8
5.0	8.0
6.0	2.2
7.0	1.8

Explain the effect of pH on the retention factor for 2-aminobenzene.

20. Haddad and associates report the following retention factors for the reversed-phase separation of salicylamide and caffeine [Haddad, P.; Hutchins, S.; Tuffy, M. *J. Chem. Educ.* **1983**, *60*, 166-168].

%v/v methanol	30%	35%	40%	45%	50%	55%
k_{sal}	2.4	1.6	1.6	1.0	0.7	0.7
k_{caff}	4.3	2.8	2.3	1.4	1.1	0.9

(a) Explain the trends in the retention factors for these compounds.

(b) What is the advantage of using a mobile phase with a smaller %v/v methanol? Are there any disadvantages?

21. Suppose you need to separate a mixture of benzoic acid, aspartame, and caffeine in a diet soda. The following information is available.

	t_r in aqueous mobile phase of pH			
compound	3.0	3.5	4.0	4.5
benzoic acid	7.4	7.0	6.9	4.4
aspartame	5.9	6.0	7.1	8.1
caffeine	3.6	3.7	4.1	4.4

(a) Explain the change in each compound's retention time.

(b) Prepare a single graph that shows retention time versus pH for each compound. Using your plot, identify a pH level that will yield an acceptable separation.

22. The composition of a multivitamin tablet is determined using an HPLC with a diode array UV/Vis detector. A 5-µL standard sample that contains 170 ppm vitamin C, 130 ppm niacin, 120 ppm niacinamide, 150 ppm pyridoxine, 60 ppm thiamine, 15 ppm folic acid, and 10 ppm riboflavin is injected into the HPLC, giving signals (in arbitrary units) of, respectively, 0.22, 1.35, 0.90, 1.37, 0.82, 0.36, and 0.29. The multivitamin tablet is prepared for analysis by grinding into a powder and transferring to a 125-mL Erlenmeyer flask that contains 10 mL of 1% v/v NH_3 in dimethyl sulfoxide. After sonicating in an ultrasonic bath for 2 min, 90 mL of 2% acetic acid is added and the mixture is stirred for 1 min and sonicated at 40°C for 5 min. The extract is then filtered through a 0.45-µm membrane filter. Injection of a 5-µL sample into the HPLC gives signals of 0.87 for vitamin C, 0.00 for niacin, 1.40 for niacinamide, 0.22 for pyridoxine, 0.19 for thiamine, 0.11 for folic acid, and 0.44 for riboflavin. Report the milligrams of each vitamin present in the tablet.

23. The amount of caffeine in an analgesic tablet was determined by HPLC using a normal calibration curve. Standard solutions of caffeine were prepared and analyzed using a 10-µL fixed-volume injection loop. Results for the standards are summarized in the following table.

concentration (ppm)	signal (arb. units)
50.0	8.354
100.0	16925
150.0	25218
200.0	33584
250.0	42002

The sample is prepared by placing a single analgesic tablet in a small beaker and adding 10 mL of methanol. After allowing the sample to dissolve, the contents of the beaker, including the insoluble binder, are quantitatively transferred to a 25-mL volumetric flask and diluted to volume with methanol. The sample is then filtered, and a 1.00-mL aliquot transferred to a 10-mL volumetric flask and diluted to volume with methanol. When analyzed by HPLC, the signal for caffeine is found to be 21 469. Report the milligrams of caffeine in the analgesic tablet.

24. Kagel and Farwell report a reversed-phase HPLC method for determining the concentration of acetylsalicylic acid (ASA) and caffeine (CAF) in analgesic tablets using salicylic acid (SA) as an internal standard [Kagel, R. A.; Farwell, S. O. *J. Chem. Educ.* **1983**, *60*, 163–166]. A series of standards was prepared by adding known amounts of ace- tylsalicylic acid and caffeine to 250-mL Erlenmeyer flasks and adding 100 mL of methanol. A 10.00-mL aliquot of a standard solution of salicylic acid was then added to each. The following results were obtained for a typical set of standard solutions.

	milligrams of		peak height ratios for	
standard	ASA	CAF	ASA/SA	CAF/SA
1	200.0	20.0	20.5	10.6
2	250.0	40.0	25.1	23.0
3	300.0	60.0	30.9	36.8

A sample of an analgesic tablet was placed in a 250-mL Erlenmeyer flask and dissolved in 100 mL of methanol. After adding a 10.00-mL portion of the internal standard, the solution was filtered. Analysis of the sample gave a peak height ratio of 23.2 for ASA and of 17.9 for CAF.

(a) Determine the milligrams of ASA and CAF in the tablet.

(b) Why is it necessary to filter the sample?

(c) The directions indicate that approximately 100 mL of methanol is used to dissolve the standards and samples. Why is it not necessary to measure this volume more precisely?

(d) In the presence of moisture, ASA decomposes to SA and acetic acid. What complication might this present for this analysis? How might you evaluate whether this is a problem?

25. Bohman and colleagues described a reversed-phase HPLC method for the quantitative analysis of vitamin A in food using the method of standard additions Bohman, O.; Engdahl, K. A.; Johnsson, H. *J. Chem. Educ.* **1982**, *59*, 251–252]. In a typical example, a 10.067-g sample of cereal is placed in a 250-mL Erlenmeyer flask along with 1 g of sodium ascorbate, 40 mL of ethanol, and 10 mL of 50% w/v KOH. After refluxing for 30 min, 60 mL of ethanol is added and the solution cooled to room temperature. Vitamin A is extracted using three 100-mL portions of hexane. The combined portions of hexane are evaporated and the residue containing vitamin A transferred to a 5-mL volumetric flask and diluted to volume with methanol. A standard addition is prepared in a similar manner using a 10.093-g sample of the cereal and spiking with 0.0200 mg of vitamin A. Injecting the sample and standard addition into the HPLC gives peak areas of, respectively, 6.77×10^3 and 1.32×10^4. Report the vitamin A content of the sample in milligrams/100 g cereal.

26. Ohta and Tanaka reported on an ion-exchange chromatographic method for the simultaneous analysis of several inorganic anions and the cations Mg^{2+} and Ca^{2+} in water [Ohta, K.; Tanaka, K. *Anal. Chim. Acta* **1998**, *373*, 189–195]. The mobile phase includes the ligand 1,2,4-benzenetricarboxylate, which absorbs strongly at 270 nm. Indirect detection of the analytes is possible because its absorbance decreases when complexed with an anion.

(a) The procedure also calls for adding the ligand EDTA to the mobile phase. What role does the EDTA play in this analysis?

(b) A standard solution of 1.0 mM $NaHCO_3$, 0.20 mM $NaNO_2$, 0.20 mM $MgSO_4$, 0.10 mM $CaCl_2$, and 0.10 mM $Ca(NO_3)_2$ gives the following peak areas (arbitrary units).

ion	HCO_3^-	Cl^-	NO_2^-	NO_3^-
peak area	373.5	322.5	264.8	262.7
ion	Ca^{2+}	Mg^{2+}	SO_4^{2-}	
peak area	458.9	352.0	341.3	

Analysis of a river water sample (pH of 7.49) gives the following results.

ion	HCO_3^-	Cl^-	NO_2^-	NO_3^-
peak area	310.0	403.1	3.97	157.6
ion	Ca^{2+}	Mg^{2+}	SO_4^{2-}	
peak area	734.3	193.6	324.3	

Determine the concentration of each ion in the sample.

(c) The detection of HCO_3^- actually gives the total concentration of carbonate in solution ($[CO_3^{2-}]+[HCO_3^-]+[H_2CO_3]$). Given that the pH of the water is 7.49, what is the actual concentration of HCO_3^-?

(d) An independent analysis gives the following additional concentrations for ions in the sample: $[Na^+]$ = 0.60 mM; $[NH_4^+]$ = 0.014 mM; and $[K^+]$ = 0.046 mM. A solution's ion balance is defined as the ratio of the total cation charge to the total anion charge. Determine the charge balance for this sample of water and comment on whether the result is reasonable.

27. The concentrations of Cl^-, NO_2^-, and SO_4^{2-} are determined by ion chromatography. A 50-µL standard sample of 10.0 ppm Cl^-, 2.00 ppm NO_2^-, and 5.00 ppm SO_4^{2-} gave signals (in arbitrary units) of 59.3, 16.1, and 6.08 respectively. A sample of effluent from a wastewater treatment plant is diluted tenfold and a 50-µL portion gives signals of 44.2 for Cl^-, 2.73 for NO_2^-, and 5.04 for SO_4^{2-}. Report the parts per million for each anion in the effluent sample.

28. A series of polyvinylpyridine standards of different molecular weight was analyzed by size-exclusion chromatography, yielding the following results.

formula weight	retention volume (mL)
600000	6.42
100000	7.98
30000	9.30
3000	10.94

When a preparation of polyvinylpyridine of unknown formula weight is analyzed, the retention volume is 8.45 mL. Report the average formula weight for the preparation.

29. Diet soft drinks contain appreciable quantities of aspartame, benzoic acid, and caffeine. What is the expected order of elution for these compounds in a capillary zone electrophoresis separation using a pH 9.4 buffer given that aspartame has pK_a values of 2.964 and 7.37, benzoic acid has a pK_a of 4.2, and the pK_a for caffeine is less than 0. Figure **15.2.3** provides the structures of these compounds.

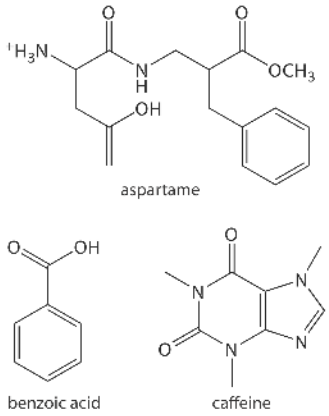


Figure 15.2.3
. Structures for the compounds in Problem 29.

30. Janusa and coworkers describe the determination of chloride by CZE [Janusa, M. A.; Andermann, L. J.; Kliebert, N. M.; Nannie, M. H. *J. Chem. Educ.* **1998**, *75*, 1463–1465]. Analysis of a series of external standards gives the following calibration curve.

$$\text{area} = -883 + 5590 \times \text{ppm } Cl^-$$

A standard sample of 57.22% w/w Cl^- is analyzed by placing 0.1011-g portions in separate 100-mL volumetric flasks and diluting to volume. Three unknowns are prepared by pipeting 0.250 mL, 0.500 mL, an 0.750 mL of the bulk unknown in separate 50-mL volumetric flasks and diluting to volume. Analysis of the three unknowns gives areas of 15 310, 31 546, and 47 582, respectively. Evaluate the accuracy of this analysis.

31. The analysis of NO_3^- in aquarium water is carried out by CZE using IO_4^- as an internal standard. A standard solution of 15.0 ppm NO_3^- and 10.0 ppm IO_4^- gives peak heights (arbitrary units) of 95.0 and 100.1, respectively. A sample of water from an aquarium is diluted 1:100 and sufficient internal standard added to make its concentration 10.0 ppm in IO_4^-. Analysis gives signals of 29.2 and 105.8 for NO_3^- and IO_4^-, respectively. Report the ppm NO_3^- in the sample of aquarium water.

