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THE BASICS OF CHEMICAL INSTRUMENTATION VOLUME I - SEPARATION METHODS

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ABSTRACT

This report is intended to provide an introduction to chemical instrumentation to workers whose training lies outside the areas of experimental chemistry. It is especially geared toward scientific managers in government and industrial laboratories who must interact on a daily basis with chemical professionals, and who often have highly sophisticated analytical chemistry laboratories under their jurisdiction. This first report deals with instrumental separation methods. After an introduction to the nature of mixtures, the topics of gas chromatography, high performance liquid chromatography, supercritical fluid chromatography and electrophoresis are discussed. These sections are followed by an appendix covering calibration methods. A glossary is provided in which terms not covered in the body of the report are defined.

Key words: chemical instrumentation; chromatography; electrophoresis; gas chromatography; liquid chromatography; supercritical fluid chromatography; separation methods.

PREFACE

This work and its companion volume on spectroscopic methods are intended to provide an introduction to chemical instrumentation for workers whose fields lie outside of experimental chemistry. This includes scientists and engineers whose interests and endeavors are principally in theoretical areas. The book is also intended to meet the needs of scientific managers in government and industrial laboratories, as well as the technical staffs involved with corporate and government patent law.

Theoretically inclined scientists and engineers often find the need to collaborate with experimentalists in the testing of theories and models. Since questions of chemical purity are almost universal in experimental science, such collaboration will often bring the theorist into contact with chemical analysis. This book attempts to serve their needs by providing a simple discussion of each major technique without an overemphasis on the fine details of hardware. At the same time, however, the text does not oversimplify the techniques. In this respect, we attempt to cure the "black box syndrome," in which theoreticians often do not have sufficient appreciation of the complexities of analytical instruments and methods development.

It is very common that middle and upper level managers in government and industry have in their chains of command

some of the most sophisticated and costly analytical laboratories in the world. It is also quite common that these managers have little or no formal training in experimental chemistry. Many are physicists or engineers, yet they must interact on a daily basis with personnel who specialize in chemical instrumentation.

The managerial function will usually include presentations to technical audiences and higher levels of management (sometimes to Congress), preparation of proposals, and the supervision of resource allocation. These tasks will often require a basic familiarity with chemical instrumentation. Clearly, these managers do not have sufficient time to read through the excellent undergraduate or graduate level texts now available that cover instrumentation. Indeed, these texts usually accompany a one– or two–semester course of study, and contain laboratory exercises and problem sets. Such a treatment may seem a bit too detailed and intensive for an executive who is continually pressed for time. This book will answer their needs by providing a concise, conceptual discussion. Mathematical expositions of each technique's theory of operation are intentionally kept to a minimum, since the desire is to convey an intuitive "feel" for the processes involved.

Another important audience for which this book is intended is the community of attorneys involved in patent law, a vital part of commerce. Many patent attorneys and counselors have undergraduate degrees in chemical fields, but have little time to remain current. This book affords such professionals a basic understanding of modern instrumental techniques, including approaches such as supercritical fluid chromatography, a field which is relatively new.

The basic philosophy which is stressed throughout the book is one of simplicity. The reader is urged to use the simplest approach possible to solve a particular problem, while still maintaining the required degree of accuracy. This involves choosing not only the simplest instrumental method which is suitable, but also the simplest calibration method which will appropriately answer the analytical questions which are posed. This philosophy was eloquently expressed by Russel B. Scott (1902 – 1967), a world–renowned low temperature physicist at the National Bureau of Standards, who once stated "If you can do an experiment using a piece of copper tube and a few pipe fittings, and still get the accuracy you need, then that is where you stop."

The required level of accuracy is invariably determined by the application for which the data are needed. In some cases, it may be adequate to simply determine that a particular chemical is present in a sample, while in another case, a quantitative measure in the parts per trillion range may be an absolute necessity. The difference in cost involved in qualitative identification and parts per trillion quantitation is likely to be quite significant. It is entirely appropriate and necessary to include economic considerations in the decision–making process when one specifies the required level of accuracy. In this text, we address these questions explicitly.

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Boulder, Colorado T. J. Bruno

Note: Certain commercial equipment, materials, instruments, and devices are identified in this volume in order to provide an adequate description. Such identification does not imply endorsement or recommendation by the National Institute of Standards and Technology, nor does it imply that the equipment, materials, instruments and devices are necessarily the best available for the purpose.

CONTENTS

CHAPTER ONE

THE NATURE OF MIXTURES

1.1 INTRODUCTION

When we deal with analytical separation methods, we are invariably dealing with samples which are presented as mixtures. These mixtures may be very simple, such as one solute (or sample) dissolved in a solvent of our own choosing, or quite complex, such as a potential hazardous waste sample over which we have no control or prior knowledge. Most of the mixtures we will consider for analysis in this volume will be fluids or solutions, although gaseous samples are encountered as well. In order to choose and properly apply a particular instrumental technique, we will have to understand some of the basic characteristics of fluid mixtures in general. This includes some macroscopic features such as physical properties, as well as molecular and geometrical features on the microscopic level.

1.2 INTERMOLECULAR FORCES

The nature of mixtures is profoundly influenced by interactions and forces which arise between the individual molecules which compose the mixture. Indeed, it is largely the results of these intermolecular forces that give us the observable bulk physical properties of matter that we will discuss later. To effect a separation of components of a mixture, we will need to understand and take advantage of intermolecular forces and the physical properties which result from these forces. This is not to say that the start of each chemical analysis should begin with the Schroedinger wave equation and quantum mechanics. We will instead use our intuitive grasp of the microstructure of matter to our advantage in designing effective separation schemes using appropriate instrumentation. A comparison of some important intermolecular forces and their energies with representative chemical bond energies is presented in Table 1.1.

Dispersion forces (studied by London in 1930) which arise among essentially nonpolar molecules contribute to both attraction and repulsion. They are represented in Table 1.1 as the interactions occurring between simple fluids. At any given instant, the electrons of a molecule (such as methane or carbon tetrachloride) are in some configuration which will result in an instantaneous dipole moment. A dipole moment occurs in a molecule whenever the electrons and nuclei of the atoms are arranged so that, at any given instant, part of the molecule has a net negative charge and part has a net positive charge. This instantaneous dipole moment can then electrostatically induce a dipole moment in another molecule, resulting in a weak, short–

TABLE 1.1 APPROXIMATE BONDING ENERGIES

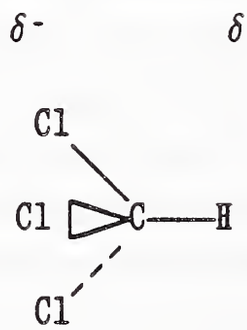
Bond	Energy $kJ \cdot mol^{-1}$
Simple Fluids	
$CH_4...CH_4$	0.5
$C_6H_6...C_6H_6$	1.6
Associating Fluids	
C—H...O	11
C—H...N	13—21
S—trinitrobenzene—mesitylene	40
Chemical Bonds	
C—C	347
O—H	465

range net attraction. This interaction is therefore alternatively called the dipole–induced dipole interaction. The attraction can be additive over several molecules, resulting in, for example, short–lived nearest neighbor ordering. As the molecules approach one another more closely, the weak attractive force gives way to a net repulsive force. The susceptibility of a molecule to induced dipole forces depends on the polarizability of the molecule. A molecule has a high polarizability when it contains groups with large, bulky electron clouds that can be easily deformed. These dispersion forces will always be present among molecules, regardless of the constituent atoms. They will be the only forces which will occur between nonpolar simple molecules such as the alkanes or the noble gases. Naturally,

molecules with more actively interacting atoms (such as oxygen, nitrogen, and in some cases the strongly electronegative halogens, for example) will display other interactions in addition to dispersion.

A compound such as formamide, CH_3NO, which has a large permanent dipole moment, will show very strong intermolecular interactions. The forces occurring between these kinds of molecules are called Keesom forces. These attractive interactions will promote self association, causing the formation of clusters of molecules. Because of this effect, these dipole–dipole forces are considered to be orientation forces, having a significant influence on long–range order (the scale is large as compared to molecular size) and structure in a fluid. Such highly polar molecules will be more strongly attracted to one another than nonpolar molecules of comparable molecular weight. Because of the strong interactions, mixtures of polar molecules can be especially difficult and challenging to separate using standard analytical techniques.

If we were to change one of the chlorine atoms of CCl_4 to a hydrogen, the formula and structure of chloroform, $CHCl_3$, results:



where δ^+ and δ^- represent fractional charges on the halogen atoms and on the hydrogen. We now notice a significant change in behavior due to the dipolar interaction known as the hydrogen bond. This will occur when a hydrogen is bonded to an atom which is in close proximity to an electronegative atom (or group of atoms) such as oxygen, nitrogen or a halogen. The hydrogen atom, being small and not shielded by a large electron cloud, can interact with nearby electron–rich groups or molecules. Thus, the hydrogen atom of chloroform will be attracted to the oxygen linkage of an ether or the carbonyl of a ketone, since they are relatively electron–rich. The hydrogen bonding potential of a particular hydrogen is controlled by the chemical neighborhood of the molecule. The electronegative environment set up by the three chlorine atoms on the carbon tends to unshield the hydrogen and make it "feel" more positively charged to its neighbors. The hydrogen bond between chloroform and a ketone would still be relatively weak, however. A very strong hydrogen bond system exists in water, where a relatively stable extended structure of four to five water molecules can exist as a cluster at ambient temperature. No such stable long–range structure exists in nonpolar fluids such as CCl_4, in which dispersion forces are the predominant interaction.

The hydrogen bond is relatively strong as intermolecular forces go, having energies of 4 to 11 kJ/mol. These interactions are highly directional in space, and may even be considered as being weak bonds. Hydrogen bonded systems will usually present problems in the design of separations, especially those

involving chromatographic techniques. Peak asymmetry (which we will discuss in detail) and the resulting quantitation problems will usually have to be addressed in analyses of systems such as these.

The stronger intermolecular interactions described above can cause the formation of intermolecular complexes. A complex is an observable, distinct pairing between molecules which is higher in interaction energy and of a longer lifetime than that which would be expected from random molecular collisions. The complex is itself a discrete chemical entity, having its own unique properties. For example, the well–known complex formed between benzene and iodine has a unique ultraviolet spectrum, having a band not present in the electronic spectra of either pure benzene or pure iodine. This complex is called a charge–transfer complex, since the movement of an electron between the benzene π–orbital system and the iodine molecule is demonstrated. The hydrogen bonded clusters of water molecules may also be considered a kind of complex. Actually, complex formation can occur in any system containing molecules which are net negative charge donors in combination with negative charge acceptors. These complexes may be weak and short lived, as in the case of chloroform with an ether, or strong enough to actually be isolated chemically. Complexation will always have a significant effect on the properties of a mixture and our ability to separate that mixture using analytical techniques.

The forces which arise between ions in aqueous solution are both very strong and long range. They will be an important factor in many separations using liquid chromatography,

especially when ion–exchange columns and ion–pairing agents are used. Ions in solution will interact strongly with dipolar molecules, causing ion–dipole interactions.

1.3 MOLECULAR GEOMETRY AND ISOMERISM

The physical arrangement of atoms in a molecule, which we shall call the molecular geometry, can also have a profound effect on the properties of a mixture. We shall consider two examples of how differences in the molecular structure or geometry of compounds can have a striking effect in one case and a very subtle effect in another.

Ethanol and dimethyl ether are isomers that have the chemical formula C_2H_6O, both with a molecular weight of 46. The following table gives some of their relevant physical properties:

	Dimethyl Ether	Ethanol
State	Gas	Liquid
Molecular Mass	46 g/mol	46 g/mol
Boiling Point	–24.5° C	78.5° C
Melting Point	–138.5° C	–117.3° C

Clearly, these two chemicals have vastly different properties despite having the same formula. The reason, as you may

suspect, relates to the previous section on intermolecular interactions. The predominant interaction between molecules of dimethyl ether is the London dispersion (or the van der Waals interaction). While these interactions are attractive, the overall effect is very weak. Ethanol, on the other hand, due to the change in molecular geometry, has the specific interaction of hydrogen bonding that we discussed earlier. As a result, it actually exists in a long–range, self–associated network. The effect of the hydrogen bond is so dramatic that it causes ethanol to exist in the liquid state (at ambient temperature and pressure) rather than in the gaseous state, as is the case for dimethyl ether. We would not expect the problem of separating a mixture of ethanol and dimethyl ether to be particularly challenging on the basis of their physical properties.

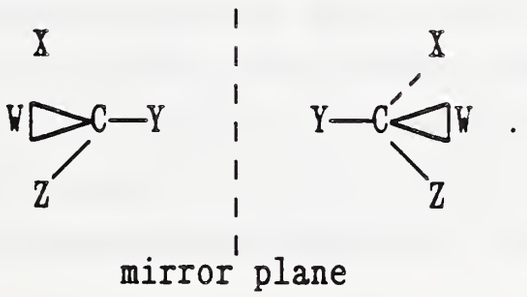
Let us now turn our attention to two other isomers, each having the formula $C_2F_3Cl_2H$ and a molecular weight of 152 g/mol. These compounds are both representatives of the newly developed ozone–benign alternative refrigerant fluids. These compounds are of interest to many sectors of industry and the environmental community, since they are not as long–lived as the fully–halogenated refrigerants which are currently thought to harm the atmospheric ozone layer. The following table lists some relevant properties of these materials.

	CF_3CHCl_2	$CClF_2CHClF$
Boiling Point	27.1° C	28° C
Melting Point	–107° C	–78° C

The slight difference in geometrical structure between these two materials has a very subtle effect on their physical properties at ambient temperature and pressure. Generally, the major intermolecular forces to be considered for both are the dispersion forces. As you may imagine, separation of these isomers for analysis does indeed cause some difficulties, especially since there is usually cross contamination of one in the other.

Our discussion of molecular geometry and isomerism would be incomplete without a few words about stereoisomerism and chirality. This is especially important in a book which treats separation methods, since many of the more vexing and rigorous analytical separations performed in recent years have concerned stereoisomers. This is especially important in the study of biomolecules for the pharmaceutical industry. Stereoisomers are isomeric species which differ only in the way the individual atoms are arranged in space. This can be contrasted with the refrigerant isomers described above, in which there were actually differences in the number of halogen species on each carbon. Stereoisomers can be subdivided into two groups, called enantiomers and diastereomers.

Isomers which are not superimposable upon their mirror image are enantiomers:



This example shows a tetrahedral carbon atom with four different substituents, W, X, Y, and Z. The solid lines indicate a bond in the plane of the page, the dotted lines a bond beneath the plane of the page and the filled triangles a bond projecting from the page. If a molecule is superimposable upon its mirror image, no isomerism exists. Molecules which cannot be superimposed on their mirror images are termed "chiral" (from the Greek work meaning handedness), while those that can are called "achiral." Carbon atoms which contain four different substituents are always chiral, and the carbon in question is called the chiral center.

If we can isolate one enantiomer from the other, an interesting optical phenomenon may be observed. The individual enantiomers can rotate the plane of polarized light to the right or left, depending on their structure. In fact, one of the isomers will rotate the plane to the right (the dextrorotary, or "+" isomer), and the other to the left (the levorotary, or "–" isomer) by the same amount. While the physics of this interesting interaction need not concern us in the design of effective analytical separations, we must have an appreciation of the consequences of the structural features of these molecules. An equimolar enantiomeric mixture (50 percent of each isomer) will not change the plane of polarized light, since the tendency to rotate to the right and left is exactly balanced. This mixture is called a "racemic modification." If there is a greater amount of one over the other, optical activity may be observed in the mixture. The direction of polarized light rotation in such a mixture will depend on the predominating enantiomer.

The physical properties of the individual enantiomers which compose the mixture will be identical. This includes density, boiling point, vapor pressure, refractive index, etc. The only difference, other than the possibility of optical activity, will be in the relative reactivity and interactions toward enantiomeric reagents. This is fortunate, since it will allow us to carry out separations of chiral materials using the chromatographic methods discussed in this book.

Diastereomers are a class of stereoisomers which are not mirror image isomers, as opposed to being simply not superimposable. These isomers are much simpler to deal with from a separation point of view. The physical properties of diastereomers are always different, in many cases strikingly so. The chemical properties of diastereomers, while similar, will also be different. It will be much easier to successfully apply the methods of chromatography to mixtures of diastereomers than to mixtures of enantiomers.

1.4 PHYSICAL PROPERTIES

Our discussion thus far has centered around individual molecules in mixtures, and how microscopic properties of molecules influence mixture behavior. In general, we do not have the means to examine and analyze individual molecules, but only large collections of molecules. The molecular and structural features which we have discussed interact in a kind of cooperative phenomenon to produce what we call the physical

properties of materials. These properties are quite different for the gaseous, liquid and solid states of matter. From the standpoint of analytical separations, a number of physical properties are very important indicators. Several of these are summarized in Table 1.2, which provides a listing of the typically expected values of these properties for the different states of matter. We will describe some important general aspects of these physical properties here, but will leave detailed discussions until the appropriate techniques are described. Naturally, many other physical properties can be of value in the design of effective analytical strategies. We have chosen to focus on just a few of them to illustrate their importance.

The density of a material is a fundamental property which is related to the closeness of the individual molecules (the interatomic distance, shown in the last column of Table 1.2). The presence of intermolecular interactions such as hydrogen bonding will influence the magnitude of the density. The density of gases tends to be relatively low; those of liquids and supercritical fluids are higher (and are comparable in magnitude), and the densities of solids are relatively high. The dependence of density on pressure and temperature is very striking for the fluid phases (gas, liquid, and supercritical fluid). This dependence is depicted in the P–V–T (pressure–volume–temperature) surfaces which we will encounter in Chapter 4, when we discuss supercritical fluid chromatography. We will make use of density in many ways in chemical analysis, since this property will allow the prediction and control of many aspects of sample solubilization.