32. Suggest conditions to separate a mixture of 2-aminobenzoic acid (pK_{a1} = 2.08, pK_{a2} = 4.96), benzylamine (pK_a = 9.35), and 4-methylphenol (pK_a = 10.26) by capillary zone electrophoresis. Figure ***PageIndex4*** provides the structures of these compounds.

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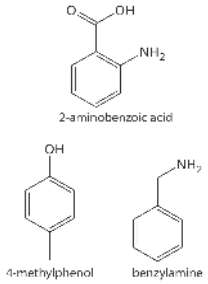


Figure 15.2.4. Structures for the compounds in Problem 32.

33. McKillop and associates examined the electrophoretic separation of some alkylpyridines by CZE [McKillop, A. G.; Smith, R. M.; Rowe, R. C.; Wren, S. A. C. *Anal. Chem.* **1999**, *71*, 497–503]. Separations were carried out using either 50-µm or 75-µm inner diameter capillaries, with a total length of 57 cm and a length of 50 cm from the point of injection to the detector. The run buffer was a pH 2.5 lithium phosphate buffer. Separations were achieved using an applied voltage of 15 kV. The electroosmotic mobility, μ_{eof}, as measured using a neutral marker, was found to be 6.398×10^{-5} $cm^2\ V^{-1}\ s^{-1}$. The diffusion coefficient for alkylpyridines is 1.0×10^{-5} $cm^2\ s^{-1}$.

(a) Calculate the electrophoretic mobility for 2-ethylpyridine given that its elution time is 8.20 min.

(b) How many theoretical plates are there for 2-ethylpyridine?

(c) The electrophoretic mobilities for 3-ethylpyridine and 4-ethylpyridine are 3.366×10^{-4} $cm^2\ V^{-1}\ s^{-1}$ and 3.397×10^{-4} $cm^2\ V^{-1}\ s^{-1}$, respectively. What is the expected resolution between these two alkylpyridines?

(d) Explain the trends in electrophoretic mobility shown in the following table.

alkylpyridine	μ_{ep} ($cm^2\ V^{-1}\ s^{-1}$)
2-methylpyridine	3.581×10^{-4}
2-ethylpyridine	3.222×10^{-4}
2-propylpyridine	2.923×10^{-4}
2-pentylpyridine	2.534×10^{-4}
2-hexylpyridine	2.391×10^{-4}

(e) Explain the trends in electrophoretic mobility shown in the following table.

alkylpyridine	μ_{ep} ($cm^2\ V^{-1}\ s^{-1}$)
2-ethylpyridine	3.222×10^{-4}
3-ethylpyridine	3.366×10^{-4}
4-ethylpyridine	3.397×10^{-4}

(f) The pK_a for pyridine is 5.229. At a pH of 2.5 the electrophoretic mobility of pyridine is 4.176×10^{-4} $cm^2\ V^{-1}\ s^{-1}$. What is the expected electrophoretic mobility if the run buffer's pH is 7.5?

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CHAPTER OVERVIEW

16: Molecular Mass Spectrometry

Mass spectrometry is an analytical technique that ionizes chemical species and sorts the ions based on their mass-to-charge ratio. In simpler terms, a mass spectrum measures the masses within a sample. Mass spectrometry is used in many different fields and is applied to pure samples as well as complex mixtures. A mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio. These spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules, such as peptides and other chemical compounds.

16.1: Mass Spectrometry - The Basic Concepts
16.2: Ionizers
16.3: Mass Analyzers (Mass Spectrometry)
16.4: Ion Detectors
16.5: High Resolution vs Low Resolution
16.6: The Molecular Ion (M^+) Peak
16.7: Molecular Ion and Nitrogen
16.8: The M+1 Peak
16.9: Organic Compounds Containing Halogen Atoms
16.10: Fragmentation Patterns in Mass Spectra
16.11: Electrospray Ionization Mass Spectrometry

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16.1: Mass Spectrometry - The Basic Concepts

This page describes how a mass spectrum is produced using a mass spectrometer.

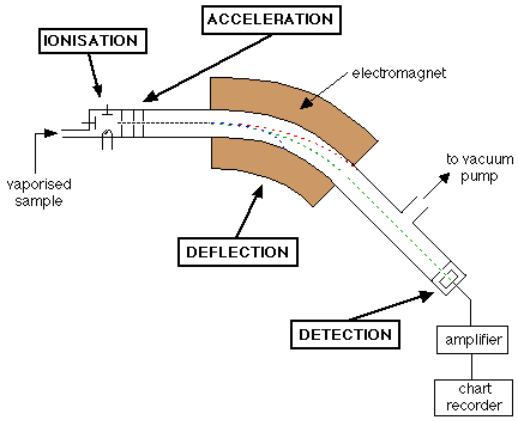
An outline of what happens in a mass spectrometer

Atoms can be deflected by magnetic fields - provided the atom is first turned into an ion. Electrically charged particles are affected by a magnetic field although electrically neutral ones aren't.

The sequence is :

- **Stage 1: Ionization**: Gas phase particles of the sample are ionized through a collision with a high energy electron yielding a positive ion.
- **Stage 2: Acceleration:** The ions are accelerated so that they all have the same kinetic energy and directed into a mass analyzer.
- **Stage 3: Separation according to the mass-to charge-ratio (m/ze) of the ions:** The ions are sorted according to their (m/ze).
- **Stage 4: Detection**: The beam of ions passing through the mass analyzer is detected as a current.

A diagram of a magnetic sector mass spectrometer



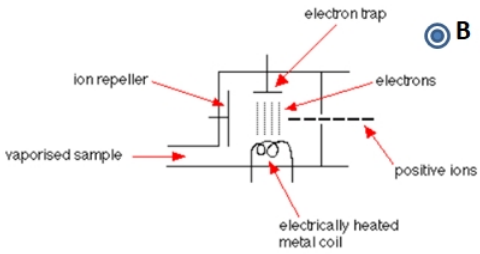
Understanding what's going on

The need for a vacuum

It's important that the ions produced in the ionization chamber can travel from the ionizer, where they are created, through the mass filter, to the detector. The mean free path is the average distance a particle travels before it suffers a collision with another particle. The mean free path is a concept often presented when discussing the Kinetic Molecular Theory in a first year chemistry course. The mean free path for a nitrogen molecule at room temperature and 1 atm pressure is 95 nm (9.5×10^{-9} m). In a room temperature vacuum chamber the mean free path of a nitrogen molecule is 7.2×10^{-5} m when the pressure is 1 mm Hg (1 Torr or 0.0013 atm), 7.2×10^{-2} m when the pressure is 0.001 mm Hg (1 mTorr or 1.3×10^{-6} atm), and 72 cm when the pressure is 1×10^{-6} mm Hg (1×10^{-6} Torr or 1.3×10^{-9} atm). Given the typical dimensions of a mass analyzer and the larger cross section for collisions for an ion relative to a neutral molecule, mass spectrometers need to be operated under condition of high vaucuum, 10^{-9} atm or lower.

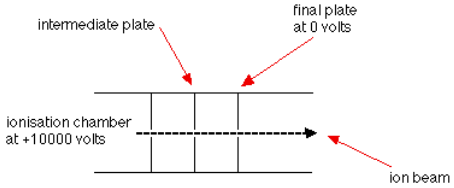
Ionization

The vaporized sample passes into the ionization chamber. In the ionization chamber electrons are produced from a heated filament (coil) by thermionic emission. The electrons are accelerated from the electrically heated metal coil towards the electron trap plate.



The particles in the sample (atoms or molecules) bombarded by the stream of energetic electrons leading to the loss of one or more electrons from the sample particles to make positive ions. Most of the positive ions formed will carry a charge of +1 because it is much more difficult to remove further electrons from an already positive ion. These positive ions are directed out of the ionization chamber towards the mass analyzer by the ion repeller.

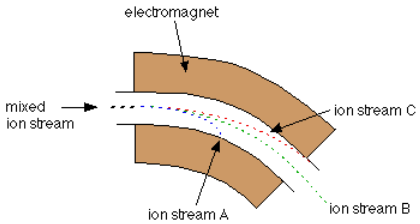
Acceleration



The positive ions are repelled out of the ionizer by the repeller and are accelerated by away additional electron optic plates. The ions are "all" accelerated to the same energy which is based on the difference between the birth potential for the ion and the final acceleration plate. The quotation marks around the worrd all are to indicate there is always some spread in the energy of the accelrated ions.

The Mass Analyzer - sorting the ions according to their mass-to-charge ratio (m/ze)

The mass analyzer is a instrument component that sorts the ions coming from the ionizer according to their (m/ze). In the instrument pictured in this section the mass analyzer is a magnetic sector mass analyter.Different ions are deflected by the magnetic field by different amounts. The magnetic sector mass analyzer is based on deflection of an ion in a magnetic field which follows the right hand rule



Ions with (m/ze) will pass though the analyzer provided the equation below is satisfied. In this equation, B is the magnetic field strength, V is the kinetic energy of the entering ions, and r is the radius of curvature for the path through the magnetic field

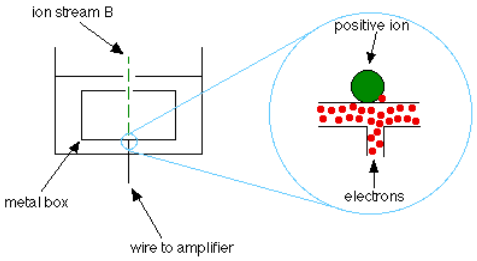
$$\frac{m}{ze} = \frac{B^2r^2}{2V}$$

Ions with (m/ze) for which the magnetic field is too strong will bend such that they will collide with the inner wall and be neutralized (stream A). Ions with (m/ze) for which the magnetic field is too weak will bend such that they will collide with the out wall and be neutralized (stream B)

V and r are fixed for a particular instrument and the mass range (range of (m/ze) is scanned by varying the magnetic field strength, B.

Detection

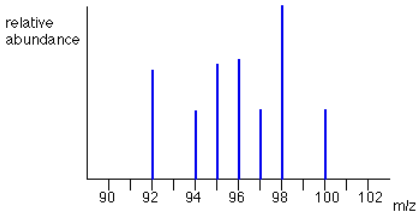
Only ions with (m/ze) satisfying the equation above wil pass through the mass analyzer to the ion detector. th detector pictured below is a Farady Cup detector.



When an ion hits the metal surface on the inside of the Faraday Cup detector the charge of the ion is neutralized by an electron. The flow of electrons in the wire is detected as an electric current which can be amplified and recorded. The more ions arriving, the greater the current.

What the mass spectrometer output looks like

The output from the chart recorder is usually simplified into a "stick diagram". This shows the relative current produced by ions of different m/ze values. The stick diagram for a collection molybdenum ions, (Mn^+) looks similar to the image below:



You may find diagrams in which the vertical axis is labeled as either "relative abundance" or "relative intensity". Whichever is used, it means the same thing. The vertical scale is related to the current received by the chart recorder - and so to the number of ions arriving at the detector: the greater the current, the more abundant the ion.

The collection of peaks in the pictured mass spectrum is due to the natural abundance of the ions found in a large collection of molybdenum (Mo) ions. The singly charged ion of the most abundant isotope of Mo has a (m/ze) = 98. Other ions coming from the isotopes of Mo have (m/ze) values of 92, 94, 95, 96, 97 and 100.