STATE OF MATTER	DENSITY (Kg/m^3)	VAPOR PRESSURE (Pa)	DIFFUSIVITY (m^2/s)	THERMAL CONDUCTIVITY ($W/m \cdot K$)	SOUND VELOCITY (m/s)	DYNAMIC VISCOSITY (Pa x s)	INTER MOLECULAR DISTANCE (Mean Free Path) (m)
GASES	10^{-1}–1.0		10^{-8}–10^{-9}	7.4×10^{-4} to 1.9×10^{-1}	150–1300	1×10^{-5} to 3×10^{-5}	3.6×10^{-8} to 13.3×10^{-8}
LIQUIDS	10^3–10^4	2.4×10^5 to 4.2×10^6	5×10^{-10}	0.094 to 0.668	900–1900	2×10^{-4} to 1.0	2.56×10^{-10} to 4.07×10^{-10}
SUPERCRITICAL FLUIDS	3×10^2 to 10^3		10^{-7}	0.043 to 0.17	200–1000	2.5×10^{-5} to 1.7×10^{-4}	
SOLIDS	10^3–10^4	10^{-4}–10^{-9}		10^2 to 7.0×10^1	10^3–10^4		0.5×10^{-10} to 3.0×10^{-10}

The vapor pressure may be termed a two–phase property, since it is a gaseous pressure which always exists above the surface of a condensed phase (liquid or solid). For this reason, no value range is provided for either the gas or supercritical fluid state, since these are both single–phase systems. The vapor pressure is a useful parameter in the development of many analytical methods for liquid samples. Since vapor pressure is directly related to the normal boiling point of a liquid, we can use it as a design parameter for many chromatographic separations. From a practical point of view, we will have to consider the effect of the sample vapor pressure on sampling and on the instrumentation we choose to apply.

The diffusivity, thermal conductivity, and viscosity are a related set of physical properties which are called transport properties. They describe the efficiency of either heat or mass transfer that can be accomplished in a given state of matter. The diffusivity is an important property to consider in all types of chromatographic analyses (Chapters 2, 3 and 4), since it will describe the tendency of a solute to disperse into the carrier and stationary phase. The viscosity is also important in chromatography, since in simple terms, it describes the resistance of a fluid to flow. The thermal conductivity is mentioned since instrumental methods often depend upon the efficient transfer of heat to a sample, as in some injection and detection techniques in chromatography. All of the transport properties are dependent on temperature, pressure, and density. Thus, by properly adjusting experimental variables, we can often "tune" these physical properties to aid us in obtaining the

desired analytical procedures and results. This is analogous to the use of our knowledge of intermolecular interactions (such as hydrogen bonding) to choose chromatographic columns.

SUGGESTED READING

Molecular Complexes and Intermolecular Forces:

L. Andrews, Molecular Complexes in Organic Chemistry, Holden–Day, San Francisco, 1964.

J. Rose, Molecular Complexes, Pergamon, Oxford, 1967.

M. D. Joesten and L. J. Schaad, Hydrogen Bonding, Marcel Dekker, New York, 1974.

J. N. Israelachvili, Intermolecular and Surface Forces, Academic Press, Orlando, 1985.

Isomers and Stereochemistry:

J. March, Advanced Organic Chemistry, 3rd Edition, John Wiley and Sons (Wiley Interscience), New York, 1985.

Thermophysical Properties:

R. C. Reid, J. M. Prausnitz, and B. E. Poling, The Properties of Gases and Liquids, 4th Edition, McGraw–Hill, New York, 1987.

CHAPTER TWO

GAS CHROMATOGRAPHY

2.1 INTRODUCTION

The term "gas chromatography" is a broad label which includes many separate, distinct techniques and approaches. The successful use of gas chromatography (GC) to solve an analytical problem will require an understanding of the many differences and tradeoffs in approach and instrumentation. We therefore have a set of decisions and choices to make. We will have to decide beforehand what exactly the questions are which we want answered, and to what level of certainty. Only then can we attempt to provide the answers using this technique (or any analytical technique, for that matter). Our earlier discussion on the nature of mixtures will play a vital role in guiding us to the optimal (note that the word "correct" is avoided) combination of hardware and method that will provide the answers we want.

Our discussion of gas chromatography will be very detailed for two reasons. First, GC is the most important and economical of all the separation methods. Second, many of the underlying principles and approaches which pertain to GC also pertain to liquid and supercritical fluid chromatography, which are discussed in following chapters.

We must first understand that "gas chromatography" broadly covers three major separation–analysis methods. All of these methods share the common characteristic of a gaseous mobile phase or eluent that carries the solute or sample through the stationary phase, where separation occurs. While the eluent gas chosen is usually helium, nitrogen or sometimes hydrogen[1], one may choose many other carrier gases (or gas mixtures) depending on the problem to be solved. We can gain a basic understanding of the chromatographic process by considering the series of "snapshots" in Figure 2.1. Note that each frame of our chromatographic movie is divided in half, one part marked M (for mobile phase) and the other marked S (for stationary phase). The moving carrier is always present in the mobile phase part of the figure as it represents a steady flow of the gas. The stationary phase contains an interacting (or active) species, labeled D, which is affixed to a stationary base (hence the name stationary phase).

The first frame of our movie shows the relatively uninteresting situation of carrier gas flowing through the stationary phase. The second frame shows the appearance of A,

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[1]Hydrogen is a flammable gas which easily forms explosive mixtures with air and thus must be handled with extreme care, preferably in an explosion–proof laboratory.

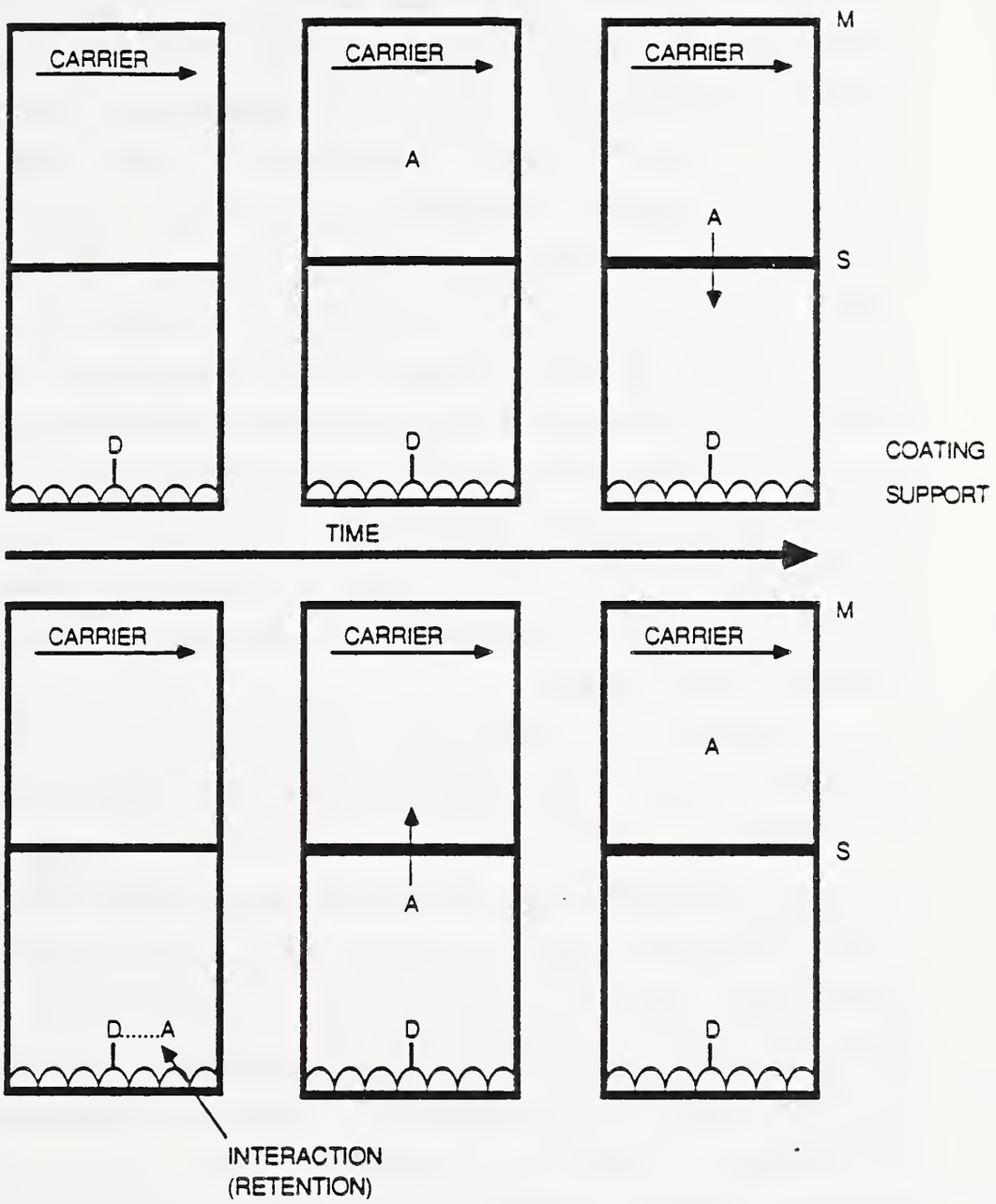


Figure 2.1 A "chromatographic movie" showing the processes which occur in a column.

our sample. The nature of the sample, whether it is a polar mixture or an inert gas, need not concern us just yet. We will also put off the very important question of "just how did the sample get in there?" Subject to both thermodynamic and kinetic considerations, some of our sample will cross the imaginary line and enter the environment of the stationary phase, as in Frame 3. Depending on what kind of intermolecular forces are present between A and D, an interaction of some kind (weak, strong or in between) will occur, as seen in Frame 4. Here is where our discussion of intermolecular forces becomes important. The success of any gas chromatographic method will very often depend upon our skill in using these forces as a tool. The stronger the interaction between A and D, the longer that interaction will last. We do wish to recover and detect our sample, so we design the interaction to ultimately release A, as shown in frames 5 and 6.

In reality, our movie will be replayed many hundreds of times as the sample is carried through the stationary phase by the carrier gas. Although we have not mentioned temperature, we have assumed implicitly that it has been constant during our "chromatographic movie." As we might expect, raising the temperature would be like shifting our movie into "fast forward;" the A—D interaction would in general be shorter in duration, and the elution time faster. Just a brief comment on the notation of Figure 2.1 may be in order. The A and D moieties represent electron pair acceptor and donor species, respectively, as in our discussion of intermolecular complexes. In this figure we have chosen to make the stationary phase the donor, but the reverse situation may be just as likely.

So far, so good, but what about a mixture with, say, ten components? An example might be a homologous series of hydrocarbons. The strength of the interactions of each of the individual components with D will be different, even if only slightly. It would be difficult to imagine a mixture containing components which all have identical chemical and physical properties. Naturally, the more strongly interacting components will form longer–lived interactions than those which are weaker. These components will be "retained" in the stationary phase longer, and will be released from the stationary phase later and therefore detected later than other components. As we have mentioned, temperature also makes a difference, and we will always use it to our advantage in the design of a separation. In general, the separation which will occur between two given components will be greater at a lower temperature. While our treatment so far is a drastic oversimplification, we will make do for now and add details when needed as our discussion progresses.

We can better understand the separation process by referring to the hypothetical chromatogram shown in Figure 2.2. The chromatogram is presented as a graph of detector signal level (here represented as millivolts) as a function of time. Two peaks have emerged, corresponding to two separated components, a and b . These two peaks serve to illustrate three important chromatographic parameters: efficiency, selectivity, and resolution.

The efficiency of a column is a kinetic (rate dependent) parameter which describes the column's ability to separate components sharply as a function of the sample residence time

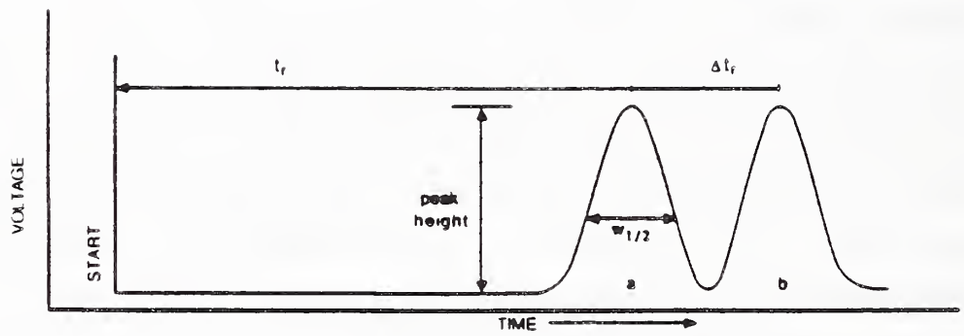


Figure 2.2 An idealized chromatogram illustrating the concepts of retention time and peak width.

in the column. The historical analogy between distillation and chromatography resulted in means of expressing column efficiency using chemical engineering parlance. The concept of the "theoretical plate," or more simply, the plate number is really a measure of solute zone spreading or dispersion. A

theoretical plate can be thought of as an imaginary zone or volume element in which rapid equilibration of the solute between the mobile and stationary phases can take place. For the idealization of a Gaussian–shaped peak, the number of plates contained on a given column is given by:

$$n = 5.545 \, (t_r/w_{1/2})^2 \qquad (2.1)$$

where t_r is the retention time of the solute, and $w_{1/2}$ is the peak width (at half the peak height) expressed in units of time. The larger the value for the dimensionless number n, the higher is the column efficiency. An alternative expression for efficiency is the "height equivalent to a theoretical plate," h, which is found from:

$$h = L/n \qquad (2.2)$$

where L is the length of the column. The efficiency of the column increases as h decreases. It must be emphasized that these simple equations are strictly applicable only to the Gaussian curve. For asymmetric peaks the equations become more complex and approximate, subject to many simplifying assumptions.

There are many factors which affect the efficiency of a column for the separation of a given solute. The major factors are the mobile phase velocity through the column, the nature and thickness of the stationary phase film (if any), the viscosity

of the mobile phase, and column internal diameter. These factors, along with parameters such as solute–solvent diffusivity, can be controlled in order to optimize separations. The situation can become complex, however, since most of these factors are interdependent, many are temperature dependent, and changing one parameter may cause a change in another.

The efficiency of a column can be predicted quantitatively using the Van Deemter equation:

$$h = A + B/u + C \cdot u \quad . \tag{2.3}$$

In this equation, A is a geometrical constant which describes the multiple paths which carrier and solute can take in a packed column (we will discuss the different column types in more detail later). This is sometimes called the eddy current term because of the electronic analogy of current flow inhomogeneity. This term is minimized by keeping the particle size of the packing as small as possible (approximately 200 μm) without causing an excessively high pressure drop across the column. As we will see later, the value of this term can be made negligible by using an open tubular column (or capillary column). The constant B describes the relative diffusion of the solute in the gas and stationary phase. It is most significant at low carrier gas velocity (u in the equation above). The C term represents the resistance to mass transfer of the solute at the solute/stationary phase interface. Slow mass transfer, of course, means longer analysis times. A plot of h versus carrier gas velocity (Figure 2.3) illustrates the Van Deemter equation and

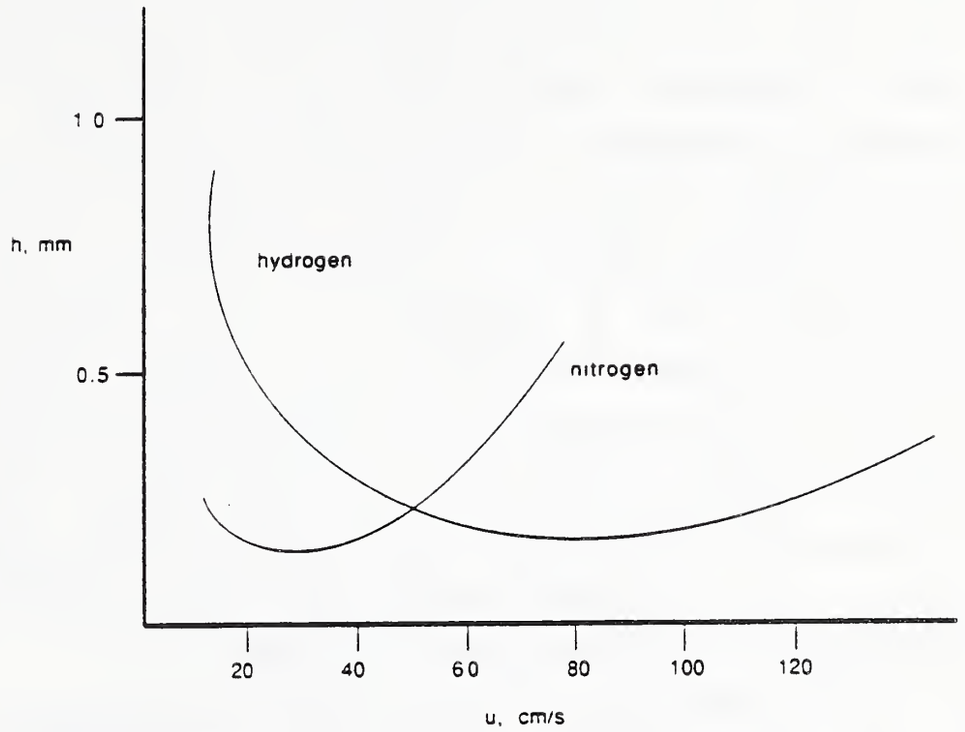


Figure 2.3 Plots of the results from the Van Deemter equation for hydrogen and nitrogen.

one of its major consequences. The plot shows a broad minimum which corresponds to the optimal carrier gas velocity. Typical values for practical operating linear velocities range from 8 to 12 cm/s, depending on the carrier gas used.

Selectivity is an essentially thermodynamic parameter which refers to the ability of a column to isolate two (or more) solutes into distinct bands, such as the peaks a and b on Figure 2.2. The selectivity is a function of the relative

magnitudes of the intermolecular forces between the solute and the stationary and mobile phases. This is where our discussion of intermolecular forces comes into play. For example, a relatively polar stationary phase will interact more strongly with polar solutes than with simple alkanes. The choice of stationary phase is therefore a very important step in the design of a chromatographic analysis. Several retention index systems have been developed to aid in the choice of stationary phase and provide adequate selectivity. The scaling systems of Kovats, of Rohrschneider, and of McReynolds have all attempted to address this problem. The most extensive tabulation available is for the system developed by McReynolds, which provides an index of retention for a set of reference compounds on a given stationary phase as compared with the nonpolar hydrocarbon, squalane. The McReynolds constant system can provide a good starting point in the choice of stationary phase.

The ultimate goal in a chromatographic measurement is to isolate a sample into successive bands corresponding to the individual components of the mixture. A measure of the isolation between components is the peak resolution, R:

$$R = \frac{\Delta t_r}{\frac{1}{2}\left[w^a_{1/2} + w^b_{1/2}\right]} \tag{2.4}$$

where Δt_r is the difference in retention time of two successive peak maxima, and $w^a_{1/2}$ and $w^b_{1/2}$ are their corresponding peak widths. Resolution is improved by increasing the peak separation (i.e., improving selectivity) or by decreasing the peak

widths (i.e., improving column efficiency). It is important to realize that a given column may have good selectivity while having poor efficiency, and vice versa. An optimized separation will produce both well–separated and sharp peaks on the chromatogram, and do so while minimizing the retention time.

Resolution is adequate when the degree of separation allows the particular analytical problem being considered to be solved. It may not be necessary to obtain optimum "baseline resolved" peaks in every situation. One must factor in the economic considerations of time and material costs. Quite often reasonable quantitative accuracy can be obtained with three percent overlap of two successive peaks. This corresponds to peak maxima separation of approximately $2w_{1/2}$ for two adjoining Gaussian–like peaks of equal size.

Now for some more choices and decisions. We will divide gas chromatography into three convenient and practical classifications: gas liquid chromatography, gas solid chromatography, and capillary gas chromatography. There are other classification schemes we could use, but this one will serve us well for the present. While the instrumental details of the three classes are unique and must be understood to properly use the methods, many of the features are common to all three.

Figure 2.4 shows a "generic" schematic of instrumental features common to all gas chromatographs. The carrier gas is usually supplied from a high pressure (approximately 15 to 16 MPa, or 2200 to 2400 psi) cylinder equipped with a two–stage pressure regulating valve. The pressure of this high purity gas (most often 99.995 percent purity or better) is usually regulated down to between 0.4 and 0.7 MPa (i.e., 60 and

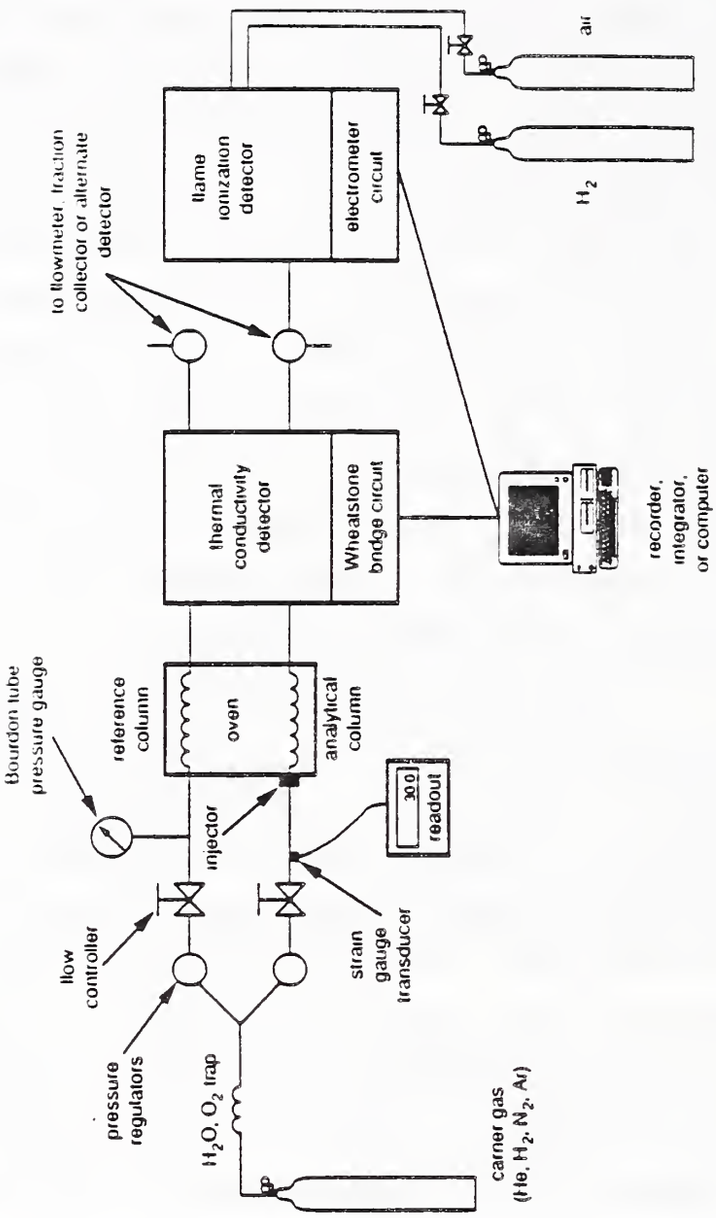


Figure 2.4 A schematic diagram showing the essential components of a typical gas chromatograph.

100 psi). A set of traps to remove trace quantities of water and oxygen are installed after the regulator, which are themselves followed by particulate filters. Trace water is effectively removed with a short length of tube filled with 5A molecular sieve, which is a synthetic zeolite (calcium alumino silicate). Commercial traps are available which contain redox resins to remove oxygen. These traps can also remove traces of carbon dioxide and carbon monoxide. The filters which follow the traps are usually sintered metal fitted disks having a 3 μm pore size.

The gas stream is usually split at this point because many common detectors require two streams, one for the sample and one with pure carrier as a reference for property comparison. We will discuss the more common detectors in detail later on. After the division of flow, the gas is passed through another stage of regulation. This is done using precision pressure controllers followed by needle valves (flow controllers), or by using a set of mass flow controllers. For work requiring the highest accuracy, these valves are maintained at constant temperature in a separate oven. Pressure measurement is usually provided by precision gauges or sometimes strain gauge transducers having an electronic readout. The flowrate of the carrier gas through the column is an important parameter which we have under our control. Flowrate is often measured at the column exit using a soap–film flowmeter, or *in situ* using a rotameter or anemometer. Increasing the flowrate will generally have much the same effect as increasing the column temperature, but not always. Usually, the samples will elute faster, and the peaks will be more closely spaced using a higher carrier gas flowrate.

The sample line contains an injection device which introduces sample into the carrier stream while causing minimal flow disturbance or pressure pulsation. Sample introduction is a very important and tricky part of the chromatographic system hardware. The choice and method of operation of this device will very often mean the success or failure of an analysis, especially for quantitative measurements and in the analysis of trace levels of components. We will spend a good deal of time discussing the injector when we treat GC hardware in more detail. Following the injector in the flow stream is the column oven area. This temperature–controlled region is usually a forced–air oven (only rarely is a liquid bath used, most often for physicochemical meaasurement work) large enough to hold several chromatographic columns, but not so large that it has an inconveniently long thermal lag time. This will allow the oven to respond quickly to temperature adjustments. The temperature should be stable to ±0.5° C for analytical work, and should ideally be programmable. Temperature programming allows the oven temperature to vary during an analysis, according to some desired scheme. For example, we may "program" the column oven temperature to begin at, say, 100° C, but to increase to 150° C at the rate of 2° C per minute.

Not all gas chromatography is done at elevated temperatures; occasionally one must resort to low temperatures (–100 to 0° C) to separate some stubborn mixtures, especially those involving gases and volatile organics. This can be done by pumping a cryogenic liquid (such as liquid nitrogen) into the oven, or by permanently mounting a Ranque–Hilsch vortex tube on the oven. A vortex tube is a unique refrigeration device

which can produce temperatures as low as –40° C simply using a source of compressed air.

Following the column oven are the detection devices. Now our earlier discussion of bulk properties will come into play, since we need an understanding of the behavior and properties of our sample components to choose an appropriate detector. Our generic gas chromatograph has two detectors; a thermal conductivity detector and a flame ionization detector, the responses of which may be logged on a computer or on an electronic recorder/integrator. Most modern gas chromato–graphs are equipped with multiple detectors, in order to make the instrument more versatile. We will discuss all of the more common detectors in detail, including some of the more useful optimization strategies.

2.2 GAS–LIQUID CHROMATOGRAPHY

Gas–liquid chromatography (GLC), as we will describe it, is characterized by a stationary phase consisting of a liquid which is coated or immobilized on an "inert" packed bed of supporting material. The liquid coating is what we previously called D, the stationary, interacting species in our "chromatographic movie." This liquid may be materials such as a heavy hydrocarbon (for example, squalane), a light polymeric liquid (such as polyethylene glycol), or a heavier silicone fluid (for example, polymethyl siloxane). The choice of stationary phase liquid, which we will discuss shortly, depends on the sample we wish to analyze. The inert bed of support material is usually a

diatomaceous earth, although in some circumstances glass beads or Teflon particles may be chosen. Diatomaceous earth, which consists of the skeletal remains of small prehistoric algae called diatoms, is about 88 percent silica. The materials used for chromatographic supports are purified by acid washing, and the surface is deactivated by a process called silanization in which active sites on the surface are capped synthetically. While this treatment cannot make the support surface perfectly inert, the deactivated surface is very suitable as a support for the analysis of all except the most polar of materials, such as alcohols and organic acids.

Even the most highly deactivated surface will be affected by the phenomenon of adsorption. Adsorption, which we will discuss in more detail in the section on gas–solid chromatography, is a universal adhesion that occurs between the mobile phase (both the carrier and sample molecules) and the surface of the stationary phase. A number of different supports may be chosen, differing mainly in density and available surface area. The choice, of course, depends upon the individual application, but the variety of supports provides a degree of flexibility.

The liquid is easily coated on the support by dissolving the liquid phase in a slight excess of a suitable solvent and adding the support to the mixture. The solvent is then removed in a rotary evaporator, leaving the support coated with the liquid. This may seem unusual, since the coated support will look and feel quite dry. What we mean, of course, is that there are layers of molecules of the liquid on the support, and these layers are liquid–like in their structure (remember the representative properties given in Table 1.2). The coated support is poured

into a tube which will serve as the column. The tube can be made of copper, stainless steel, nickel, glass, Teflon or some more specialized material. The diameter of this tubing can range from 0.64 to 0.16 cm in diameter, and from 2 to 10 m in length. Packed columns are relatively inexpensive and simple to make up in the laboratory. The many possible choices of liquid coatings are both a blessing and a curse. It is possible to design a column to perform some very specific separations, but the sheer number of possible coatings can be bewildering.

The chemical nature of the coated liquid imposes a restriction on the operating temperature range. At lower temperatures, many liquids will become very viscous or even solidify. At higher temperatures, the liquid will "bleed" from the support as a result of the increased vapor pressure. This is clearly unfavorable, since not only does the separation power of the column decrease, but also the detector response will become noisy due to the continual passage of extraneous material. Detector fouling can also result from column bleed, which can cause significant instrument down–time. Some liquids will even decompose at higher temperatures, also leading to detector fouling. An understanding of the bulk and molecular properties of the sample will guide the analyst toward a good choice of stationary phase. Quite often, however, one or more "survey runs" with several columns at several temperatures may be needed to arrive at an optimal combination of liquid phase and operating conditions for a particular analysis.

An important characteristic of gas–liquid chromatography is the notion that the physical processes involved are actually solution processes, with the sample being absorbed or dissolved

by the stationary liquid phase and later released through a "vaporization" step. All of the thermodynamic treatments applicable to solutions can be applied to gas–liquid chromatography, including considerations of enthalpy, entropy, equilibrium constants and activity coefficients. In fact, gas–liquid chromatography has been used for many years as a tool for the study of solution thermodynamics. One can actually measure thermodynamic properties of solution, such as enthalpies and entropies of solution and vaporization, activity coefficients, excess functions, and transport phenomena. Thus, for gas–liquid chromatography, our "chromatographic movie" of the previous section describes the repeated solvation and evaporation of the sample into and out of the liquid phase.

2.3 GAS–SOLID CHROMATOGRAPHY

Most of the instrumental requirements of gas–solid chromatography (GSC) are very similar to those of gas–liquid chromatography. The main difference is in the nature of the stationary phase, and the processes which occur in that phase. For clarity and convenience, we can divide the kind of stationary phases available into three types: adsorption columns, size exclusion columns, and porous polymer columns. Naturally, many of the same physical processes occur in all of these stationary phases.

The first kind of column we will consider is the adsorption column. We stated earlier that adsorption was a universal

adhesion process which will always occur at the boundary of any liquid or solid with another medium such as a gas. The difference between adsorption and absorption (or dissolution) is depicted humorously in the now–classical cartoon shown in Figure 2.5. The process is considered universal in that all solids, whether porous or nonporous, metal or nonmetal, hard or soft, will adsorb any gas, polar or nonpolar, on its surface. The attraction may be caused by physical interactions (such as van der Waals forces) or chemical interactions (possibly involving electron transfer). Adsorption is an exothermic process; when a gas is adsorbed on a solid, heat is liberated. Elementary thermodynamic considerations therefore tell us that as the temperature of a given system is increased, the amount of gas adsorbed by the solid at a given pressure will decrease. We should keep this in mind since, as we have already seen, temperature is an important chromatographic variable which we must control.

The most common packings used to prepare adsorption columns are silica, alumina, carbon blacks (especially the graphitized versions), and the synthetic zeolites (commonly called molecular sieves). The column diameters are typically the same as are used for the packings of gas–liquid chromatography, but the column length is often less. All the packings require heat activation prior to use, to drive off material which is already adsorbed. The time and temperature at which activation is performed very often will affect the chromatographic behavior of the packing, so this step requires careful attention. These adsorbents can be used successfully in the separation of lower molecular weight saturated, unsaturated,

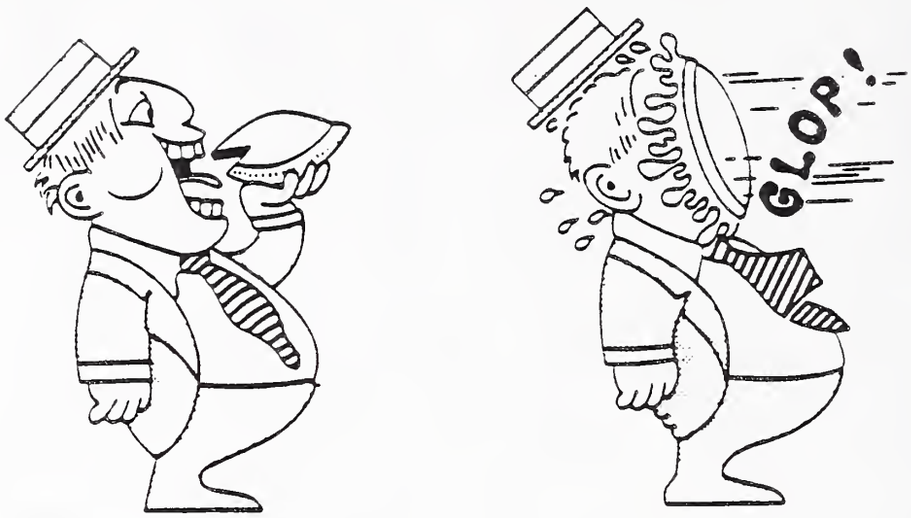


Figure 2.5 The difference between ABsorption and ADsorption .

aromatic, and halogenated hydrocarbons, as well as many permanent gas separations.

Adsorbent packings are very easy to prepare in the laboratory since the coating steps required for GLC columns are not required. Alternatively, as with the more common GLC columns, they can be purchased commercially made up and ready to install in the chromatograph. The adsorbents can be used at higher temperatures than most other chromatographic stationary phases, since there is practically no danger of decomposition or bleeding. The absence of bleeding is very favorable from a detection standpoint, since the column will not contribute "impurity noise" to the signal baseline. These phases can also be used in subambient temperature work, since there is no viscosity or solidification effect as there can be with coated

liquid phases. With the one exception of the carbon black packings, the adsorption phases are much more resistant to degradation by trace amounts of oxygen in the carrier gas than other phases. Naturally, carbon is subject to attack by oxygen, especially at elevated temperatures.

The user must be aware of some pitfalls associated with the use of adsorbent columns. Significant nonuniformity may exist between different lots of silica and alumina adsorbents. Many of the columns will adsorb some samples so strongly that release will not occur without reactivation of the material at high temperature. An example is the nearly permanent adsorption of carbon dioxide by most of the zeolite molecular sieve materials. They will also adsorb trace quantities of water that may be present in the carrier and sample matrix, thus changing the retention characteristics of the packing during operation. Since the adsorption isotherms of these materials are, in general, nonlinear, changing the size of the sample will change the retention time on a given column. This will also cause peak asymmetry (such as tailing) and sometimes incomplete sample recovery from the column. Also, since the adsorptive surfaces are of high energy, many polar and moderately polar samples will show excessively long retention times on these columns.

Adsorption columns can be modified by coating the material with a small amount of a nonvolatile liquid or an inorganic salt. The most energetic sites on the surface of the adsorbent will preferentially bind to these modifiers. As a result, these sites will be unavailable to participate in retention. This technique is sometimes called gas–layer–adsorption chromatography.

The next type of column we shall consider is the size–exclusion column. The packing for this column is the same synthetic zeolite or molecular sieve which we discussed earlier as an adsorption column. The structures of these crystalline aluminosilicate materials are unique in that they contain cavities of a definite size, which can be controlled by the elemental composition of the zeolite. The cavities are roughly the same size as many smaller molecules such as water and the permanent gases. Solute molecules in this size range which can penetrate the internal pore structure will experience an increase in retention time due to entropically enhanced adsorption. The popularity of molecular sieve columns originally resulted from their ability to separate oxygen and nitrogen rapidly under reasonable chromatographic conditions. The most commonly used packings are the 5A (calcium alumino–silicate, having an average pore size of 0.5 nm), 13X (sodium alumino–silicate, with an average pore diameter of 1.0 nm), and the 3A (potassium alumino–silicate, having an average pore size of less than 0.5 nm). Molecular sieve 4A (another sodium alumino–silicate, with an average pore size of less than 0.5 nm) has also found chromatographic application.