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SECTION OVERVIEW

16.2: Ionizers

The function of an ionizer is to convert the particles in a sample into gas phase ions. In addition to the types of samples each ionizer can handle, the big distinction is whether the ionization process is hard or soft. Hard ionizers produce ions with a great deal of excess internal energy leading to fragmentation. Hard ionizers less likely to produce the molecular ion, M^+. Soft ionizers produce considerably less fragment ions and are very likely to produce the molecular ion or a quasi molecular ion. A quasimolecular ion is an ion formed by the association of the molecule, M and a known charged species, i.e. MH^+ or MNa^+.

Figure **16.2.1** gives an example of hard ionization versus soft ionization for the nerve gas agent VX.

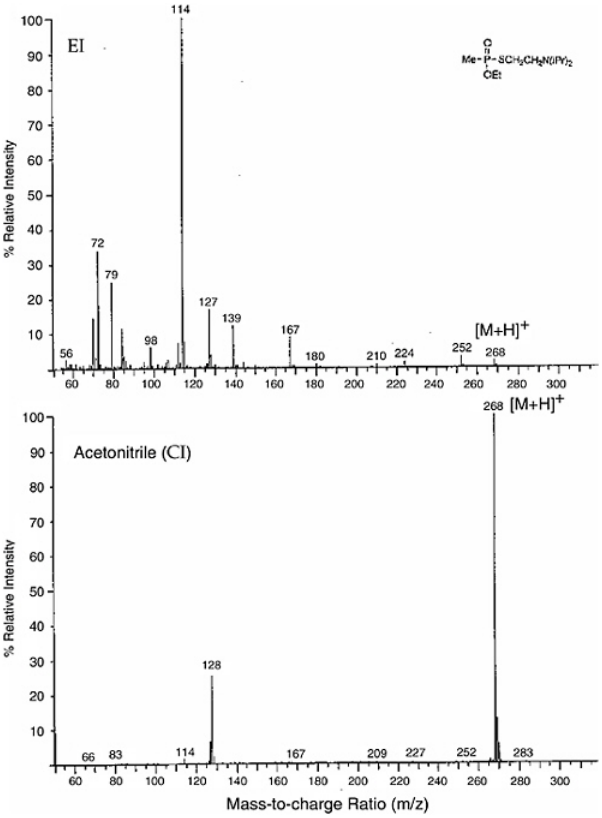


Figure **16.2.1**: The mass spectra obtained for agent VX using electron impact ionization (hard) and chemical ionization (soft). Image from www.nap.edu but presented in https://socratic.org/questions/when-...bardment-ei-ms

The intensity of the peak for the quasi molecular ion of agent VX ($VX\text{-}H^+$) is much more intense with CI and there is only one significant fragment ion (daughter ion) peak at (m/ze) = 128 coresponding to the loss of the neutral fragment associated with rupture of the S - C bond. electron impact ionization produced a spectrum with many more fragment ion peaks.

The Electron Impact Ionizer

The electron impact ionizer (EI) is a hard ionization method. As shown below in Figure **16.2.2**, electrons emitted thermionic emission from a hot filament are accelerated across the ionizer. Energetic collisions between the accelerated electrons and gas phase sample species, M. The collision results in the loss of one (or more) electrons; $M + e^- \rightarrow M^+ + 2e^-$.

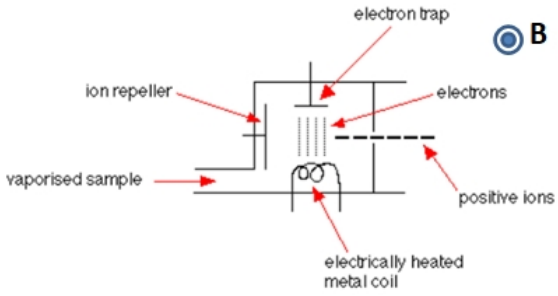


Figure **16.2.2**: A simple sketch of an electron impact ionizer. The magnetic field is increases the path of the electrons across the ionizer leading to more ionizing collisions.

A generic organic molecule has an ionization energy on the order of 5 eV (500 kJ/mole). So electrons with kinetic energies > 5 eV will produce ions with the degree of fragmentation increasing as the kinetic energy of the electrons increases. However at low electron kinetic energies all molecules are not ionized equally well. A electron kinetic energy of 70 eV is commonly employed for EI ionization and mass spectrometry libraries are based on this value

Chemical Ionization

Chemical ionization is a soft ionization method based on ion-molecule reactions occurring in an electron impact ionizer. As shown in Figure **16.2.3** reagent gas molecules are introduced at a concentration about 100X greater than the sample particles. The reagent molecules, R, are ionized by the energetic electrons to for a reagent ion, R+. The reagent ions react with the sample molecules, S, to produce the ions, S+, to be sent to the mass analyzer.

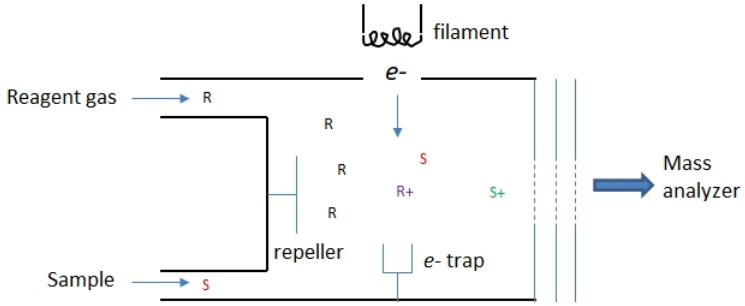


Figure **16.2.3**: A simple sketch of a chemical ionization source.

Tehe reagent ions employed in chemical ionization are commonly based on methane (CH_5^+, $C_2H_5^+$), ammonia (NH_4^+), or the nobel gases. The types of ion-molecule reactions used to produce ions in a chemical ionization source are shown in the table below. All these reaction are exothermic, however, the excess energy of the ion produced is much less than that produced by EI ionization.

Ion-Molecule Reactions used to make analyte ions from MH

$CH_5^+ + MH \rightarrow MH_2^+ + CH_4$	$\Delta H < 0$	proton transfer
$C_2H_5^+ + MH \rightarrow MH_2^+ + C_2H_4$	$\Delta H < 0$	proton transfer
$NH_4^+ + MH \rightarrow MH_2^+ + NH_3$	$\Delta H < 0$	proton transfer
$C_2H_5^+ + MH \rightarrow M^+ + C_2H_6$	$\Delta H < 0$	hydride transfer
$Ar^+ + MH \rightarrow MH^+ + Ar$	$\Delta H < 0$	charge transfer

The ΔH for proton transfer reactions range from - 0.01 to - 0.5 eV (-1 to -50 kJ/mole) while the ΔH for charge transfer reactions - 0.01 to - 0.2 eV (-1 to -20 kJ/mole). The excess energy imparted to teh ion produced and consequently the extent of ion fragmentation can be varied by the choice of reagent ions. For example, in a charge transfer ionization between a He ion (ionization energy 24.5 eV) and a sample molecule with an ionization energy of 5 eV will leave the ion produced with

approximately 19.5 eV of excess energy. Charge transfer ionization of the same molecule with argon ions (ionization energy 15.7 eV) or krypton ions (ionization energy 14 eV) will leave the ion with an excess energy of approximately 10.7 eV and 9 eV respectively

Fast Atom Bombardment (FAB)

Fast atom bombardment (FAB) is a soft ionization technique that produces positive ions, negative ions, and quasi molecular ions in an energetic collision between a fast moving atom and sample molecule contained in a viscous matrix. As shown in Figure **16.2.4**, argon ions produced via electron impact are accelerated to very large kinetic energies (8 - 35 keV)and directed towards the target copper probe tip. These fast moving ions are directed into a gas chamber containing slow moving, thermal energy, argon atoms. Electrons transferred in a net energy neutral reaction from the atoms to some of the fast moving argon ions produce fast moving argon atoms. Only fast moving atoms strike the target as any remaing fast moving ions are directed off the target path using electrostatic deflector plates.

$$Ar^{+\cdot}\,(fast) + Argon\,(thermal) \rightarrow Ar\,(fast) + Ar^{+\cdot}\,(thermal)$$

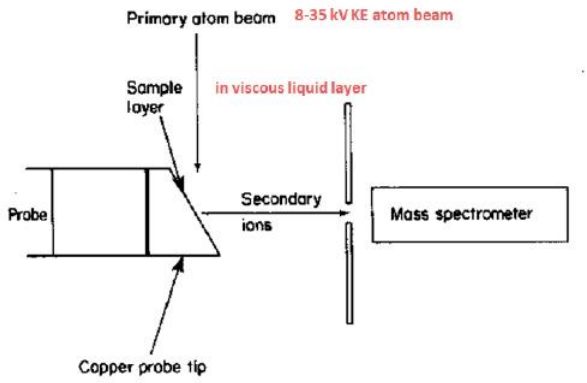


Figure **16.2.4**: A schematic illustration of a fast atom bombardment (FAB) ionization source.

While both positive ions and negative secondary ions are produced in collision between the fast argon atoms and the sample molecules in the matrix only one type of ion is sent to the mass analyzer. One can either analyze positive ions or negative ions at a time, not both. It should be noted that multiply chaged ions are often produced in a FAB source.

Matrix Assisted Laser Desorption Ionization (MALDI)

Pulsed lasers such as the nitrogen laser (337 nm) or a pulsed Nd:Yag laser (1064 nm, 532 nm or 355 nm) are capable of delivering millijoules of energy in a 5 - 10 nsec pulse of light. Consequently and as shown in Figure **16.2.5** a matrix assisted laser desorption ionization source look very much like a FAB ionization source with the fast atom beam replaced by the light beam from a pulsed laser.

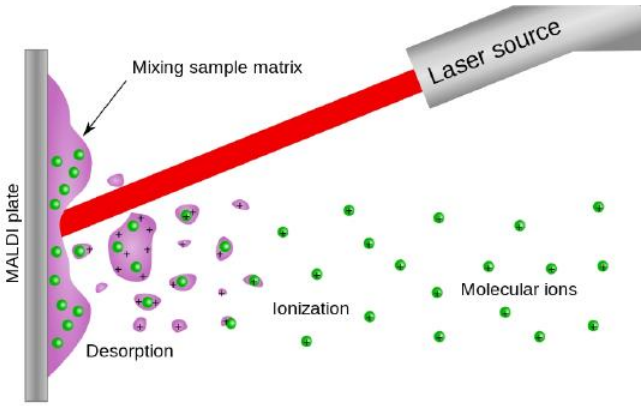


Figure (\PageIndex{5}\): An schmatic illustration of a MALDI source. Image from the Wikimedia Commons.

As with the FAB ionization source, MALDI produces both positive ions and negative secondary ions, quasi molecular ions and multiply charged ions. There are many receipies for the matrix in a MALDI source, some which add species that absorb at the wavelength of the pulsed laser.

Because of the pulsed nature of the MALDI source this type of ionizer is often coupled with a Time-of-flight (TOF) mass analyzer.