Another type of molecular sieve is the carbon molecular sieve, which is prepared from the pyrolysis of poly(vinylidene chloride). This material is very inert and nonpolar. The sieve structure results from the cross–linking of individual, small crystallites of carbon. This material has a high affinity for hydrocarbons; in fact, water will elute before methane under most chromatographic conditions. It is very useful for the

separation of light inorganics, and has been particularly valuable with mixtures containing water solutions of formaldehyde.

The final solid packing type that we will consider for application in GSC is the porous polymer phase. These packings are prepared using the method of suspension polymerization, in which monomers and crosslinking agents undergo reaction in an inert solvent. The more popular porous polymer phases are copolymers of styrene and divinylbenzene. These polymers are members of the Porapak series or the Century series of commercially available packings. The porous polymer phases are quite useful in the separation of polar compounds such as alcohols, carboxylic acids, amines and amides, aldehydes and ketones, and many organic and inorganic gases. The suspension polymerization technique produces a sponge–like structure having a uniform pore size which can be controlled by adjusting the reaction conditions. Externally, the packing resembles microscopic beads. The chromatographic behavior of the packings is a function of the chemical nature of the polymer, surface area and particle size in addition to the pore size. In general, however, retention times increase as the pore size is decreased.

Although we have chosen to discuss porous polymers under GSC, there is some doubt as to the mechanism of retention on these phases. At lower temperatures (say, below 100° C), adsorption appears to be the principle retention mechanism. At higher temperatures, the polymers behave more like a coated liquid phase (that is, the surface structure becomes liquid–like). Under these conditions, a partitioning of the sample in the

polymer appears to occur, as was depicted in our "chromatographic movie" of Section 2.1.

As with the other GSC phases, activation or conditioning (at approximately 200° C) is required prior to use. This is done in order to drive off any residual monomer or solvent, and to remove any material which may be adsorbed on the surface. All of the polymers have relatively high maximum operating temperatures (at least 250° C), and introduce little impurity noise to the detector. A somewhat different porous polymer material is Tenax–GC, which is a linear polymer of p–2,6–diphenyl–phenylene oxide. This material has a maximum operating temperature of 375° C, but it can be run at 400° C for short periods. It is useful in the separation of high boiling, polar compounds such as diols, methyl esters of dicarboxylic acids, aldehydes and ketones, amines and amides, and phenols. Short cartridges of Tenax–GC are also used as sampling sorbents for the collection of volatile or gaseous samples. These sorbent columns are especially useful in environmental work, and play a role in many standard procedures set by regulatory agencies.

Some minor operational disadvantages may be experienced in the use of the polymeric phases. It is sometimes difficult to pack a porous polymer into a metal column due to the effects of static charge. This is especially pronounced in dry climates. It is therefore often necessary to purchase columns already prepared. Another problem occurs when some of the polymers are used over a large temperature range. The polymer beads tend to swell, thus changing the flow characteristics of a column. This can lead to less than optimum detector performance.

2.4 OPEN TUBULAR COLUMN CHROMATOGRAPHY

The final classification of gas chromatography we will consider is capillary or open tubular column chromatography. This technique centers around the use of columns consisting of long (30 to 60 m), coiled, small diameter (0.25 to 0.05 mm inside diameter) capillary tubes, usually made of fused silica coated with a protective layer of polyimide. In recent years, fused silica open tubes have been coated with a thin layer of aluminum in order to extend the operating temperature, since the polyimide will degrade at approximately 325° C. The stationary phase is coated as a film on the inside surface of the tube, rather than a coated packed bed of support particles. The flow heterogeneities associated with packed columns are thus eliminated, along with the A term (eddy diffusion) of the Van Deemter equation. Capillary columns are available with efficiencies as high as 600,000 plates, in contrast to the typical maximum of 10,000 for packed columns. Capillary column chromatography is useful for all types of separations except permanent gas analysis. Of course, preparative scale separations are also not suited to capillary columns due to the very low solute capacity. The technique is especially suited for the separation of complex mixtures containing upwards of a hundred individual components. In general, separations on capillary columns can be performed at temperatures averaging 30° C lower than would be required using a liquid phase coated on a solid support.

The extremely high efficiencies of capillary columns make stationary phase selectivity a less important consideration in the design of an analysis. The most common liquid phases used

with open tubular columns are the methyl silicones and their derivatives. An open tubular column coated with cross–linked polymethyl silicone is essentially a boiling point separation column (using primarily London dispersion forces as the separation mechanism), while phenyl– and cyano–substituted polymers provide for more specific polar interactions. These liquids are coated to the desired thickness (typically 0.3 to 1 μm, although even thicker films are possible) and immobilized by cross–linking the fluid to form a polymer network. The silicone–based phases have high thermal stability, and can be operated at temperatures in excess of 275° C.

The capillary column length is a relatively unimportant parameter in the design of effective analyses. The parameter of film thickness is very important, however. An increase of 0.3 μm in film thickness can produce the same increase in resolution as quadrupling the column length, without the fourfold increase in analysis time that the longer column would entail.

There are a number of disadvantages in the use of open tubular columns which must be addressed. The manufacture of these columns is a nontrivial process which cannot usually be duplicated successfully in the laboratory. The commercially available columns tend to be very expensive relative to packed columns. Since the columns are so small in size, their sample capacities are also quite small. To prevent column overload, a flow splitter (to be discussed in more detail in the next section) on the injection port is almost always necessary. The splitter discards most (90 to 99 percent) of the sample injected from the syringe. For trace analysis work, an injector capable of

operating in "splitless mode" during injection is usually used. These injectors are more complex and more expensive than the simpler devices used with packed columns, and require a bit more skill to use properly. An additional consequence of the low sample capacity is the requirement for the most sensitive detectors available. These are usually limited to the flame ionization, thermionic, electron capture and mass selective detectors. The small diameter of the open tubular column also requires a higher degree of stability in the oven temperature than is required for a packed column. The stationary phase films in the columns are prone to oxidative damage, and therefore require a constant flow of oxygen–free carrier at temperatures very much above ambient.

2.5 SAMPLE INTRODUCTION

A major consideration in the design of effective GC analyses is the method used to introduce sample into the carrier stream. This involves the appropriate choice of chromatographic injector, and the optimal operation of the device. If this initial step is not handled properly, the outcome can be excessive experimental error (reaching order of magnitude levels), incorrectly assigned peaks, unexpected "ghost" peaks, or even no peaks at all. We actually have a two–step process to deal with, and we will discuss each step separately. The first step is to get our sample into the appropriate injector, and the second is to properly use that injector to apply the sample to the column.

The delivery of liquid samples to the injector is conveniently performed using a graduated glass–barrel syringe that usually has a total capacity of 10 μL. When we use packed columns, injection volumes may be as high as 5 μL, while the lower capacity of open tubular columns dictates volumes of between 0.5 and 2 μL. For the handling of volatile liquids and solvents, it is possible to cool the syringe to –40° C using a vortex tube, which supplies cold air to a glass jacket surrounding the syringe. This approach can also aid in the analysis of thermally sensitive mixtures. There are a number of pitfalls to be aware of concerning the use of chromatographic syringes. The influence of the needle volume on sample size, typically 0.8 μL, must be considered. It is therefore desirable to "bracket" the sample solution between two plugs of air or pure solvent in the syringe in order to achieve the best reproducibility. Some solutes can be adsorbed on the ground glass parts of the interior of the syringe and thereby lost from the sample. In addition, catalysis by this glass surface is not unknown. Another difficulty, called sample discrimination, occurs with all heated injectors such as flash vaporizers. This will be discussed in more detail in the next section.

The application of solids directly into an injector presents many problems. Fortunately, many solid samples can be dissolved in a suitable solvent and handled the same way as a liquid sample. When this is impossible, the best choice is an encapsulation method. The sample is placed in a capsule which can be opened inside of the injector. Capsules made of Wood's metal (which is melted at 60.5° C), glass (which is mechanically

crushed), or gold (which is pierced by a hollow "thorn") have been used with varying degrees of success.

The transfer of a gaseous sample to the chromatographic injector must involve the consideration of temperature and pressure since these variables have a profound effect on the density (and therefore the number of moles) of a gas. We learned this in the first chapter, when we discussed bulk properties. The simplest and least expensive method to transfer a gas to the injector is the use of a gas–tight syringe having a volume between 0.001 and 50 mL. If the room temperature is constant, a reproducibility of between 2 and 5 percent is possible.

A better method of gas sampling involves the use of a thermostatted sampling valve with a fixed volume sample loop as the injector. Such a valve is schematically shown in Figure 2.6. The valve consists of a stainless–steel body which accomodates the necessary transfer lines, and a channeled polyimide (usually) rotor which changes the flow path. In the fill position, the gas to be analyzed is allowed to flow into the sample loop. Enough sample gas should be allowed to flow through the valve to ensure that any residual carrier gas is swept from the loop and rotor. While residual carrier gas will not show up on the chromatogram, its presence will impair quantitative performance since carrier molecules will displace molecules of sample. This will cause problems in achieving good reproducibility among a set of replicate analyses. To assist in carrier removal, some operators plumb a vacuum line into the vent manifold. In the injecting position, the sample gas is swept

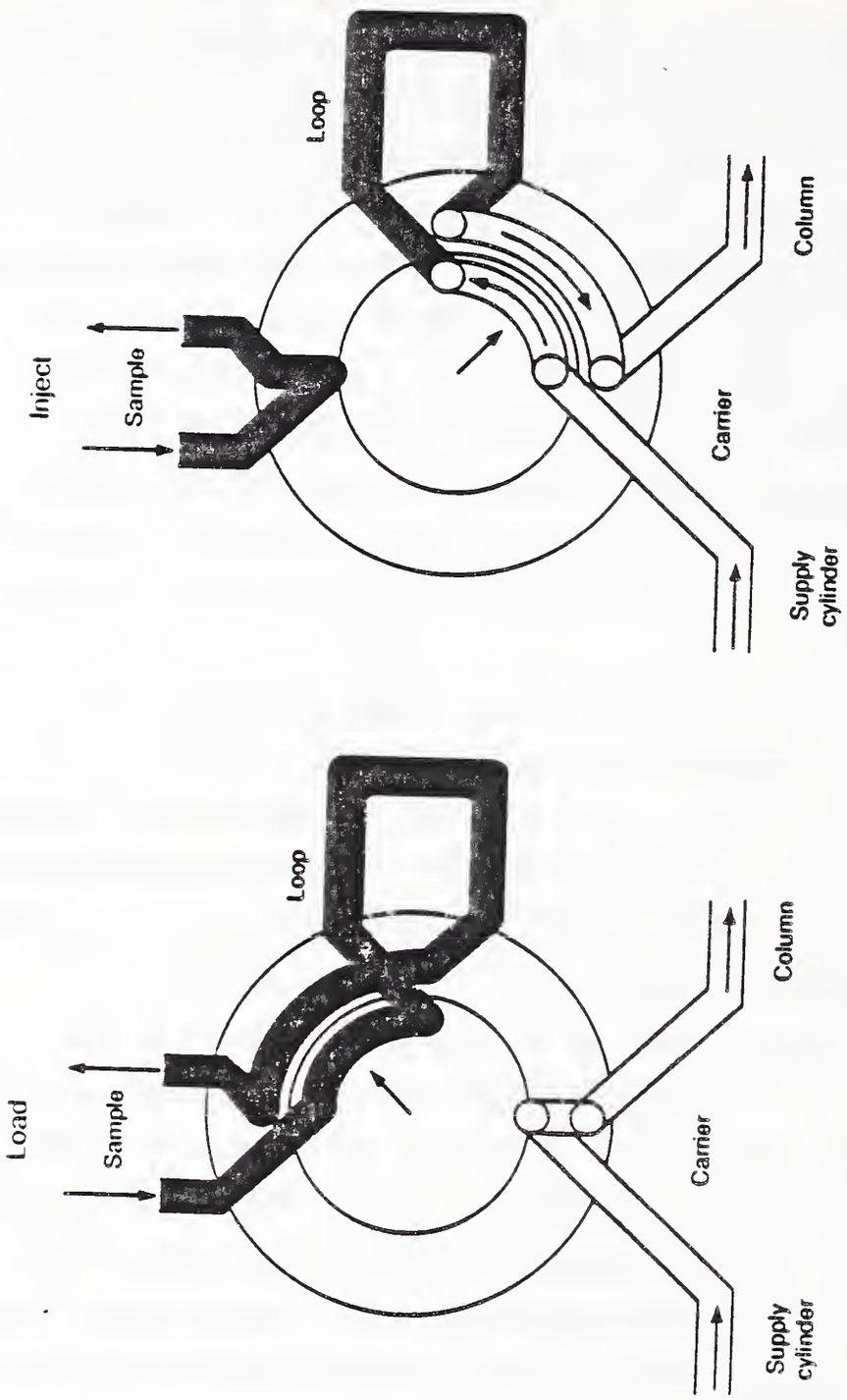


Figure 2.6 A schematic diagram of the sampling valves typically used in gas analysis by gas chromatography. Figure 2.6a shows the valve in the fill position; Figure 2.6b shows the inject position.

into the column by the carrier gas. The valve is usually maintained at an elevated temperature in a separate oven. Temperatures of 150 to 250° C are normally employed to prevent the adsorption of sample gas on the surface of the loop or rotor. The rotors are usually made from a thermally stable polyimide material which retains mechanical integrity over many cycles. Using a sampling valve is the most accurate way to introduce gaseous or very volatile samples into the chromatograph. The total volume of sample injected is very reproducible since the volume of the loop and rotor do not change from run to run. The injection process is very reproducible since the valves are usually actuated using an electronically controlled solenoid or a pneumatic cylinder. This provides for very rapid injection, which minimizes the pressure pulse caused by the momentary disruption of the carrier gas flow. The use of helium as the pneumatic actuation gas usually provides the fastest switching, especially if the pneumatic actuator is equipped with high–flow pilot valves.

2.5.1 Flash Vaporizer

The most common type of chromatographic injector used with all types of packed columns is the flash vaporization injector, shown schematically in Figure 2.7a. The injector consists of a heated cylinder containing a concentrically positioned liner, usually made of glass or quartz. At the top of the inlet is a self–sealing silicone rubber septum, through which the syringe needle penetrates to introduce the sample. The carrier gas inlet is usually at the base of the injector, and the gas

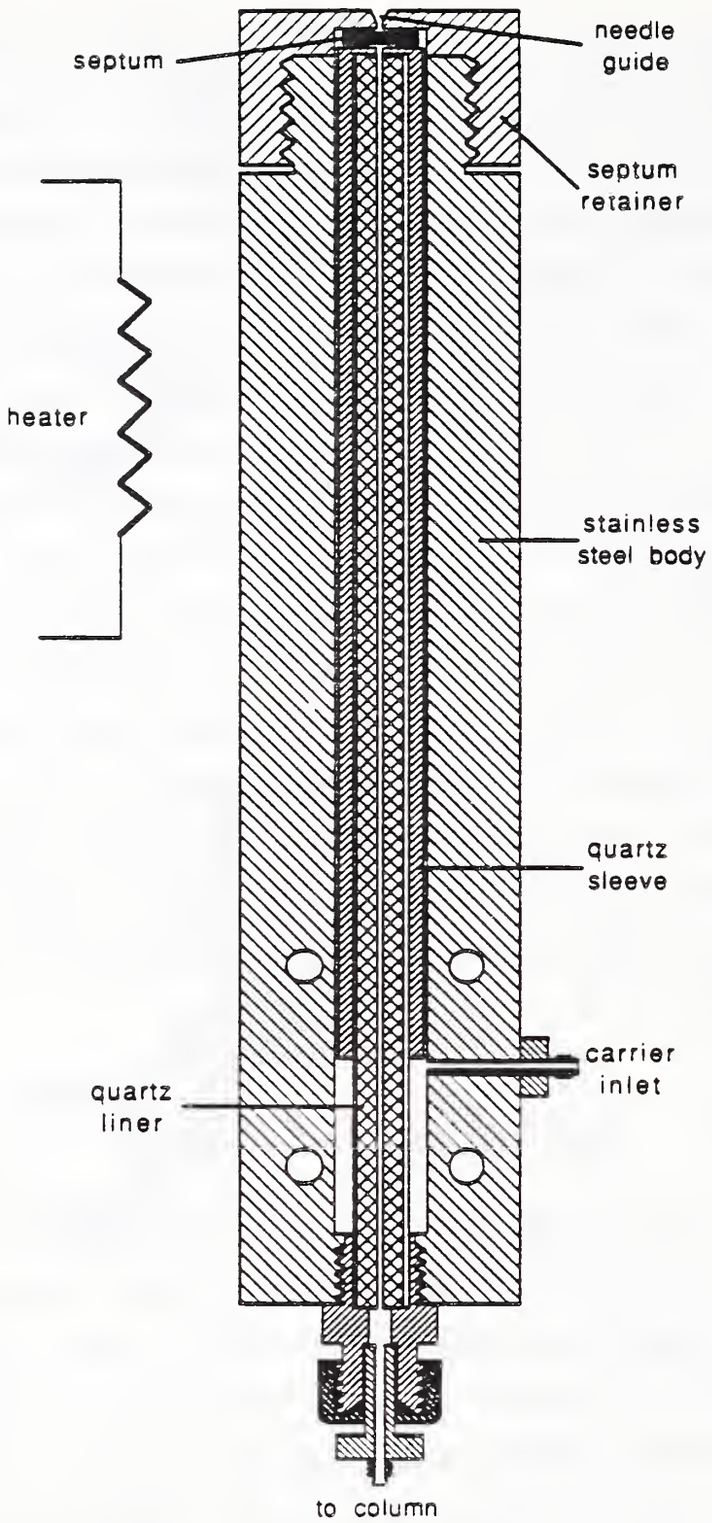


Figure 2.7a Schematic diagram of a flash vaporization injector.

is warmed as it travels to the top, thus providing a preheated flow stream. Flash vaporizers are relatively high volume devices, sometimes having internal volumes as high as several milliliters. They are usually maintained at a high temperature (upwards of 200° C) to ensure complete vaporization of the sample. This can be a disadvantage for a number of reasons. Septum bleed, responsible for unassignable "ghost" peaks, is always more severe at higher temperatures. This is aggravated by the large volume of the injector, much of which may be poorly swept by the carrier gas. In addition, flash vaporizers cannot be used with heat sensitive samples. They all suffer from a problem called "sample discrimination," which is especially pronounced when the sample consists of a complex mixture of components with widely varying boiling points. The more volatile components of the sample will be more easily vaporized; therefore the sample delivered to the column may be of different composition than the one applied by the syringe. This makes the flash vaporizer a marginal choice for the quantitative analysis of complex mixtures. On the positive side, flash vaporizers are low–cost devices and are easy to maintain. A single unit can be used with packed columns made of different materials and of different diameters.

2.5.2 Splitter Injector

Injection of samples into open tubular columns is a somewhat more demanding process than injection onto packed columns. As we discussed in the previous section, the sample capacity of open tubular columns is much lower than that of

packed columns. This requires much smaller injector volume, since sample volumes will rarely be larger than 2 μL, and usually closer to 0.5 μL. To handle this situation, the splitter injector (shown schematically in Figure 2.7b) was developed. This injector consists of a heated liner (like the flash vaporizer) where the sample is introduced by syringe and vaporized. A small plug of glass wool or chromatographic support is located downstream from this area in the split liner, to facilitate mixing of the vaporized sample with the carrier. The flow of the mixture is then split into two streams, one entering the capillary column and the other vented through a back pressure regulator. Typical split ratios (ratio of vented flow/column flow) are between 10:1 and 1000:1, controllable by the back pressure regulator. This method of decreasing the column flow is done without affecting the column pressure, and ensures that column overload does not occur. Sample transfer to the column is fast, resulting in little band broadening due to the injector volume.

Splitter injectors are more complex and costly than the flash vaporizers used for packed columns. All splitters will cause sample discrimination to some extent, although the problem can be minimized more easily than with the flash vaporizer. The elevated temperatures make the device unsuitable for use with thermally labile samples. Obtaining reproducible data with a splitter can be problematical, since the split ratio which is set by the operator before a run is not the ratio which is obtained when sample appears at the splitter. The actual split ratio which results is a complex function of sample volume and boiling point range, solvent characteristics, injector temperature, syringe technique, and the magnitude of the pressure pulse produced

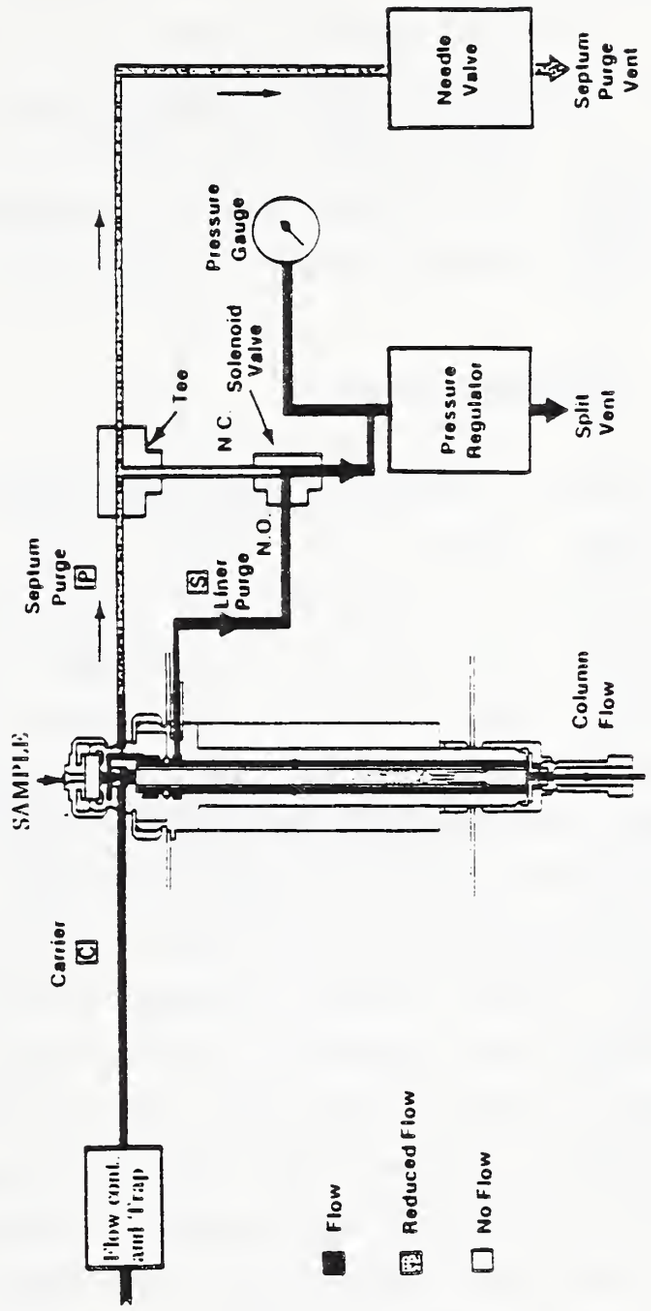


Figure 2.7b Schematic diagram of a splitter injector for capillary columns (N.O. = Normally Open, N.C. = Normally Closed). Courtesy of Hewlett–Packard Corporation.

upon vaporization. Many of these variables are hardly reproducible or predictable. For the best results, one should reproduce the injection process precisely from run to run, especially in terms of liquid volume injected and the time taken for injection. It is also advisable, where possible, to use internal standardization as the calibration method (a complete discussion on the calibration process is provided in Appendix I).

2.5.3 Splitless Injector

An alternative injector for open tubular columns is the splitless injector (Figure 2.7c), which is similar enough in external construction to the splitter that a single injection port can be modified to provide both split and splitless modes. As with the splitter, small (0.5 to 1.0 μL) volumes of liquid are injected, making it attractive for trace analysis. This injector uses a straight glass or quartz liner in place of the split liner, and a solenoid valve diverts the purge flow during injection so all carrier and sample present in the liner is deposited into the column. The injection process is therefore much slower (the rapid vaporization step required for the other injectors is not needed) than that used in split mode, and is usually done at lower temperatures. This makes the splitless injector attractive for the injection of thermally labile samples, those samples with components which elute near the tail of the solvent front, and samples that are very dilute. The lower temperature is used to refocus the sample volume, which is then deposited as a whole into the column. An alternative to this thermal refocusing is to make use of the so–called "solvent effect" in which the solvent

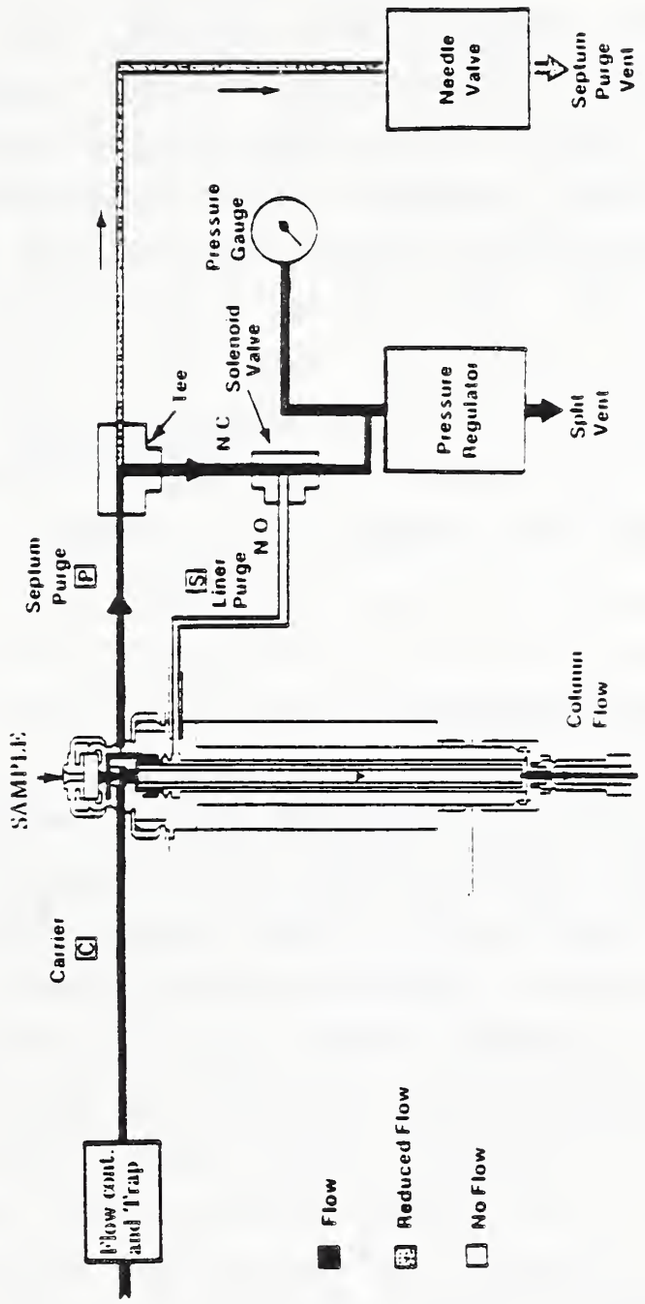


Figure 2.7c. Schematic diagram of a splitless injector for capillary columns (N.O. = Normally Open, N.C. = Normally Closed). Courtesy of Hewlett–Packard Corporation.

plug is used as a secondary stationary phase to retard and reform the solute components at the head of the column.

The splitless injector, while relatively simple in construction and easy to maintain, can be difficult to operate and optimize. The use of cool refocusing or the solvent effect usually requires careful adjustment of the chromatographic pneumatics, a trial–and–error process which can take longer than the analysis itself. The sample size must be reproducible within experimental error for the most precise quantitative work. Septum bleed can be a serious problem even with lower operating temperatures, since any septum impurities present in the liner during splitless injection will be deposited on the column.

2.5.4 On–Column Injector

An injector which can be adapted for both packed and larger diameter open tubular columns is the cool on–column injector. Using this device, the sample is syringe–deposited directly into the packing or inside the capillary column. An on–column injector is inherently simple, and is often found as a homemade device modified from a flash vaporizer. Injection is done quickly with the temperature maintained at or below the boiling point of the solvent. Sample volumes are usually 2 μL or less. This method of injection is well suited to trace analysis, or work involving dilute solutions, since all of the sample is deposited on the column. The effects of catalytic or thermal degradation on the sample are minimal, as is sample discrimination. It is generally the most precise injection

method, and is relatively easy to implement and optimize, especially with packed columns.

The application of on–column injection to the smaller diameter capillary columns can be impossible, however. Another disadvantage is that involatile material can be deposited at the head of the column, accumulating over time. Also, this injector does not lend itself well to automation.

2.6 DETECTORS

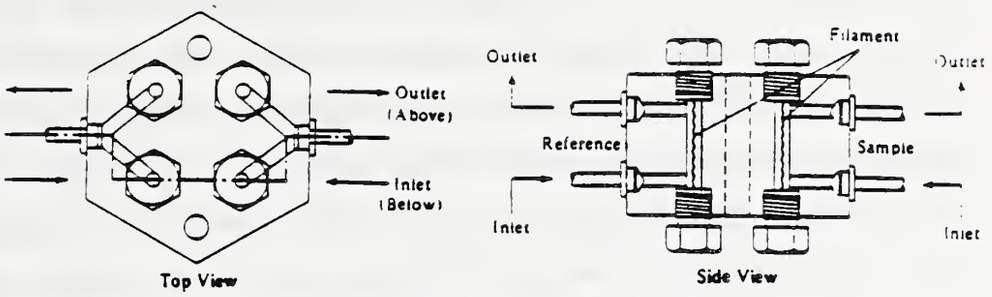
The function of the detector is, of course, to provide a recordable, reproducible, quantitative response to the presence of separated components as they emerge from the column exit. We would like the response of the detector to produce a signal that is proportional to the quantity of each component, and linear over the concentration range of interest to us. Some of the detection devices used in gas chromatography are based on technology dating from the 1880's, while others depend on advances that are barely five years old. The nature of the sample, the required level of sensitivity and the mode of chromatography we wish to employ will play major roles in our choice of detector.

2.6.1 Thermal Conductivity Detector

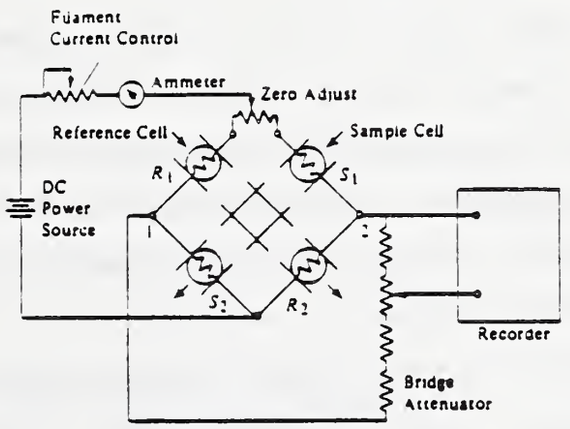
The first detection device we will consider is the thermal conductivity detector (TCD), which is based on very old measurement technology. This detector measures the change in

a bulk transport property of the gas stream, and is therefore universal in response. There are two basic TCD varieties available, hot wire based and thermistor based detectors.

The most commonly used TCD is the hot wire version or katharometer, shown schematically in Figure 2.8, along with the relevant Wheatstone bridge circuit. The carrier gas is passed over short sections of coiled wire made from gold or rubidium sheathed tungsten. Two cell compartments contain wires bathed by the sample stream, while an additional two compartments accept the flow from a reference column. The wires in these four compartments form the legs of a Wheatstone bridge circuit, with the chromatographic integrator or recorder in place of the electrometer. The wire is electronically heated to between 300 and 450°C, but the continuous stream of carrier serves to remove much of the heat from the cell. High thermal conductivity gases such as hydrogen and helium are especially efficient heat removers. When sample elutes from the column and enters the sample part of the detector cell, there will be a differential change in the rate at which heat is removed from the two sets of hot wires. This is due to the different (usually lower) thermal conductivity of the sample relative to that of the carrier. This leads to an increase in temperature of the wires in the sample stream, and consequently an increase in their electrical resistance. It is this electronic measurement of resistance which provides the output. Alternatively, the wires can be maintained at a constant temperature (regardless of the presence or absence of sample) using a feedback circuit. The response signal is then the measurement of the marginal change in current applied to the wires which is needed to maintain the



(a)



(b)

Figure 2.8 A schematic diagram of the essentials of a thermal conductivity detector, showing a typical cell configuration (a), and a control and sensing circuit (b). (Courtesy of the Gow—Mac Instrument Company.)

isothermal condition of the wires. This eliminates the temperature gradient at the wire supports that occurs when the wire temperature is allowed to change.

A hot wire TCD is prone to oxidation damage, and therefore must not be operated without the constant flow of carrier gas. The sheathed tungsten wires are susceptible to attack by sulfur–containing compounds, and extensive use with halogenated compounds will require special nickel alloy wires. In addition, the volume of gas required for the TCD is relatively high, even for the newer micro–scale cells. This usually limits the usefulness of the hot wire TCD for applications with packed columns only, where flow rates on the order of 20 to 40 mL/min are possible.

The other type of TCD is one based on thermistor elements instead of hot wires. The thermistors are semiconductors in which the resistance decreases sharply with increasing temperature. This type of TCD has a somewhat higher sensitivity, but at a cost of operating temperature range. The thermistors will fail at temperatures much above 150° C; thus the detector cannot be used with high boiling point solutes. The thermistor TCD is, however, very useful for work on gaseous samples. The thermistor elements are much less prone to oxidative damage than the hot wires, but they tend to be sensitive to reducing agents. This usually precludes the use of hydrogen as the carrier gas. Since the thermistors are small, it is often possible to build detectors of much lower internal volume than the hot wire based cells. This allows the use of thermistor detectors with larger bore capillary columns.

When carriers other than hydrogen or helium are used with a TCD, there is the possibility of obtaining negative peaks on the chromatogram. This is because many materials have

higher thermal conductivities than the other common carrier gases such as nitrogen and argon. This is a problem since integrators and chromatographic computer software usually cannot process negative peaks. The TCD is a flow sensitive detector which cannot be used easily for analyses that require the column temperature to be programmed. The temperature–dependent viscosity of the carrier gas will change the flow rate to the detector and thereby cause severe baseline drift, even if mass flow controllers are used on the carrier pneumatics. While the sensitivity of the thermal conductivity detector is somewhat low as compared to other detectors, it does offer some unique advantages which make it very popular. The TCD will respond to water, carbon dioxide, carbon monoxide, and permanent gases. It is in low cost, easy to operate, and requires very little maintenance to provide good performance.