Thermospray

The thermospray ion source is one of the first sources designed specifically to couple a HPLC or syringe pumped capillary to a mass spectrometer. As shown in Figure (\PageIndex{6}\) a sample solution is pumped into a heated stainless-steel capillary, rapid evaporation of solvent from the liquid surface occurs, resulting in an ultrasonic spray of vapor and charged droplets. Disintegration of the charged droplets occurs repetitively due to continuous evaporation of solvent and the Coulombic repulsion between like charges. The process eventually causes ions,as well as neutral molecules, to be released from the surface of the microdroplets. The ions are extracted and accelerated toward the analyzer by an electrostatic system voltage.

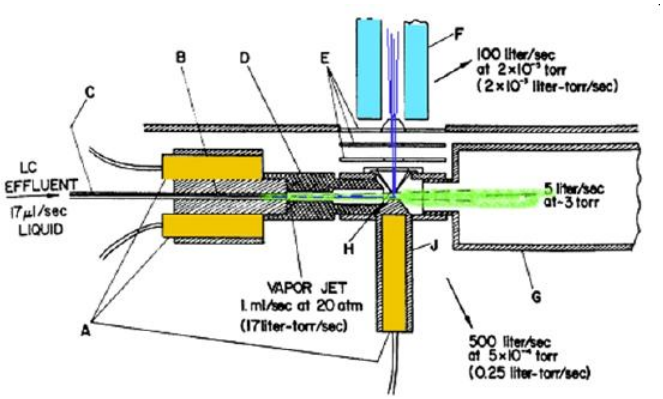


Figure (\PageIndex{6}\): A schematic illustration of a thermospray ionization source . In this figure **A** 100 W cartridge heaters, **B** copper block, **C** stainless steel capillary 0.15 mm id, **D** copper tube, **E** ion lenses, **F** quadrupole mass analyzer. Ref.: C. R. Blakley, M. L. Vestal, *Anal. Chem*. **55**, 750 (1983).

Thermospray is a soft ionization technique producing both positive ions and negative secondary ions, quasi molecular ions and multiply charged ions. When applied to studies of proteins, biological macromolecules with multiple acid or base groups, the distribution of charges on the ions of the same species can be greatly influenced by the pH of the buffer solution.

The thermospray source is more of historical importance as the electrospray source and the atmospheric pressure interface are much more commonly found in today's instruments.

Atmospheric Pressure Ionization (API)

In addition to the electrospray ion source described in a later subsection in this chapter, the atmospheric pressure ionization interface is widely used to couple HPLC's and syringe pumped capillaries to mass spectrometers. As shown in Figure (\PageIndex{7}\) a small flow solution is passed through a heated zone as in a thermospray source and nebullized. The resulting aerosol mist is passed by a sharp needle held at 2 - 4 kV producing a corona discharge. The resulting solvated ions are allowed to lose solvent molecules by evaporation and a sample of the ion cloud is passed into the mass analyzer.

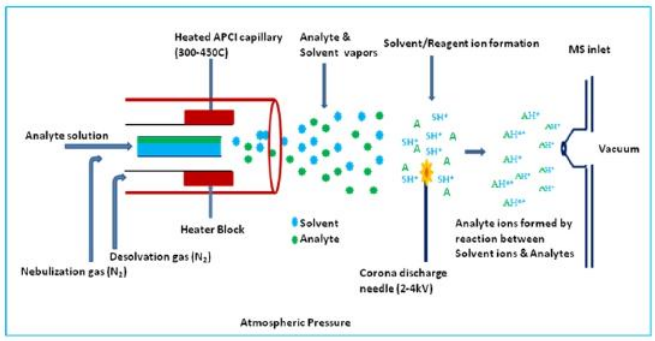


Figure (\PageIndex{7}\): A scehmatic illustration of a atmospheric pressure interface. Image from https://www.chem.pitt.edu/facilities...y-introduction.

As with the thermospray and electrospray API is a soft ionization technique producing both positive ions and negative secondary ions, quasi molecular ions and multiply charged ions. When applied to studies of proteins, biological macromolecules with multiple acid or base groups, the distribution of charges on the ions of the same species can be greatly influenced by the pH of the buffer solution.

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16.3: Mass Analyzers (Mass Spectrometry)

Mass spectrometry is an analytic method that employs ionization and mass analysis of compounds to determine the mass, formula and structure of the compound being analyzed. A mass analyzer is the component of the mass spectrometer that takes ionized masses and separates them based on charge to mass ratios and outputs them to the detector where they are detected and later converted to a digital output.

Introduction

There are six general types of mass analyzers that can be used for the separation of ions in a mass spectrometry.

1. Quadrupole Mass Analyzer
2. Time of Flight Mass Analyzer
3. Magnetic Sector Mass Analyzer
4. Electrostatic Sector Mass Analyzer
5. Quadrupole Ion Trap Mass Analyzers
6. Ion Cyclotron Resonance

Quadrupole Mass Analyzer

The DC bias will cause all the charged molecules to accelerate and move away from the center line, the rate being proportional to their charge to mass ratio. If their course goes off too far they will hit the metal rods or the sides of the container and be absorbed. So the DC bias acts like the magnetic field B of the mass spec and can be tuned to specific charge to mass ratios hitting the detector.

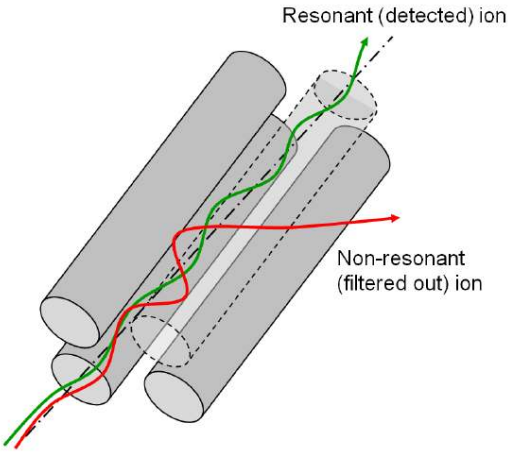


Figure 1: A quadrupole

The two sinusoidal electric fields at 90 orientation and 90 degrees degrees phase shift will cause an electric field which oscillates as a circle over time. So as the charged particles fly down toward the detector, they will be traveling in a spiral, the diameter of the spiral being determined by the charge to mass ratio of the molecule and the frequency and strength of the electric field. The combination of the DC bias and the circularly rotating electric field will be the charge particles will travel in a spiral which is curved. So by timing the peak of the curved spiral to coincide with the position of the detector at the end of the quadrupole, a great deal of selectivity to molecules charge to mass ratio can be obtained.

TOF (Time of Flight) Mass Analyzer

TOF Analyzers separate ions by time without the use of an electric or magnetic field. In a crude sense, TOF is similar to chromatography, except there is no stationary/ mobile phase, instead the separation is based on the kinetic energy and velocity of the ions.

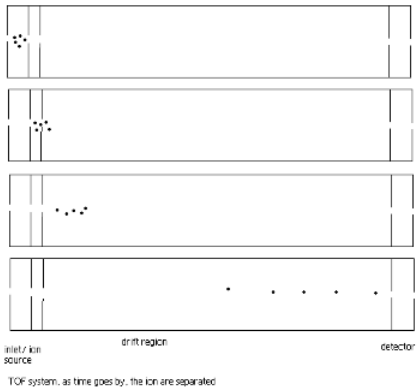


Figure 2: A TOF system. As time evolves, the ions (formed at the source) are separated.

Ions of the same charges have equal kinetic energies; kinetic energy of the ion in the flight tube is equal to the kinetic energy of the ion as it leaves the ion source:

$$KE = \frac{mv^2}{2} = zV \tag{1}$$

The time of flight, or time it takes for the ion to travel the length of the flight tube is:

$$T_f = \frac{L}{v} \tag{2}$$

- with L is the length of tube and
- v is the velocity of the ion

Substituting Equation 1 for kinetic energy in Equation 2 for time of flight:

$$T_f = L\sqrt{\frac{m}{z}}\sqrt{\frac{1}{2V}} \propto \sqrt{\frac{m}{z}} \tag{3}$$

During the analysis, L, length of tube, the Voltage from the ion source V are held constant, which can be used to say that time of flight is directly proportional to the root of the mass to charge ratio.

Unfortunately, at higher masses, resolution is difficult because flight time is longer. Also at high masses, not all of the ions of the same m/z values reach their ideal TOF velocities. To fix this problem, often a **reflectron** is added to the analyzer. The reflectron consists of a series of ring electrodes of very high voltage placed at the end of the flight tube. When an ion travels into the reflectron, it is reflected in the opposite direction due to the high voltage.

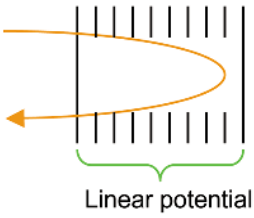


Figure 3: A reflection. from Wikipedia.

The reflectron increases resolution by narrowing the broadband range of flight times for a single m/z value. Faster ions travel further into the reflectrons, and slower ions travel less into the reflector. This way both slow and fast ions, of the same m/z value, reach the detector at the same time rather then at different times, narrowing the bandwidth for the output signal.

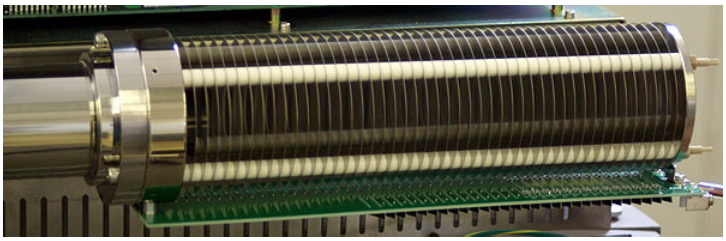


Figure 4. A photo of a reflectron. An ion mirror (right) attached to a flight tube (left) of the reflectron. Voltages applied to a stack of metal plates create the electric field reflecting the ions back to the flight tube. In this particular design, gaps between the mirror electrodes are too large. This can lead to a distortion of the field inside the mirror caused by a proximity of metal surface of the enveloping vacuum tube. from Wikipedia.

Magnetic Sector Mass Analyzer

Similar to time of flight (TOF) analyzer mentioned earlier,in magnetic sector analyzers ions are accelerated through a flight tube, where the ions are separated by charge to mass ratios. The difference between magnetic sector and TOF is that a magnetic field is used to separate the ions. As moving charges enter a magnetic field, the charge is deflected to a circular motion of a unique radius in a direction perpendicular to the applied magnetic field. Ions in the magnetic field experience two equal forces; force due to the magnetic field and centripetal force.

$$F_B = zvB = F_c = \frac{mv^2}{r} \tag{4}$$

The above equation can then be rearranged to give:

$$v = \frac{Bzr}{m} \tag{5}$$

If this equation is substituted into the kinetic energy equation:

$$KE = zV = \frac{mv^2}{2} \tag{6}$$

$$\frac{m}{z} = \frac{B^2r^2}{2V} \tag{7}$$

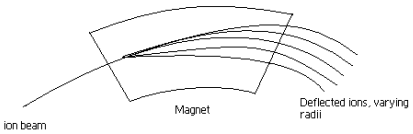


Figure 5: A magnetic sector separator.