2.6.2 Flame Ionization Detector

The flame ionization detector (FID) is as popular for routine analysis as the TCD. In fact, it is rare to find a gas chromatograph which is not equipped with both of these detectors. It is relatively simple in construction, and is easy to operate and maintain. The FID may be considered universal in response for organic compounds. A typical flame ionization detector is shown schematically in Figure 2.9. A hydrogen – air flame is maintained above the tube which delivers carrier and sample from the column. The flame itself, in the absence of an organic sample species, contains few ions and has a high electrical resistance. When a sample containing carbon–

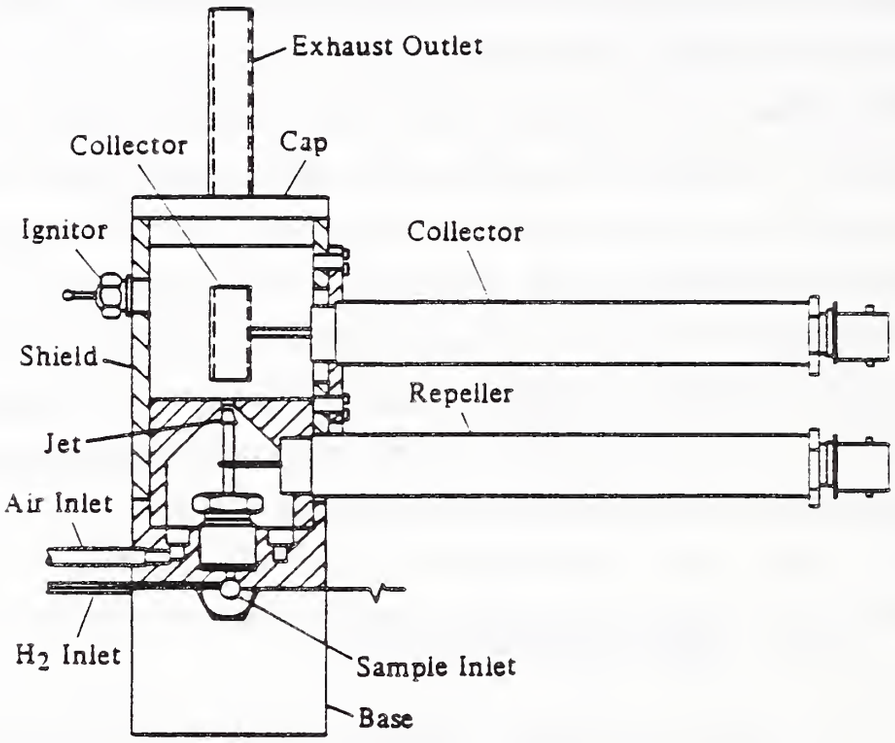


Figure 2.9 A schematic diagram of a flame ionisation detector. (Courtesy of the Gow–Mac Instrument Company.)

hydrogen bonds leaves the column and enters the flame it is burned, and a cascade of ions is produced. This increases the electrical conductivity of the flame by many orders of magnitude. A collector electrode above the flame is maintained at a potential of 180 to 300 V above that of the sample delivery tube. This system of electrodes, in combination with a sensitive electrometer, measures the flame conductivity and produces the response signal.

The FID is one of the most sensitive and fast responding detectors available. It can easily sense the presence of 10^{-11} g of hydrocarbon, and the response will be linear over a wide concentration range. The high sensitivity makes the FID very suitable for use with capillary columns, where very small amounts of material are analyzed. The device has high baseline stability and shows little or no sensitivity to changes in the carrier gas flow rate. It can therefore be used in temperature programmed applications without the viscosity–induced disruptions which plague the TCD. The dead volume of the detector is very small, since the sample is released directly into the flame.

Since any heated metal object will be a source of thermal electrons, the FID performance is optimized at lower detector temperatures. The body of the detector is usually heated just enough to prevent sample or water condensation. Water is a product of combustion of the hydrogen–air flame, and must not be allowed to drip back into the base of the detector, since it will cause temperature fluctuations and may even extinguish the flame.

The FID will not show a response to water, carbon dioxide, permanent gases and most inorganic species. This is a drawback, although very often the chromatogram is "cleaner" due to the absence of these peaks. When the FID is used with samples dissolved in chlorinated solvents, soot formation in the flame can be a problem. Soot can also form from a low level bleed of silicone liquid phases, resulting in an insulating deposit of silica in the detector. The response of the FID to species containing heteroatoms (organic nitrogen and phosphorus compounds, for example) can be unpredictable. It is sometimes difficult to keep the flame lit when eluting these materials. For this reason, most detectors can be operated with the igniter energized and continuously glowing. The potential of thermal electron interference makes this a last resort, however.

Several important operational disadvantages of the FID must be considered. Since a hydrogen flame is used in the detector, a slight but finite explosion hazard exists. An FID would not generally be usable in an explosion–proof laboratory (as defined in Class A, Group b of the National Electrical Code). The detector is destructive to the sample and cannot be followed downstream by another detector. It is not usually easy to determine the carrier gas flow rate at the exit of an FID; one is often forced to disconnect the column exit for this measurement.

2.6.3 Thermionic Detector

A modification of the FID is the thermionic detector (Figure 2.10), a device which is specific for nitrogen or phosphorus compounds. It is similar in construction to the FID,

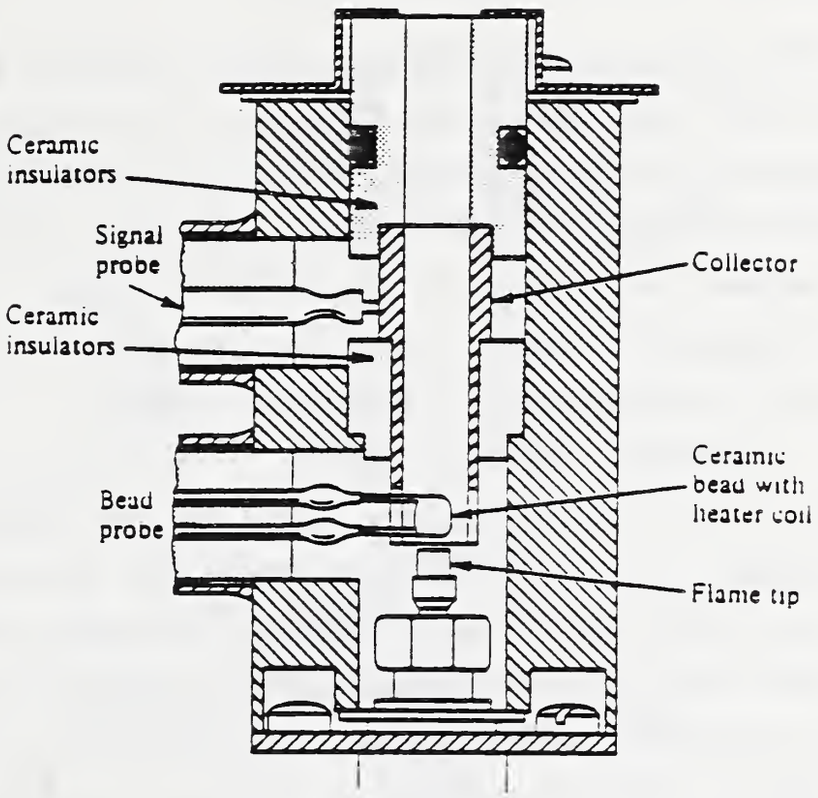


Figure 2.10 A schematic diagram of a thermionic detector. (Courtesy of Varian Associates.)

except that a small pellet of alkali metal salt is held in the vicinity of the flame. The salts of potassium, rubidium, cesium and sodium have been used. The detector is very sensitive for certain compounds, approaching the level of the electron capture

detector (see following section). The use of this detector is confined mainly to the analysis of pesticides.

2.6.4 Electron Capture Detector

The electron capture detector (ECD) is certainly a unique device, since it has been called the best and the worst detector available for gas chromatography. The love–hate relationship of analytical chemists with this detector is no doubt the result of the extreme sensitivity of the ECD. While it is a chromatographers' dream come true for some ultra–trace analyses, the device can be a nightmare to optimize.

A simplified schematic diagram of an electron capture detector is shown in Figure 2.11. There has been surprisingly little change in the design of these devices ever since the first viable unit was made in 1958. Naturally, significant advances have been made in the sensing and control electronics since that time. A radioactive source ionizes the carrier gas as it leaves the column. The carrier used must be either pure nitrogen or argon that contains between 5 and 10 percent methane, since these gases are most easily ionized. The carrier gas must be ultra–pure, because any contaminants will interfere with optimum operation. The radioactive source most used today is a ^{63}Ni foil, although some older models use adsorbed tritium, ^{3}H. The ionization of the carrier gas caused by the β–radiation produces a high background level of current measured across the anode and cathode. A sample eluting from the column enters this region, and will participate in ionization processes. If the sample contains atoms that will absorb electrons (such as

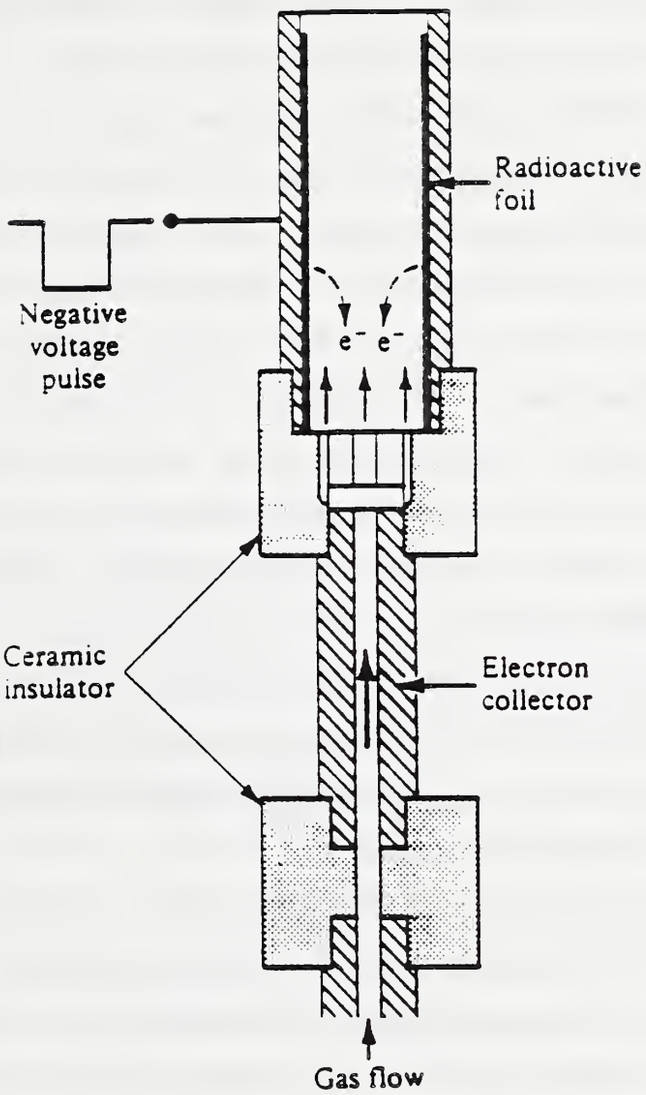


Figure 2.11 A schematic diagram of an electron capture detector. (Courtesy of Varian Associates.)

halogens, nitrogen or organometallic moities), the background ion current will drop sharply. It is this dramatic decrease in the current which provides the high level of response from the ECD.

The response sensitivity of the ECD is strongly dependent on the sample itself, and increases with the electronegativity of the functional groups. Some "ideal" cases (for example, the analysis of sulfur hexafluoride) have been reported in which an ECD provided a response to 10^{-14} g/s of sample. The response to an aliphatic hydrocarbon will be very slight, however. The response will also be related to the operating temperature, which is usually maintained at a level just high enough to prevent sample condensation. The presence of trace quantities of interfering species (such as oxygen, which is an electron absorber) will also affect sensitivity. Even the careful control of these factors does not guarantee reproducible results between separate ECD's, however. It is very important to use non–bleeding stationary phases, such as the cross–linked methyl silicones. Septa used in the injectors should be heat–conditioned to minimize bleed, and the pneumatic lines should be baked out to drive off adsorbed impurities.

As can be seen, it is a tricky business to optimize the performance of the electron capture detector. Even in the ideal situation, disadvantages persist. The linearity of the detector over sample concentration ranges is comparatively low, being two to four orders of magnitude. Once can compare this with the eight–decade linearity of the flame ionization detector. This is not a fatal flaw, however; the main consequence of the short linear range is the need for a more careful and extensive calibration (see Appendix I).

2.6.5 Mass Selective Detector

The routine use of a mass spectrometer as a detector for gas chromatography began the trend toward what have come to be called hyphenated techniques (GC–MS). This combination is one of the most powerful analytical techniques available. This is because mass spectrometry is the single most useful tool for organic structure elucidation and compound identification.

It is difficult to consider the mass spectrometer (or mass selective detector, MSD, a term that will often be used interchangeably) in the same vein as the TCD or FID. The cost and complexity of the combination requires us to consider the GC–MS instrument as a system unto itself. A schematic diagram of such a system is provided in Figure 2.12. Only a brief outline of the mass spectrometer will be given here, since this topic is covered in Volume II of this text. The mass spectrometer consists of a vacuum chamber pumped to a level of 1.3×10^{-3} to 1.3×10^{-4} Pa (10^{-5} to 10^{-6} Torr). This can be accomplished using a turbomolecular pump or a diffusion pump, both of which must be backed by a mechanical rough pump which produces 0.13 Pa (10^{-3} Torr). Inside the vacuum chamber is the ion source, the mass filter and the electron multiplier detector. The ion source usually consists of an "electron gun" filament which ionizes the sample eluting from the chromatograph. Actually, only a small fraction of the sample is ionized. Most of the sample leaving the column will be pumped away without ever encountering an electron from the source. Some instruments allow the use of chemical ionization reagent gases as an alternative to electron impact ionization. The ion

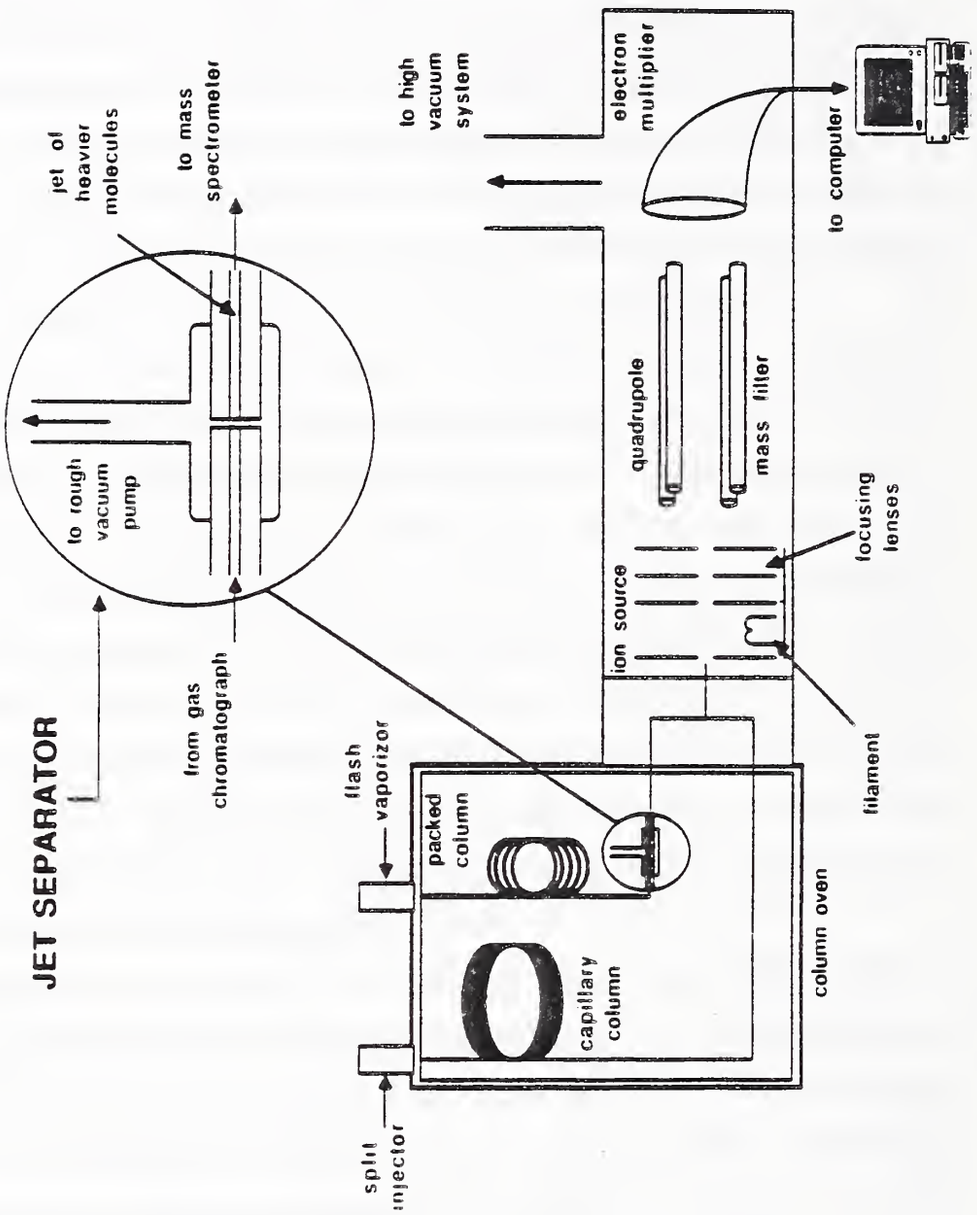


Figure 2.12 A schematic diagram showing the essential components of a GC–MS system.

source also contains a series of electronic lenses which accelerate and focus the positive ions which are formed by the electrodes. The beam of ions is passed to the mass filter, which is most often of the quadrupolar type. The electron multiplier amplifies the electron current signal, and a computer system is required to process this signal into the familiar mass histogram format. Usually, the output consists of a total ion chromatogram, which is displayed as peaks as a function of time, just as the output from any GC detector.

The tremendous advantage of the GC–MS system is that one can choose any peak on the chromatogram, and at the punch of a button, have a mass spectrum of that peak. The computer allows manipulation of spectra, such as the addition, subtraction, and averaging of spectra, and the searching of measured spectra against libraries. This is of great value in the qualitative identification of eluted samples. These libraries can be built up by the user or purchased from the manufacturer. The commercial libraries are usually based on the 49,000–entry library developed jointly by the U.S. National Bureau of Standards (now the National Institute of Standards and Technology), the U.S. Environmental Protection Agency (EPA), and the Mass Spectrometry Data Center of the Royal Society of Chemistry (England).

The mass selective detector can be used in one of two modes: mass scanning mode or single ion monitoring mode. When operating in scan mode, the MSD will record complete mass spectra over the molecular weight range selected by the operator. It is usual to keep the molecular mass range which is scanned as small as possible without sacrificing needed

information, since a finite time is require to sweep the spectrum. This means if a large molecular weight range is scanned, there will be relatively fewer spectra obtained per second than if a smaller range is scanned. Single ion monitoring is used primarily for high sensitivity quantitative analysis. Instead of scanning over hundreds of molecular mass units, one (or several) individual molecular mass values are selected for continuous intensity measurement. When the MSD is used in this fashion, the sensitivity approaches that of the electron capture detector.

The MSD is one of the most expensive detectors available for the gas chromatograph. It can easily cost three or four times more than the chromatograph itself. It is relatively complex and requires some degree of training in order to be used effectively. The interface between the chromatograph and the high vacuum of the MSD requires some discussion as well. The low mass flow rate of a capillary column can be accommodated directly by most MSD pumping systems, but packed columns usually require the use of a jet separator or some other device to divert and vent the bulk of the carrier flow. A jet separator, shown on the inset of Figure 2.11, draws off most of the column flow in low vacuum. The solute molecules, being much heavier than the carrier, remain roughly focused at the center of the jet and are drawn into the ion source of the MSD by the high vacuum. The use of a jet separator results in some degradation of performance of the overall GC–MSD system. The major manifestations of this are lower sensitivity and resolution.

Another consideration in the operation of a MSD is maintenance which, relative to other GC detectors, is hardly routine. Pumping system service, source cleaning, filament

replacement, and electron multiplier replacement must be done periodically. This is usually an all–day job requiring extreme care, ultra–clean conditions, and a very steady hand.

2.7 DATA COLLECTION

As shown in Figure 2.4, the output from whatever detector is chosen is fed into a data collection system which can consist of a strip chart recorder, a digital electronic integrator, or a computer driven with special peak–processing software. A strip chart recorder is not usually used in analytical applications of GC, but is still quite useful in physicochemical work in which the main experimental parameter is the retention time or retention volume. For analytical work, the use of a dedicated electronic integrator is now standard. The purpose of the integrator is to measure the area under each chromatographic peak, since this quantity is most easily related to the quantity of sample. The relationship between the peak area and sample quantity or concentration is obtained by the calibration method, discussed more fully in Appendix I. The analog output of the detector is converted to a digital signal by the integrator, and is internally manipulated as area slices in units of voltage, as depicted in Figure 2.13. Most of the modern integrators allow calculation of concentration directly after the appropriate calibration method is chosen and the standard values are stored in the internal memory. Calculation of peak height and peak widths is also standard, thus making theoretical plate height calculations a simple matter. Many integrators can provide

programming (usually in BASIC) and disk storage of chromatographic data as options.

Using appropriate software, a personal computer may be used to process chromatographic data. The programs are quite sophisticated, and allow deconvolution of very complex chromatograms. Data from different detectors can be processed and displayed in real time in separate windows on the computer screen. The cost of a personal computer plus the necessary chromatographic software is usually much higher than even the best chromatographic integrator. Unless there is a compelling reason for using a computer, the use of an integrator is to be preferred on economic grounds. As was mentioned earlier, the use of a computer with a dedicated software system is necessary, however, when using the mass selective detector.

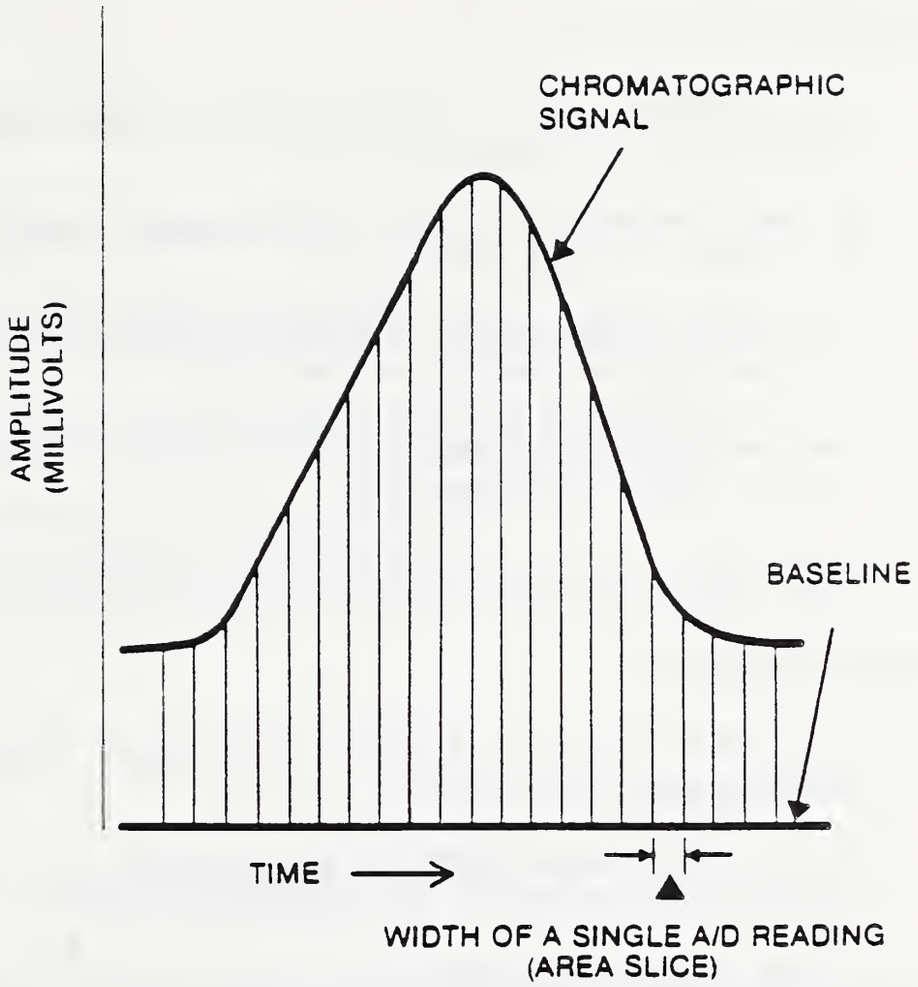


Figure 2.13 A schematic diagram illustrating the process of peak integration for sample quantitation. (Courtesy of Hewlett—Packard Corporation.)

SUGGESTED READING

Introductory Treatments

H. M. McNair and E. J. Bonelli, Basic Gas Chromatography, Varian Aerograph, Walnut Creek, 1969.

A. Braithwaite and F. J. Smith, Chromatographic Methods, 4th Ed., Chapman and Hall, London, 1985.

J. M. Miller, Chromatography: Concepts and Contrasts, John Wiley and Sons (Wiley Interscience), New York, 1987.

J. E. Willet, Gas Chromatography, John Wiley and Sons (on behalf of ACOL, London), Chichester, 1987.

P. A. Sewell and B. Clark, Chromatographic Separations, John Wiley and Sons (on behalf of ACOL, London), Chichester, 1987.

Detailed Treatments

C. F. Poole and S. A. Schuette, Contemporary Practice of Chromatography, Elsevier, 1984.

R. L. Grob, Ed., Model Practice of Chromatography, John Wiley and Sons (Wiley Interscience), New York, 1985.

E. Katz, Quantitative Analysis Using Chromatographic Techniques, John Wiley and Sons, Chichester, 1987.

M. L. Lee, F. J. Yang, and K. D. Bartle, Open Tubular Column Gas Chromatography, John Wiley and Sons (Wiley Interscience), New York, 1984.

Special Topics of Interest

C. J. Cowper and A. J. DeRose, The Analysis of Gases by Chromatography, Pergamon Press, Oxford, 1983.

R. J. Laub and R. L. Pecsok, Physicochemical Applications of Gas Chromatography, John Wiley and Sons (Wiley Interscience), New York, 1978.

CHAPTER THREE

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.1 INTRODUCTION

The systematic study of chromatography began with the work of Mikhail Semenovich Tsvett (1872 – 1919). He described his initial research on the application of adsorption phenomena to the separation of biochemicals at a meeting of the Warsaw Society of Naturalists in 1903. At this lecture, he described his separations of plant pigments on more than 100 adsorbents in funnels flushed with liquids. Thus, although most of the chromatographic analysis performed today and reported in the literature is done using gas chromatography, the very first work done was in liquid chromatography.

The obvious difference between liquid chromatography and gas chromatography is the use of a liquid as the mobile phase carrier or eluent. We recall, from our discussion of gas chromatography, that interactions of the solute with the carrier gas were of little consequence. As long as the solute is present as a vapor, it is miscible in all proportions with the carrier gas. The gas does not actually participate in the partitioning process with the stationary phase. With a liquid as the eluent, we have the potential of intermolecular interactions (such as hydrogen bonding, complexation, and charge transfer, which were discussed in Chapter 1) of the solute with both the mobile and stationary phases. This adds another dimension of both flexibility and complexity to this technique as compared to gas chromatography. While gas chromatography depends on sample volatization, liquid chromatography is done using samples in solution at near–ambient temperature. This makes liquid chromatography amenable to the analysis of heat–sensitive compounds, macromolecules, natural products and biochemicals which cannot be easily studied using GC methods.

Modern liquid chromatography is largely the result of significant developments in instrumentation which boosted efficiency and sensitivity as compared with the classical column techniques. Advances in solvent pumps, injectors and detectors and in the available stationary phases resulted in what we now call high performance liquid chromatography (HPLC). We can divide HPLC into four modes: liquid–liquid chromatography (LLC), liquid–solid chromatography (LSC), ion–exchange chromatography (IEC), and size–exclusion chromatography (SEC, sometimes called gel permeation chromatography).

3.2 Liquid–Liquid Chromatography

The most important and commonly used modes of HPLC are the varieties of liquid–liquid chromatography, in which the stationary phase consists of a liquid or, more often, a species having liquid–like structure, fixed on a support. This includes the chemically bonded phases used in reverse phase HPLC, which we will discuss later. Separation is achieved by the partitioning of the solute between the liquid mobile phase and the "liquid" stationary phase. Earlier adaptations actually employed a liquid coating (water, for example) on silica, with chloroform as the mobile phase. As you might imagine, much of the water was stripped from the column by the mechanical shear forces of the mobile phase acting on the liquid coating as it passed through the packed bed. Modern adaptations generally employ a liquid chemically bonded to a specially treated support. Most of the supports currently used consist of a specially treated silica having a tightly controlled particle size distribution.

The partitioning process can be described by the chromatographic parameter called the capacity factor, k:

$$k = (t_{r1} - t_u)/t_u \tag{3.1}$$

where t_{r1} is the retention time of a retained solute and t_u is the elution time of an unretained reference compound. The physical meaning of Equation 3.1 can be understood by considering Figure 3.1, in which two eluted solute peaks plus an unretained

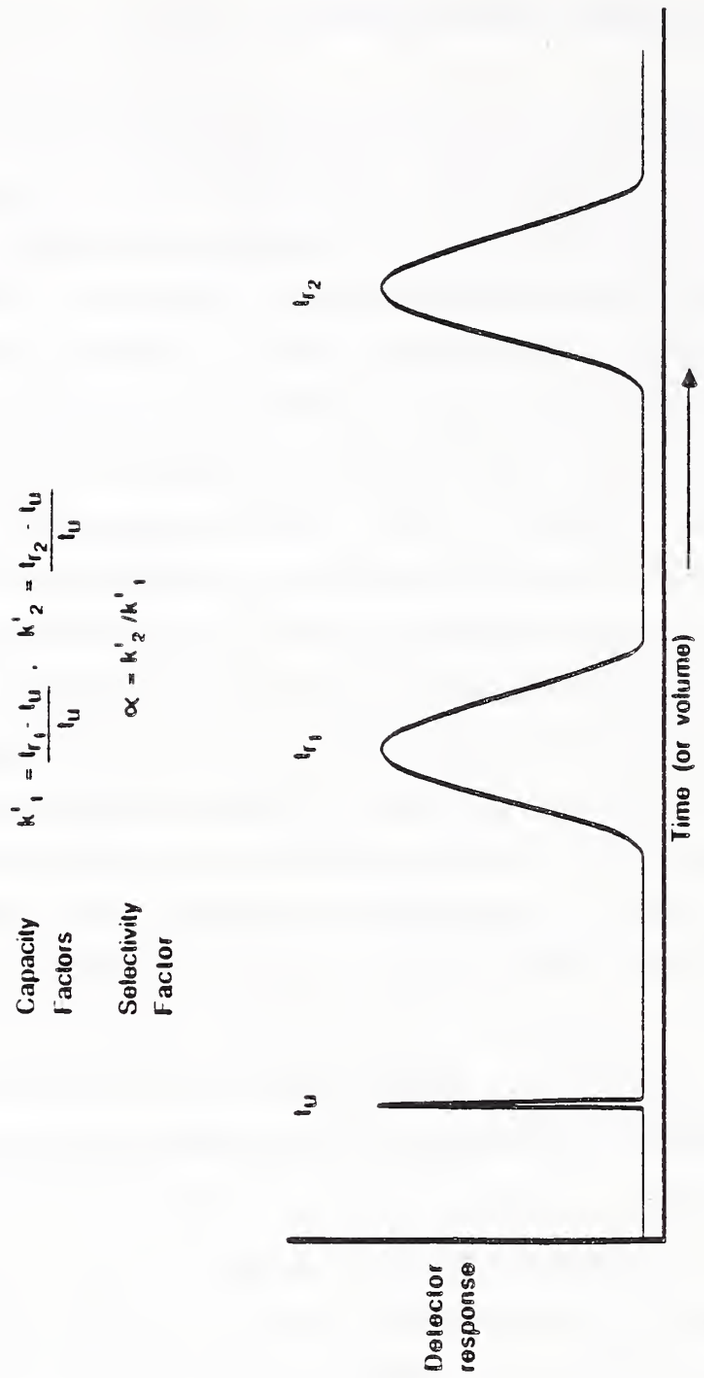


Figure 3.1 An idealised chromatogram illustrating the concepts of capacity and selectivity.

reference "peak" is shown. The capacity factor, being dimensionless, could also be expressed in terms of the solvent volume required for elution instead of the retention time. Thus, the position of the unretained reference compound in a sense represents the void volume of the column. The relationship of the two retained peaks to one another is represented by the separation factor, α, defined as the ratio of the capacity factors (with the larger one in the numerator). By now you have no doubt noticed the strong similarity of this discussion to our description of the retention mechanisms of GC. The separation factor gives us a means of judging the resolving power of a given column/mobile phase combination. The capacity factor and separation factor ignore the effects of peak dispersion. As with GC, this is a function of column efficiency, which we describe in terms of the theoretical plate height or total number of theoretical plates on the column. The equation for the number of plates on the column is very similar to the one used in GC:

$$N = 5.54 \left(V_r / w_{1/2}\right)^2 \qquad (3.2)$$

where V_r is the volume of solvent required to elute the peak and $w_{1/2}$ is the width of the peak at one half of its height. Typical HPLC columns available commercially are 25 cm long and have 25,000 to 50,000 plates, although some specialty packings and microbore columns have considerably more. One way to increase the separation factor is to increase the efficiency (as described by the plate number) of the column. This can be done

by using smaller particles for the packing, using a slower mobile phase flowrate, or increasing the column length. The other way is to adjust the selectivity by using the solvent system to control the partition process. It is this aspect of HPLC which requires skill and experience. Indeed, the brute force method of trial–and–error may be the only way to begin the optimization process for tricky separations.

3.3 LIQUID–SOLID CHROMATOGRAPHY

Liquid–solid chromatography was the mode originally used by Tsvett in his investigation of plant pigments. Separation is by the reversible adsorptive interaction of the solute with a solid adsorbent, and is dependent on the relative polarities of the solute and adsorbent. The commonly used adsorbents are silica gel, alumina, glass beads and polymer beads. Highly selective and reproducible separations can be done using this mode. It is most valuable when the sample is soluble in nonpolar or moderately polar solvents. The stationary phases used in LSC separate solute mixtures into functional group classes. These phases are also useful in the separation of mixtures of isomers having similar properties.

3.4 ION–EXCHANGE CHROMATOGRAPHY

Modern ion–exchange chromatography (IEC), which is used in the analysis of ionized or ionizable compounds, is the result of

nearly thirty years of technology. It is very useful in the separation of biochemicals such as amino acids, nucleic acids, and proteins by taking advantage of electrostatic (charge–charge) interactions. The ion exchange phase consists of a polymer or silica support backbone to which the ion exchange functional groups are attached. These bound functional groups consist of an ion and a counterion, the latter being exchangeable with an ion of the same sign in the solute. Ion exchange media used to exchange cations bear negatively charged groups and are most often sulfonate based. Ion exchange media used to exchange anions bear positively charged groups, and usually consist of quaternary ammonium groups. Complexing agents such as citrate buffers or EDTA (ethylene diamine tetraacetic acid) are sometimes added to the mobile phase to influence the separation.

3.5 SIZE EXCLUSION CHROMATOGRAPHY

Size exclusion chromatography is different in mechanism from most other types of chromatographic methods we have considered, since it is essentially noninteractive. Separation is effected on the basis of size of the solute molecules, and is not due to intermolecular forces or interactions. The column consists of a stationary phase with pore structure of controllable size. Smaller molecules, which can enter the pores, will be retarded strongly while larger molecules which cannot enter the pores will elute more quickly. Molecules of intermediate size will, of course, experience moderate retention. The method is

used mainly for compounds having molecular masses above 2000 g/mol (up to 8×10^5 g/mol), such as polymers and larger biomolecules. Solvent selection is relatively simple due to the noninteractive nature of the separation mechanism. A single solvent is required to dissolve the sample and does not participate in partition. The method is fast, and high efficiency separations are possible between narrow molecular weight ranges. The technique is also applied as a physical method to determine the molecular weight distribution (polydispersivity) of polymeric materials.