Basically the ions of a certain m/z value will have a unique path radius which can be determined if both magnetic field magnitude B, and voltage difference V for region of acceleration are held constant. when similar ions pass through the magnetic field, they all will be deflected to the same degree and will all follow the same trajectory path. Those ions which are not selected by V and B values, will collide with either side of the flight tube wall or will not pass through the slit to the detector. Magnetic sector analyzers are used for mass focusing, they focus angular dispersions.

Electrostatic Sector Mass Analyzer

Is similar to time of flight analyzer in that it separates the ions while in flight, but it separates using an electric field. Electrostatic sector analyzer consists of two curved plates of equal and opposite potential. As the ion travels through the electric field, it is deflected and the force on the ion due to the electric field is equal to the centripetal force on the ion. Here the ions of the same kinetic energy are focused, and ions of different kinetic energies are dispersed.

$$KE = zV = \frac{mv^2}{2} \tag{8}$$

$$F_E = zE = F_c = \frac{mv^2}{R} \tag{9}$$

Electrostatic sector analyzers are energy focusers, where an ion beam is focused for energy. Electrostatic and magnetic sector analyzers when employed individually are single focusing instruments. However when both techniques are used together, it is called a double focusing instrument., because in this instrument both the energies and the angular dispersions are focused.

Quadrupole Ion Trap Mass Analyzers

This analyzer employs similar principles as the quadrupole analyzer mentioned above, it uses an electric field for the separation of the ions by mass to charge ratios. The analyzer is made with a ring electrode of a specific voltage and grounded end cap electrodes. The ions enter the area between the electrodes through one of the end caps. After entry, the electric field in the cavity due to the electrodes causes the ions of certain m/z values to orbit in the space. As the radio frequency voltage increases, heavier mass ion orbits become more stabilized and the light mass ions become less stabilized, causing them to collide with the wall, and eliminating the possibility of traveling to and being detected by the detector.

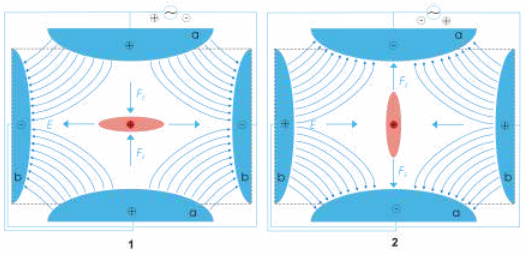


Figure 5: Scheme of a Quadrupole ion trap of classical setup with a particle of positive charge (dark red), surrounded by a cloud of similarly charged particles (light red). The electric field E (blue) is generated by a quadrupole of endcaps (a, positive) and a ring electrode (b). Picture 1 and 2 show two states during an AC cycle. from Wikipedia.

The quadrupole ion trap usually runs a mass selective ejection, where selectively it ejects the trapped ions in order of in creasing mass by gradually increasing the applied radio frequency voltage.

Ion Cyclotron Resonance (ICR)

ICR is an ion trap that uses a magnetic field in order to trap ions into an orbit inside of it. In this analyzer there is no separation that occurs rather all the ions of a particular range are trapped inside, and an applied external electric field helps to generate a signal. As mentioned earlier, when a moving charge enters a magnetic field, it experiences a centripetal force making the ion orbit. Again the force on the ion due to the magnetic field is equal to the centripetal force on the ion.

$$zvB = \frac{mv^2}{r} \tag{10}$$

Angular velocity of the ion perpendicular to the magnetic field can be substituted here $w_c = v/r$

$$zB = mw_c \tag{11}$$

$$w_c = \frac{zB}{m} \tag{12}$$

Frequency of the orbit depends on the charge and mass of the ions, not the velocity. If the magnetic field is held constant, the charge to mass ratio of each ion can be determined by measuring the angular velocity ω_c. The relationship is that, at high w_c, there is low m/z value, and at low ω_c, there is a high m/z value. Charges of opposite signs have the same angular velocity, the only difference is that they orbit in the opposite direction.

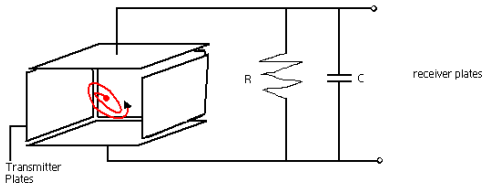


Figure 6: An ICR trap.

To generate an electric signal from the trapped ions, a vary electric field is applied to the ion trap

$$E = E_o \cos(\omega_c t) \tag{13}$$

When the ω_c in the electric field matches the ω_c of a certain ion, the ion absorbs energy making the velocity and orbiting radius of the ion increase. In this high energy orbit, as the ion oscillates between two plates, electrons accumulate at one of the plates over the other inducing an oscillating current, or current image. The current is directly proportional to the number of ions in the cell at a certain frequency.

In a Fourier Transform ICR, all of the ions within the cell are excited simultaneously so that the current image is coupled with the image of all of the individual ion frequencies. A Fourier transform is used to differential the summed signals to produce the desired results.

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SECTION OVERVIEW

16.4: Ion Detectors

The simplest ion detector is the **Faraday Cup Detector** pictured in Figure 1. In this detector an ion beam strikes the inner metal surface and is neutralized by electrons. The small electron current is amplified and converted into a voltage. The electron current is proportional to the number of ions striking the surface. The Faraday Cup detector is akin to the vacuum phototube in that at most one electron flow for each ion striking the surface. Hence it is not a very sensitive ion detector.

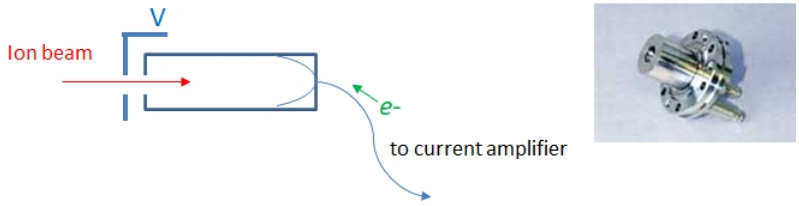


Figure 1: A simple sketch of a Faraday cup detector and a picture of an actual one.

A more commonly encountered ion detector is the **channeltron detector** shown in Figure 2. In a channeltron an ion strikes the inner surface and produces a secondary electron. Because of the arrangement of the electrostatic potentials, the secondary electron is directed further into the channel where it strikes the inner surface again producing 1 -3 secondary electron. The process continues for each of the secondary electrons generated and the charge packet moves further towards the end of the channel. A channeltron is akin to a photomultipler tube but rather than a discrete dynode array a channeltron is a continuous dynode array. Like a photomultiplier tube a channeltron has gain. On average each ion striking the channeltron is converted into 10^7 or 10^8 electrons.

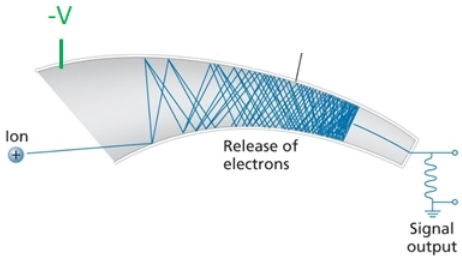


Figure 2: a illustration of a channeltron ion detector.

Another ion detector related to the channeltron is the microchannel plate (MCP) detector. A microchannel plate is a thin disc composed of many individual channeltrons. Each disc has a gain of about 10^3 and typically two plates are used in tandem to achieve a gain approaching that of a channeltron. MCPs are widely used in physics and physical chemistry to spatially detect the path of ions. MCPs are also in fast oscilloscopes to intensify images.

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16.5: High Resolution vs Low Resolution

Two common categories of mass spectrometry are high resolution mass spectrometry (HRMS) or exact mass mass spectrometry and low resolution mass spectrometry (LRMS). Not all mass spectrometers simply mass-to-charge ratios (m/ze) as whole numbers. High resolution mass spectrometers can measure mass so accurately that they can detect the minute differences in (m/ze) between two ions that, on a regular low-resolution instrument, would appear to be identical.

The reason is because each ion has a (m/ze) that is based on the atoms in the ion. In your first year chemistry course you learned that the masses of atoms of the same element can differ because not all atoms of the same element have the same number of neutrons. Hence, the masses that appear in the periodic table are not useful to calculate the exact (m/ze) of an ion because they are average mass for a large number of atoms of an element taking inot account the relative natural abundances of each isotope.

- An atom of ^{12}C has a mass of 12.00000 amu while an atom of ^{13}C has a mass of 13.00335 amu
- An atom of ^{16}O weighs 15.99491 amu while an atom of ^{17}O has a mass of 116.99913 amu
- An atom of ^{14}N weighs 14.00307 amu while an atom of ^{15}N has a mass of 15.00011 amu
- An atom of ^{1}H weighs 1.00782 amu while an atom of ^{2}H has a mass of 2.01410 amu

As a result, on a high resolution mass spectrometer, 2-octanone, $C_8H_{16}O$ with atoms of only the most abundant isotopes, has a molecular weight of 128.12018 instead of 128. Naphthalene, $C_{10}H_8$, again with only atoms of only the most abundant isotopes has a molecular weight of 128.06264. Thus a high resolution mass spectrometer can not only distinguish between these two ions it is also able to supply an the molecular formula for the ion because of the unique combination of masses that result.

In reality the resolution required to distinguish the molecular ions for 2-octanone and naphthalene is only 128 / (128.12018-128.06264) ~ 2200, well within the resolution of most mass analyzers. However, being able to discern the formula of the ion is only possible with an accurate mass analyzer capable of knowing the (m/ze) to roughly 1 part in 150,000.

- In LRMS, the molecular weight is determined to the nearest amu. The type of instrument used here is more common because it is less expensive and easier to maintain.
- In HRMS, the molecular weight in amu is determined to several decimal places. That precision allows the molecular formula to be narrowed down to only a few possibilities.

HRMS relies on the fact that the mass of an individual atom does not correspond to an integral number of atomic mass units.

Problem MS7.

Calculate the high-resolution molecular weights for the following formula.

1. $C_{12}H_{20}O$ and $C_{11}H_{16}O_2$
2. $C_6H_{13}N$ and $C_5H_{11}N_2$

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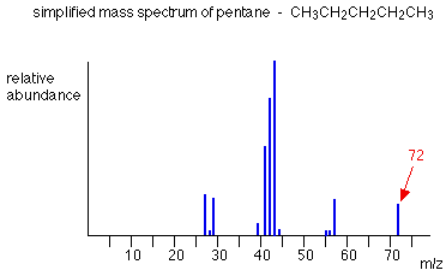
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16.6: The Molecular Ion (M^+) Peak

This page explains how to find the relative formula mass (relative molecular mass) of an organic compound from its mass spectrum. It also shows how high resolution mass spectra can be used to find the molecular formula for a compound.

When the vaporised organic sample passes into the ionization chamber of a mass spectrometer, it is bombarded by a stream of electrons. These electrons have a high enough energy to knock an electron off an organic molecule to form a positive ion. This ion is called the molecular ion. The molecular ion is often given the symbol M^+ or $M^{\cdot+}$ - the dot in this second version represents the fact that somewhere in the ion there will be a single unpaired electron. That's one half of what was originally a pair of electrons - the other half is the electron which was removed in the ionization process. The molecular ions tend to be unstable and some of them break into smaller fragments. These fragments produce the familiar stick diagram. Fragmentation is irrelevant to what we are talking about on this page - all we're interested in is the molecular ion.

In the mass spectrum, the heaviest ion (the one with the greatest m/z value) is likely to be the molecular ion. A few compounds have mass spectra which don't contain a molecular ion peak, because all the molecular ions break into fragments. For example, in the mass spectrum of pentane, the heaviest ion has an m/z value of 72.



Because the largest m/z value is 72, that represents the largest ion going through the mass spectrometer - and you can reasonably assume that this is the molecular ion. The relative formula mass of the compound is therefore 72.

Finding the relative formula mass (relative molecular mass) from a mass spectrum is therefore trivial. Look for the peak with the highest value for m/z, and that value is the relative formula mass of the compound. There are, however, complications which arise because of the possibility of different isotopes (either of carbon or of chlorine or bromine) in the molecular ion. These cases are dealt with on separate pages.

Using a mass spectrum to find a molecular formula

So far we've been looking at m/z values in a mass spectrum as whole numbers, but it's possible to get far more accurate results using a high resolution mass spectrometer. You can use that more accurate information about the mass of the molecular ion to work out the molecular formula of the compound.

For normal calculation purposes, you tend to use rounded-off relative isotopic masses. For example, you are familiar with the atomic mass numbers (Z). To 4 decimal places, however, these are the relative isotopic masses.

Isotope	Z	Mass
^{1}H	1	1.0078
^{12}C	12	12.0000
^{14}N	14	14.0031
^{16}O	16	15.9949

The carbon value is 12.0000, of course, because all the other masses are measured on the carbon-12 scale which is based on the carbon-12 isotope having a mass of exactly 12.

Using these accurate values to find a molecular formula

Two simple organic compounds have a relative formula mass of 44 - propane, C_3H_8, and ethanal, CH_3CHO. Using a high resolution mass spectrometer, you could easily decide which of these you had. On a high resolution mass spectrometer, the molecular ion peaks for the two compounds give the following m/z values:

C_3H_8		44.0624
CH_3CHO		44.0261

You can easily check that by adding up numbers from the table of accurate relative isotopic masses above.

Example 16.6.1:

A gas was known to contain only elements from the following list:

^{1}H		1.0078
^{12}C		12.0000
^{14}N		14.0031
^{16}O		15.9949

The gas had a molecular ion peak at m/z = 28.0312 in a high resolution mass spectrometer. What was the gas?

Solution

After a bit of playing around, you might reasonably come up with 3 gases which had relative formula masses of approximately 28 and which contained the elements from the list. They are N_2, CO and C_2H_4. Working out their accurate relative formula masses gives:

N_2		28.0062
CO		27.9949
C_2H_4		28.0312

The gas is obviously C_2H_4.

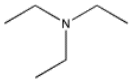
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16.7: Molecular Ion and Nitrogen

Molecular Weight: Even or Odd?



triethylamine

Figure MS6. Mass spectrum of triethylamine. Source: SDBSWeb : http://riodb01.ibase.aist.go.jp/sdbs/ (National Institute of Advanced Industrial Science and Technology of Japan, 22 August 2008)

This phenomenon is a result of the fact that the most common elements in organic compounds, carbon and oxygen, have even atomic masses (12 and 16, respectively), so any number of carbons and oxygens will have even masses. The most common isotope of hydrogen has an odd molecular weight, but because carbon and oxygen both have even valences (carbon forms four bonds and oxygen forms two), there is always an even number of hydrogen atoms in an organic compound containing those elements, so they also add up to an even numbered molecular mass.

Nitrogen has an even atomic mass (14), so any number of nitrogen atoms will add up to an even molecular mass. Nitrogen, however, has an odd valence (it forms three bonds), and as a result there will be an odd number of hydrogens in a nitrogenous compound, and the molecular mass will be odd because of the presence of an extra hydrogen.

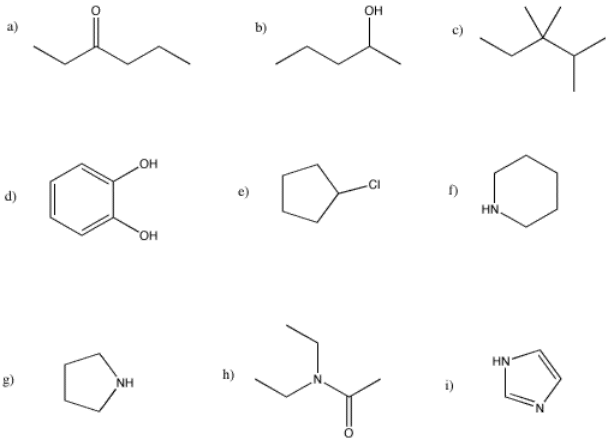
Of course, if there are two nitrogens in a molecule, there will be two extra hydrogens, so the molecular mass will actually be even. That means the rule about molecular mass and nitrogen should really be expressed as:

- odd numbers of nitrogen atoms in a molecule in an odd molecular mass.

What about those other atoms that sometimes show up in organic chemistry, such as the halogens? Halogens all have odd molecular mass (19 amu for fluorine, 35 or 37 for chlorine, 79 or 81 for bromine, and 127 for iodine). However, halogens all have a valence of 1, just like hydrogen. As a result, to add a halogen to methane, we would need to erase one of the hydrogen atoms and replace it with the halogen. Since we are just substituting one odd numbered atomic mass for another, the total molecular masss remains even.

Problem MS6.

Calculate molecular weights for the following compounds.



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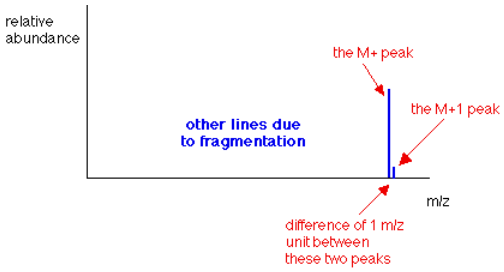
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16.8: The M+1 Peak

This page explains how the M+1 peak in a mass spectrum can be used to estimate the number of carbon atoms in an organic compound.

What causes the M+1 peak?

If you had a complete (rather than a simplified) mass spectrum, you will find a small line 1 m/z unit to the right of the main molecular ion peak. This small peak is called the M+1 peak.

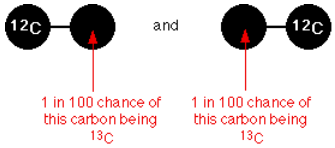


The carbon-13 isotope

The M+1 peak is caused by the presence of the ^{13}C isotope in the molecule. ^{13}C is a stable isotope of carbon - don't confuse it with the ^{14}C isotope which is radioactive. Carbon-13 makes up 1.11% of all carbon atoms. If you had a simple compound like methane, CH_4, approximately 1 in every 100 of these molecules will contain carbon-13 rather than the more common carbon-12. That means that 1 in every 100 of the molecules will have a mass of 17 (13 + 4) rather than 16 (12 + 4). The mass spectrum will therefore have a line corresponding to the molecular ion $[^{13}CH_4]^+$ as well as $[^{12}CH_4]^+$. The line at m/z = 17 will be much smaller than the line at m/z = 16 because the carbon-13 isotope is much less common. Statistically you will have a ratio of approximately 1 of the heavier ions to every 99 of the lighter ones. That's why the M+1 peak is much smaller than the M+ peak.

Using the M+1 peak

Imagine a compound containing 2 carbon atoms. Either of them has an approximately 1 in 100 chance of being ^{13}C.



There's therefore a 2 in 100 chance of the molecule as a whole containing one ^{13}C atom rather than a ^{12}C atom - which leaves a 98 in 100 chance of both atoms being ^{12}C. That means that the ratio of the height of the M+1 peak to the M peak will be approximately 2 : 98. That's pretty close to having an M+1 peak approximately 2% of the height of the M peak.

Using the relative peak heights to predict the number of carbon atoms

If you measure the peak height of the M+1 peak as a percentage of the peak height of the M peak, that gives you the number of carbon atoms in the compound. We've just seen that a compound with 2 carbons will have an M+1 peak approximately 2% of the height of the M peak. Similarly, you could show that a compound with 3 carbons will have the M+1 peak at about 3% of the height of the M peak. The approximations we are making won't hold with more than 2 or 3 carbons. The proportion of carbon atoms which are ^{13}C isn't 1% - it's 1.11%. And the approximation that a ratio of 2 : 98 is about 2% doesn't hold as the small number increases.

Consider a molecule with 5 carbons in it. You could work out that 5.55 (5 x 1.11) molecules will contain 1 ^{13}C to every 94.45 (100 - 5.55) which contain only ^{12}C atoms. If you convert that to how tall the M+1 peak is as a percentage of the M peak, you get an answer of 5.9% (5.55/94.45 x 100). That's close enough to 6% that you might assume wrongly that there are 6 carbon atoms. Above 3 carbon atoms, then, you shouldn't really be making the approximation that the height of the M+1 peak as a percentage of the height of the M peak tells you the number of carbons - you will need to do some fiddly sums!

Contributors and Attributions

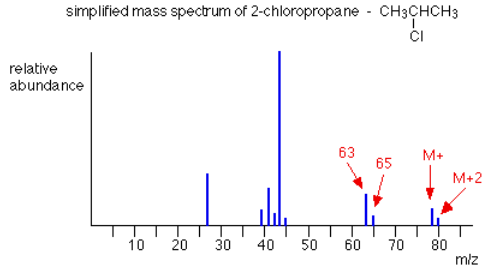
Jim Clark (Chemguide.co.uk)

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16.9: Organic Compounds Containing Halogen Atoms

This page explains how the M+2 peak in a mass spectrum arises from the presence of chlorine or bromine atoms in an organic compound. It also deals briefly with the origin of the M+4 peak in compounds containing two chlorine atoms.

One chlorine atom in a compound



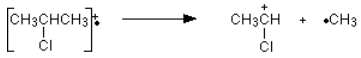
The molecular ion peaks (M+ and M+2) each contain one chlorine atom - but the chlorine can be either of the two chlorine isotopes, ^{35}Cl and ^{37}Cl.

The molecular ion containing the ^{35}Cl isotope has a relative formula mass of 78. The one containing ^{37}Cl has a relative formula mass of 80 - hence the two lines at m/z = 78 and m/z = 80.

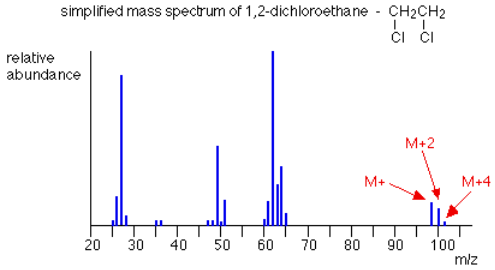
Notice that the peak heights are in the ratio of 3 : 1. That reflects the fact that chlorine contains 3 times as much of the ^{35}Cl isotope as the ^{37}Cl one. That means that there will be 3 times more molecules containing the lighter isotope than the heavier one.

So . . . if you look at the molecular ion region, and find two peaks separated by 2 m/z units and with a ratio of 3 : 1 in the peak heights, that tells you that the molecule contains 1 chlorine atom.

You might also have noticed the same pattern at m/z = 63 and m/z = 65 in the mass spectrum above. That pattern is due to fragment ions also containing one chlorine atom - which could either be ^{35}Cl or ^{37}Cl. The fragmentation that produced those ions was:



Two chlorine atoms in a compound



The lines in the molecular ion region (at m/z values of 98, 100 ands 102) arise because of the various combinations of chlorine isotopes that are possible. The carbons and hydrogens add up to 28 - so the various possible molecular ions could be:

$$28 + 35 + 35 = 98$$

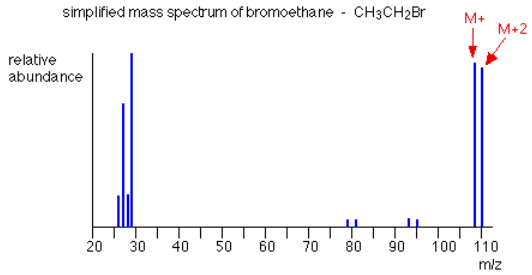
$$28 + 35 + 37 = 100$$

$$28 + 37 + 37 = 102$$

If you have the necessary math, you could show that the chances of these arrangements occurring are in the ratio of 9:6:1 - and this is the ratio of the peak heights. If you don't know the right bit of math, just learn this ratio! So . . . if you have 3 lines in the molecular ion region (M+, M+2 and M+4) with gaps of 2 m/z units between them, and with peak heights in the ratio of 9:6:1, the compound contains 2 chlorine atoms.

Compounds containing bromine atoms

Bromine has two isotopes, ^{79}Br and ^{81}Br in an approximately 1:1 ratio (50.5 : 49.5 if you want to be fussy!). That means that a compound containing 1 bromine atom will have two peaks in the molecular ion region, depending on which bromine isotope the molecular ion contains. Unlike compounds containing chlorine, though, the two peaks will be very similar in height.



The carbons and hydrogens add up to 29. The M+ and M+2 peaks are therefore at m/z values given by:

$$29 + 79 = 108$$

$$29 + 81 = 110$$

Hence, if two lines in the molecular ion region are observed with a gap of 2 m/z units between them and with almost equal heights, this suggests the presence of a bromine atom in the molecule.

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16.10: Fragmentation Patterns in Mass Spectra

This page looks at how fragmentation patterns are formed when organic molecules are fed into a mass spectrometer, and how you can get information from the mass spectrum.

The formation of molecular ions

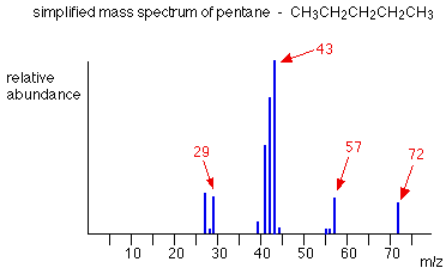
When the vaporized organic sample passes into the ionization chamber of a mass spectrometer, it is bombarded by a stream of electrons. These electrons have a high enough energy to knock an electron off an organic molecule to form a positive ion. This ion is called the **molecular ion - or sometimes the parent ion.** The molecular ion is often given the symbol $\mathbf{M}^+$ or $\mathbf{M}^{\cdot+}$- the dot in this second version represents the fact that somewhere in the ion there will be a single unpaired electron. That's one half of what was originally a pair of electrons - the other half is the electron which was removed in the ionization process.

Fragmentation

The molecular ions are energetically unstable, and some of them will break up into smaller pieces. The simplest case is that a molecular ion breaks into two parts - one of which is another positive ion, and the other is an uncharged free radical.

$$M^{\cdot+} \rightarrow X^+ + Y^{\cdot}$$

The uncharged free radical won't produce a line on the mass spectrum. Only charged particles will be accelerated, deflected and detected by the mass spectrometer. These uncharged particles will simply get lost in the machine - eventually, they get removed by the vacuum pump. The ion, X^+, will travel through the mass spectrometer just like any other positive ion - and will produce a line on the stick diagram. All sorts of fragmentations of the original molecular ion are possible - and that means that you will get a whole host of lines in the mass spectrum. For example, the mass spectrum of pentane looks like this:



It's important to realize that the pattern of lines in the mass spectrum of an organic compound tells you something quite different from the pattern of lines in the mass spectrum of an element. With an element, each line represents a different isotope of that element. With a compound, each line represents a different fragment produced when the molecular ion breaks up.

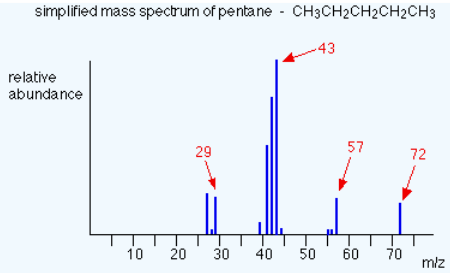
In the stick diagram showing the mass spectrum of pentane, the line produced by the heaviest ion passing through the machine (at m/z = 72) is due to the molecular ion. The tallest line in the stick diagram (in this case at m/z = 43) is called the **base peak**. This is usually given an arbitrary height of 100, and the height of everything else is measured relative to this. The base peak is the tallest peak because it represents the commonest fragment ion to be formed - either because there are several ways in which it could be produced during fragmentation of the parent ion, or because it is a particularly stable ion.

Using fragmentation patterns

This section will ignore the information you can get from the molecular ion (or ions). That is covered in three other pages which you can get at via the mass spectrometry menu. You will find a link at the bottom of the page.

Example **16.10.1**: Mass Spectrum of Pentane

Let's have another look at the mass spectrum for pentane:

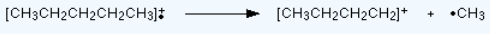


What causes the line at m/z = 57?

Solution

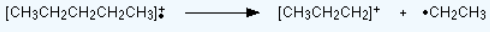
How many carbon atoms are there in this ion? There can't be 5 because 5 x 12 = 60. What about 4? 4 x 12 = 48. That leaves 9 to make up a total of 57. How about $C_4H_9^+$ then?

$C_4H_9^+$ would be $[CH_3CH_2CH_2CH_2]^+$, and this would be produced by the following fragmentation:

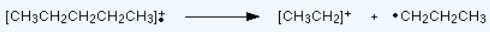


The methyl radical produced will simply get lost in the machine.

The line at m/z = 43 can be worked out similarly. If you play around with the numbers, you will find that this corresponds to a break producing a 3-carbon ion:



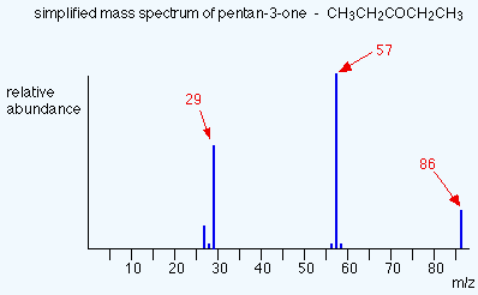
The line at m/z = 29 is typical of an ethyl ion, $[CH_3CH_2]^+$:



The other lines in the mass spectrum are more difficult to explain. For example, lines with m/z values 1 or 2 less than one of the easy lines are often due to loss of one or more hydrogen atoms during the fragmentation process.

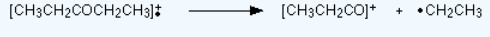
Example 16.10.2: Pentan-3-one

This time the base peak (the tallest peak - and so the commonest fragment ion) is at m/z = 57. But this isn't produced by the same ion as the same m/z value peak in pentane.

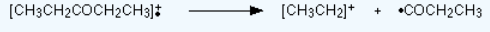


If you remember, the m/z = 57 peak in pentane was produced by $[CH_3CH_2CH_2CH_2]^+$. If you look at the structure of pentan-3-one, it's impossible to get that particular fragment from it.

Work along the molecule mentally chopping bits off until you come up with something that adds up to 57. With a small amount of patience, you'll eventually find $[CH_3CH_2CO]^+$ - which is produced by this fragmentation:



You would get exactly the same products whichever side of the CO group you split the molecular ion. The m/z = 29 peak is produced by the ethyl ion - which once again could be formed by splitting the molecular ion either side of the CO group.



Peak heights and the stability of ions

The more stable an ion is, the more likely it is to form. The more of a particular sort of ion that's formed, the higher its peak height will be. We'll look at two common examples of this.

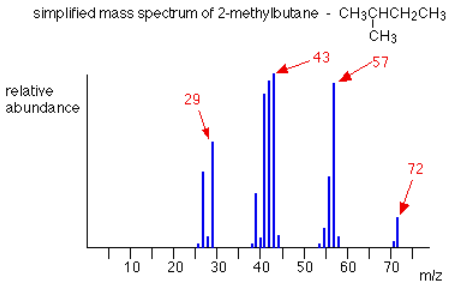
Examples involving carbocations (carbonium ions)

Summarizing the most important conclusion from the page on carbocations:

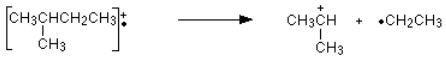
Order of stability of carbocations

primary < secondary < tertiary

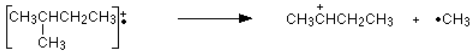
Applying the logic of this to fragmentation patterns, it means that a split which produces a secondary carbocation is going to be more successful than one producing a primary one. A split producing a tertiary carbocation will be more successful still. Let's look at the mass spectrum of 2-methylbutane. 2-methylbutane is an isomer of pentane - isomers are molecules with the same molecular formula, but a different spatial arrangement of the atoms.



Look first at the very strong peak at m/z = 43. This is caused by a different ion than the corresponding peak in the pentane mass spectrum. This peak in 2-methylbutane is caused by:



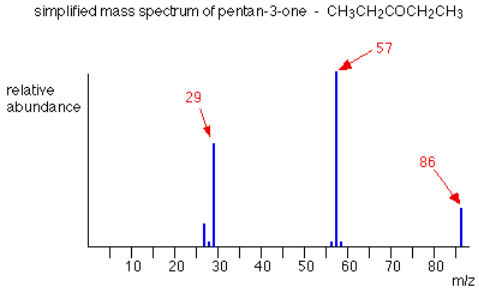
The ion formed is a secondary carbocation - it has two alkyl groups attached to the carbon with the positive charge. As such, it is relatively stable. The peak at m/z = 57 is much taller than the corresponding line in pentane. Again a secondary carbocation is formed - this time, by:



You would get the same ion, of course, if the left-hand CH_3 group broke off instead of the bottom one as we've drawn it. In these two spectra, this is probably the most dramatic example of the extra stability of a secondary carbocation.

Examples involving acylium ions, $[RCO]^+$

Ions with the positive charge on the carbon of a carbonyl group, C=O, are also relatively stable. This is fairly clearly seen in the mass spectra of ketones like pentan-3-one.



The base peak, at m/z=57, is due to the $[CH_3CH_2CO]^+$ ion. We've already discussed the fragmentation that produces this.

Using mass spectra to distinguish between compounds

Suppose you had to suggest a way of distinguishing between pentan-2-one and pentan-3-one using their mass spectra.

pentan-2-one		$CH_3COCH_2CH_2CH_3$
pentan-3-one		$CH_3CH_2COCH_2CH_3$

Each of these is likely to split to produce ions with a positive charge on the CO group. In the pentan-2-one case, there are two different ions like this:

- $[CH_3CO]^+$
- $[COCH_2CH_2CH_3]^+$

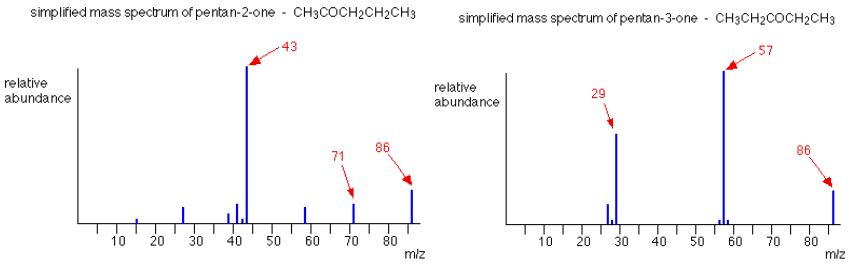
That would give you strong lines at m/z = 43 and 71.

With pentan-3-one, you would only get one ion of this kind:

- $[CH_3CH_2CO]^+$

In that case, you would get a strong line at 57. You don't need to worry about the other lines in the spectra - the 43, 57 and 71 lines give you plenty of difference between the two. The 43 and 71 lines are missing from the pentan-3-one spectrum, and the 57 line is missing from the pentan-2-one one.

The two mass spectra look like this:



Computer matching of mass spectra

As you've seen, the mass spectrum of even very similar organic compounds will be quite different because of the different fragmentations that can occur. Provided you have a computer data base of mass spectra, any unknown spectrum can be computer analysed and simply matched against the data base.

Contributors and Attributions

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16.11: Electrospray Ionization Mass Spectrometry

Electrospray ionization is a soft ionization technique that is typically used to determine the molecular weights of proteins, peptides, and other biological macromolecules. Soft ionization is a useful technique when considering biological molecules of large molecular mass, such as the aformetioned, because this process does not fragment the macromolecules into smaller charged particles, rather it turns the macromolecule being ionized into small droplets. These droplets will then be further desolvated into even smaller droplets, which creates molecules with attached protons. These protonated and desolvated molecular ions will then be passed through the mass analyzer to the detector, and the mass of the sample can be determined.

Introduction

Electrospray ionization mass spectrometry is a desorption ionization method. Desorption ionization methods can be performed on solid or liquid samples, and allows for the sample to be nonvolatile or thermally unstable. This means that ionization of samples such as proteins, peptides, olgiopeptides, and some inorganic molecules can be performed. Electrospray ionization mass spectrometry requires that a molecule be of a fairly large mass. The instrument has a small mass range that it is able to detect, so therefore the mass of the unknown injected sample can easily be determined; as it must be in the range of the instrument. This quantitative analysis is done by considering the mass to charge ratios of the various peaks in the spectrum (Figure 1). The spectrum is shown with the mass-to-charge (m/z) ratio on the x-axis, and the relative intensity (%) of each peak shown on the y-axis. Calculations to determine the unknown mass, M_r, from the spectral data can then be performed using

$$p = \frac{m}{z} \tag{16.11.1}$$

$$p_1 = \frac{M_r + z_1}{z_1} \tag{16.11.2}$$

$$p_2 = \frac{M_r + (z_1 - 1)}{z_1 - 1} \tag{16.11.3}$$

where p_1 and p_2 are adjacent peaks. Peak p_1 comes before peak p_2 in the spectrum, and has a lower m/z value. The z_1 value represents the charge of peak one. It should be noted that as the m/z value increases, the number of protons attached to the molecular ion decreases. Figure 1 below illustrates these concepts. Electrospray ionization mass spectrometry research was pioneered by the analytical chemistry professor John Bennet Fenn, who shared the Nobel Prize in Chemistry with Koichi Tanaka in 2002 for his work on the subject.

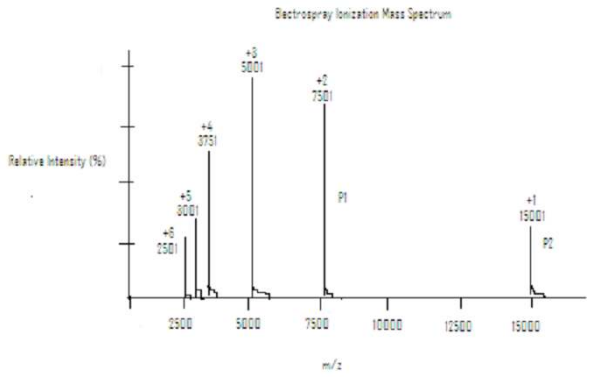


Figure 1. Mass Spectrum Example

Calculations for m/z in spectrum

$[M + 6H]^{6+}$ $[M + 5H]^{5+}$ $[M + 4H]^{4+}$

m/z = 15006/6 = 2501 m/z = 15005/5 = 3001 m/z = 15004/4 = 3751

$$[M + 3H]^{3+}\ [M + 2H]^{2+}\ [M + H]^{1+}$$

$$m/z = 15003/3 = 5001\ m/z = 15002/2 = 7501\ m/z = 15001/1 = 15001$$

Sample Preparation

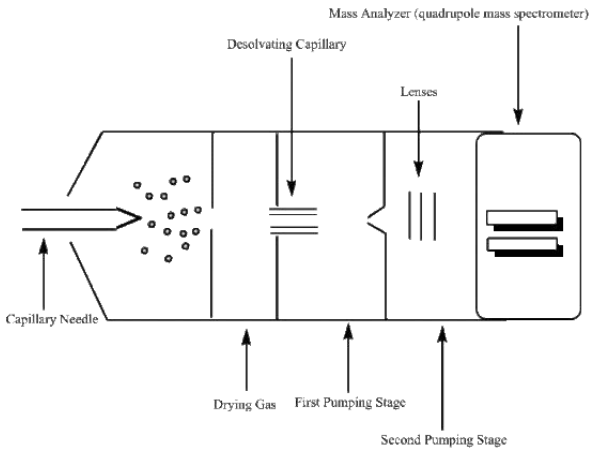
Samples for injection into the electrospray ionization mass spectrometer work the best if they are first purified. The reason purity in a sample is important is because this technique does not work well when mixtures are used as the analyte. For this reason a means of purification is often employed to inject a homogeneous sample into the capillary needle. High performance liquid chromatography, Capillary Electrophoresis, and Liquid-Solid Column Chromatography are methods of choice for this purpose. The chosen purification method is then attached to the capillary needle, and the sample can be introduced directly.

Advantages and Disadvantages

There are some clear advantages to using electrospray ionization mass spectrometry as an analytical method. One advantage is its ability to handle samples that have large masses. Another advantage is that this ionization method is one of the softest ionization methods available, therefore it has the ability to analyze biological samples that are defined by non-covalent interactions. A quadrupole mass analyzer can also be used for this method, which means that a sample's structure can be determined fairly easily. The m/z ratio range of the quadrupole instrument is fairly small, which means that the mass of the sample can be determined to with a high amount of accuracy. Finally, the sensitivity for this instrument is impressive and therefore can be useful in accurate quantitative and qualitative measurements.

Some disadvantages to electrospray ionization mass spectrometry are present as well. A major disadvantage is that this technique cannot analyze mixtures very well, and when forced to do so, the results are unreliable. The apparatus is also very difficult to clean and has a tendency to become overly contaminated with residues from previous experiments. Finally, the multiple charges that are attached to the molecular ions can make for confusing spectral data. This confusion is further fueled by use of a mixed sample, which is yet another reason why mixtures should be avoided when using an electrospray ionization mass spectrometer.

Apparatus



Capillary Needle

The capillary needle is the inlet into the apparatus for the liquid sample. Once in the capillary needle, the liquid sample is nebulized and charged. There is a large amount of pressure being applied to the capillary needle, which in effect nebulizes the liquid sample forming a mist. The stainless steel capillary needle is also surrounded by an electrode that retains a steady voltage of around 4000 volts. This applied voltage will place a charge on the droplets. Therefore, the mist that is ejected from the needle will be comprised of charged molecular ions.

Desolvating Capillary

The molecular ions are oxidized upon entering the desolvating capillary, and a continual voltage is applied to the gas chamber in which this capillary is located. Here the desolvation process begins, through the use of a dry gas or heat, and the desolvation process continues through various pumping stages as the molecular ion travels towards the mass analyzer. An example of a dry gas

would be an N_2 gas that has been dehydrated. The gas or heat then provides means of evaporation, or desolvation, for the ionized droplets. As the droplets become smaller in size, their electric field densities become more concentrated. The increase in electric field density causes the like charges to repel one another, which induces an increase in surface tension. The point where the droplet can no longer support this increase in surface tension is known as the Rayleigh limit. At this point, the droplet divides into smaller droplets of either positive or negative chrage. This process is refered to as either a coulombic explosion or the ions are described as exiting the droplet through the "Taylor cone". Once the molecular ions have reached the entrance to the mass analyzer, they have been effectively reduced through protonation.

Mass Analyzer

Mass Analyzers (Mass Spectrometry) are used to determine the mass-to-charge ratio (m/z), this ratio is used to differentiate between molecular ions that were formed in the desolvating capillary. In order for a mass-to-charge ratio to be determined, the mass analyzer must be able to separate even the smallest masses. The ability of the analyzer to resolve the mass peaks can be defined with the following equation;

$$R = \frac{m}{\Delta m} \tag{16.11.4}$$

This equation represents the mass of the first peak (m), divided by the difference between the neighboring peaks Δm. The better the resolution, the more useful the data. The mass analyzer must also be able to measure the ion currents produced by the multiply charged particles that are created in this process.

Mass analyzers use electrostatic lenses to direct the beam of molecular ions to the analyzer. A vacuum system is used to maintain a low pressure environment in order to prevent unwanted interactions between the molecular ions and any components that may be present in the atmosphere. These atmospheric components can effect the determined mass-to-charge ratio, so it is best to keep them to a minimum. The mass-to-charge ratio is then used to determine quantitative and qualitative properties of the liquid sample.

The mass analyzer used for electrospray ionization is a quadrupole mass spectrometer. A quadrupole mass spectrometer uses four charged rods, two negatively charged and two positively charged, that have alternating AC and DC currents. The rods are connected to both the positive terminal of the DC voltage and the negative terminal. Each pair of rods contains a negatively charged rod and a positively charged rod. The molecular ions are then sped through the chamber between these pairs of oppositely charged rods making use of a potential difference to do so. To maintain charge, and ultimately be readable by the detector, the molecular ions must travel through the quadrupole chamber without touching any of the four charged rods. If a molecular ion does run into one of the rods it will deem it neutral and undetectable.

Detector

The molecular ions pass through the mass analyzer to the detector. The detector most commonly used in conjunction with the quadrupole mass analyzer is a high energy dynode (HED), which is a electron multiplier with some slight variations. In an HED detector, the electrons are passed through the system at a high voltage and the electrons are measured at the end of the funnel shaped apparatus; otherwise known as the anode. A HED detector differs from the electron multiplier in that it operates at a much higher sensitivity for samples with a large mass than does the electron multiplier detector. Once the analog signal of the mass-to-charge ratio is recorded, it is then converted to a digital signal and a spectrum representing the data run can be analyzed.

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Problems

Using the above spectrum, calculate the mass of the protein. For $p_{1,}$ shown in spectrum, the m/z is 7501 and for p_2 the m/z is 15001. (Hint: Use the above equations, and the charge of p_1 for z_1)

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