The various "modes" of liquid chromatography are summarized in Figure 3.2. This flow chart provides general guidelines in selecting the appropriate method for a particular analysis. Naturally, there may be situations in which more than one approach can be used. For these analyses, one must consider factors such as applicable hardware (for example, detectors), available stationary and mobile phases, and the economics of each method.

3.6 HPLC HARDWARE

We mentioned earlier that it is the great strides in instrumental hardware which have resulted in modern high performance liquid chromatography. Figure 3.3 shows a schematic diagram of a "generic" HPLC instrument. A vessel of filtered, degassed mobile phase solvent is supplied to a precision pumping system, which has provisions for pressure and flowrate measurement and control. It is important that the pump (1) produce a uniform,

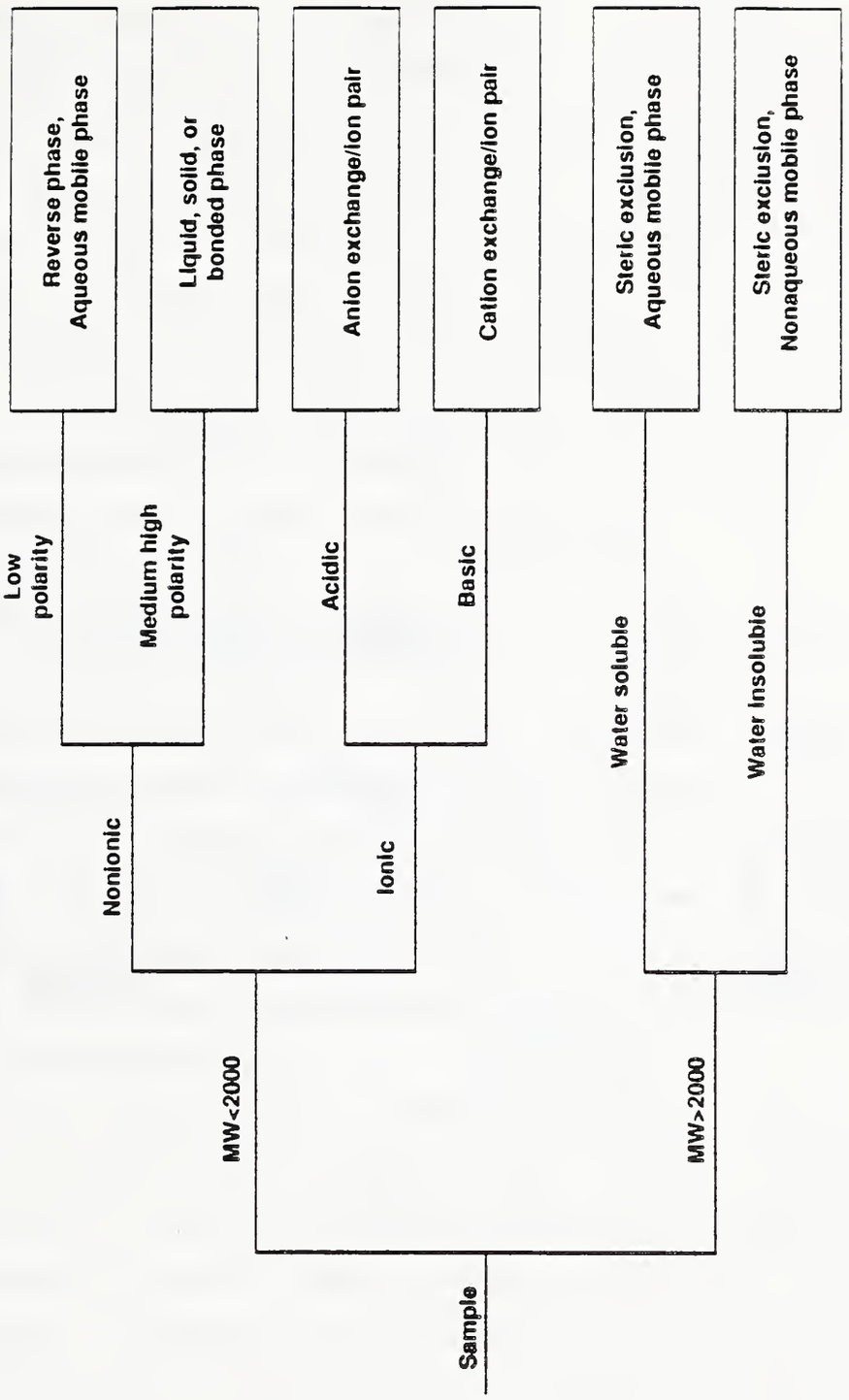


Figure 3.2 A flow chart providing guidelines for applicable techniques of liquid chromatography. (Courtesy of Millipore Corporation, Waters Division.)

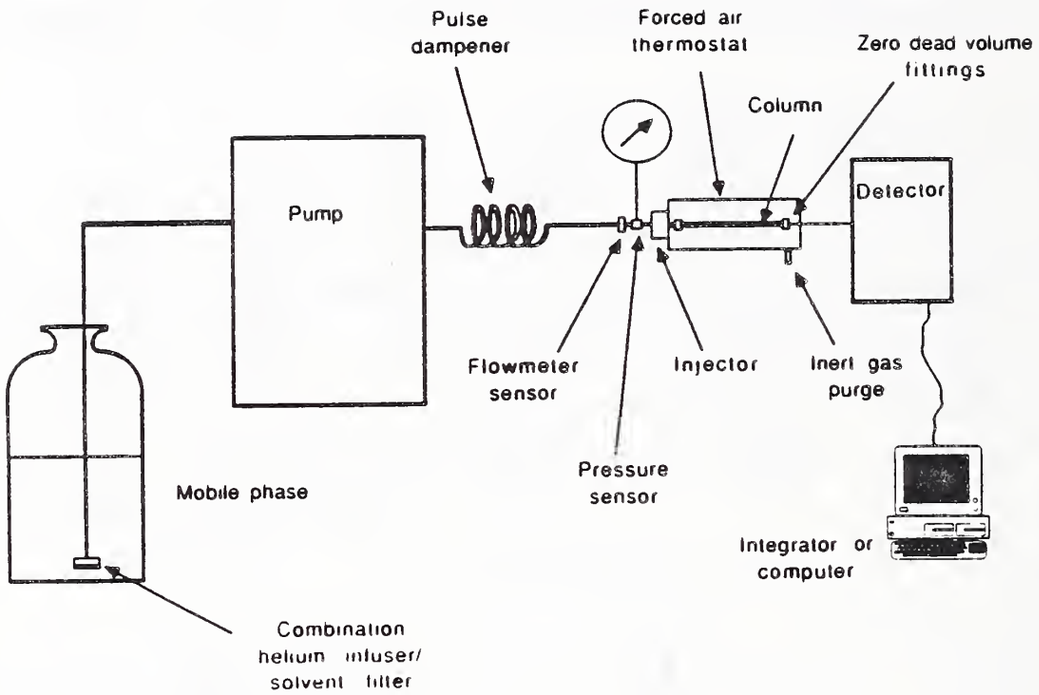


Figure 3.3 A schematic diagram showing the essential components of an instrument used for high performance liquid chromatography.

controllable flow of one or more separate solvents, (2) resist chemical attack from all solvents of interest, (3) allow easy change from one solvent (or solvent system) to another, and (4) be easy to maintain in the laboratory. The injection system follows the pump, and serves to introduce sample to the carrier stream. Injection must be performed such that the overall HPLC system will experience minimal disruption in pressure or flow. In addition, the injector must not introduce appreciable "dead volume" into the system. The column containing the stationary phase is most often made of 316 stainless steel (because of the high corrosion resistance of the alloy) and is usually 25 to 50 cm long, although newer microbore columns can be up to 100 cm in length. Again, the minimization of dead volume in the connections is a major consideration since dead volume causes peak broadening and loss of efficiency. We will discuss the stationary phases contained inside the column in more detail later. It has become common in recent years to control the column temperature using a forced air oven similar to that used in gas chromatography, although the temperatures employed are much lower.

The development of suitable detection devices has contributed the most in putting the "HP" in HPLC. Analytical chemists now have at their disposal sensitive, reasonably linear, predictable detectors which contribute minimally to peak dispersion. Although the vast majority of work is done using ultraviolet (uv) absorbence and differential refractometry detectors, devices based on fluorescence, infrared (ir) absorption, electrochemistry, and mass spectrometry are commercially available.

The data collection system is usually a computer equipped with the appropriate peak processing software, or a dedicated electronic integrator of the type used in GC (see Chapter 2). Occasionally, however, the strip–chart recorder still finds a place in HPLC work. It is not uncommon to have a fraction collector following the detector, especially in exclusion chromatography and in the study of biological samples. This allows more extensive analysis to be done on individual separated zones. A fraction collector will nearly always be used in preparative–scale work.

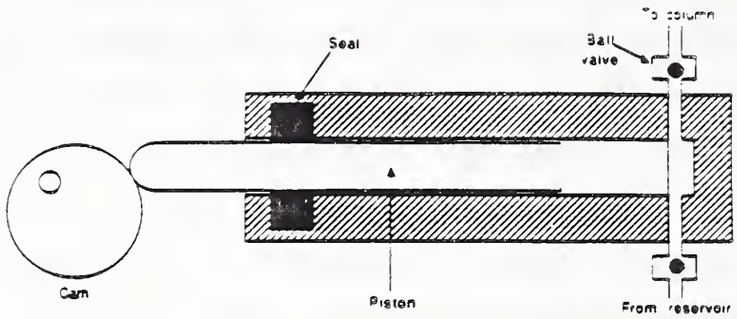
3.6.1 Solvent Pumping Systems

The HPLC solvent pumping system consists of the solvent reservoir, the pump hardware, and its controller. We mentioned earlier the solvent should be free of dissolved gas and particulate impurities. Dissolved gases, especially oxygen, can react chemically with the stationary phase in much the same way as oxygen in the GC carrier gas can cause degradation of many phases. Another possible consequence is the formation of bubbles in the pump or detector. This will show up as an unstable baseline on the integrator or recorder trace. It is therefore standard practice to degas the mobile phase solvents using low vacuum, gentle heating, agitation, the infusion of a low solubility gas (such as helium), or a combination of these. Sometimes sonolysis (the use of an ultrasound field of 35 kHz) is employed to aid in solvent degassing.

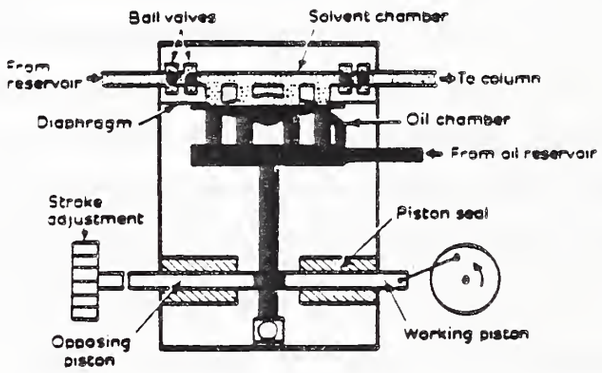
Particulate impurities on the order of 1 to 5 μm can cause plugging of delivery lines and cause serious wear on seals and

moving parts (such as check valves, pistons, etc). It is therefore necessary to filter all solvents to ensure the absence of particles at least down to a size of 0.2 μm. While many pressurization systems have been used to pump the mobile phase, we will discuss only those that currently find the widest application. Most modern pumps can be operated in either constant flow or constant pressure mode. Pressure measurement, for readout and pump control, is usually provided by a strain–gauge transducer having a small internal volume. Filters (such as a 2 μm pore disk of sintered stainless steel) are installed on the pump inlet and outlet to protect the components from wear.

The piston displacement pump, oil–driven diaphragm pump and syringe pump are shown schematically in Figure 3.4. The piston displacement pump (Figure 3.4a) consists of an eccentric cam driven, spring–loaded piston which displaces a solvent volume held between two ball–type check valves. The check valves ensure the flow is in one direction, and that solvent does not flow back into the reservoir. The flowrate from such a pump is independent of the back pressure caused by the column, and the pump will deliver fluid until the reservoir is empty. The main problem with the device is that the flow is non–uniform, having a pulsating profile of the same period as the piston stroke. Since this causes baseline noise, pulsing must be alleviated as much as possible. This has been done by operating two or three piston pump heads out of phase, by electronic pulse compensation and by the use of pulse–dampening volumes following the pump. It is never possible to completely eliminate pulsing from piston pumps, but one can reduce it to a level which does not adversely affect performance. The piston seals


Seal
Ball valve
Cam
Piston
From reservoir

(a)


Ball valves
Solvent chamber
From reservoir
To column
Diaphragm
Oil chamber
From oil reservoir
Stroke adjustment
Piston seal
Opposing piston
Working piston

(b)

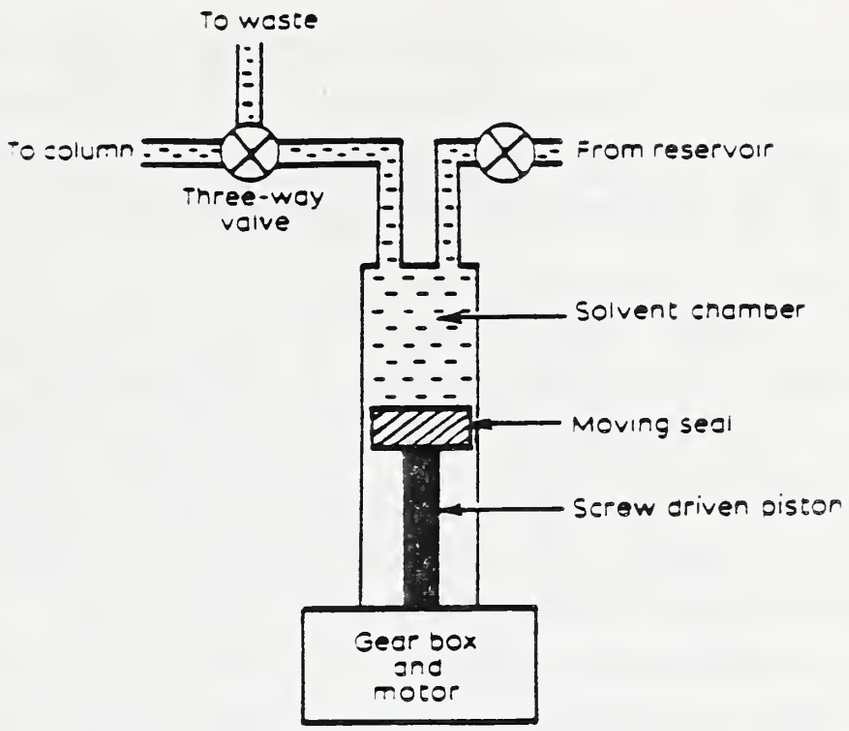


Figure 3.4 A schematic diagram of the more common solvent delivery pumps used in HPLC. Figure 3.4a A piston displacement pump. Figure 3.4b An oil—driven diaphragm pump. Figure 3.4c A syringe pump. (Reproduced with permission from Chapman and Hall, Inc.)

and check valves of this type of pump are subject to wear, and must be replaced periodically. Piston seals are especially prone to wear, and quite often particles of the seal are dislodged and cause problems downstream in the system, such as at the injector or column inlet.

The diaphragm pump (Figure 3.4b) is similar to the piston pump in that it is a continuously operating displacement pump. A flexible stainless steel diaphragm, usually driven hydraulically using an oil, moves the solvent through the pair of unidirectional check valves. The use of the diaphragm eliminates piston seals that are wetted by the mobile phase. Thus, the problem of

particles of seal material causing malfunction of check valves and plugging of columns is nonexistent. As with the piston pump, the flow from the diaphragm pump is inherently pulsating, and the same approaches to minimize the problem are required.

The syringe pump (Figure 3.4c) is a simple volume displacement device which produces a truly pulse–free flow, since it is nonreciprocating. The main drawback is that it cannot operate continuously. The piston delivers the volume in the solvent chamber, then the chamber must be refilled by opening the reservoir valve and withdrawing the piston. This can be a disadvantage, especially in preparative scale work. Syringe pumps are ideal for microbore columns, however, where the mobile phase consumption is low and recycle time is minimized.

It is possible to incorporate "solvent flow programming" into an instrument with a moderate increase in pumping system sophistication and cost. Flow programming is analogous to temperature programming in gas chromatography. This can be done by increasing the flowrate during the course of an analysis. A more effective (although more difficult and expensive) approach is to change the solvent composition during the analysis. This allows the polarity (and therefore strength) of the solvent to be increased or decreased during the run. This technique is called gradient elution, as opposed to isocratic elution in which the solvent composition remains constant during the analysis. Gradient formers are available which make the solvent mixture upstream from the pump (low pressure) or downstream from the pump (high pressure).

3.6.2 Injectors

While it is possible to use a gas–tight chromatographic syringe to directly inject a solute through a septum onto the column, this is rarely done because of the low maximum operating pressure of such a system. This is despite the inherently high efficiency of this type of on–column injection. Most injection systems on HPLC instruments make use of rotary sampling valves, as shown in Figure 3.5. The most common valve injector employs a relatively high volume sample loop (0.5 to 1 mL) which is flushed with mobile phase from the pump (Figure 3.5a). The valve is switched to the inject position and the solute, usually in a solution of the mobile phase, is applied using a syringe. The sample solution will displace some or all of the mobile phase from the loop into the waste vessel, depending upon the volume of sample solution injected. The sample in the loop is carried into the column upon switching the valve to the inject position (Figure 3.5b). A new variation especially for use with microbore columns is the internal loop valve. A small volume of sample solution is syringe–loaded to a milled groove on the valve rotor, thus allowing the efficient injection of very small samples.

3.6.3 Detectors

The most popular detectors used in HPLC are ultraviolet–visible photometers or spectrophotometers. These devices measure the degree to which a sample solution will absorb ultraviolet or visible light. Chromophoric groups on molecules

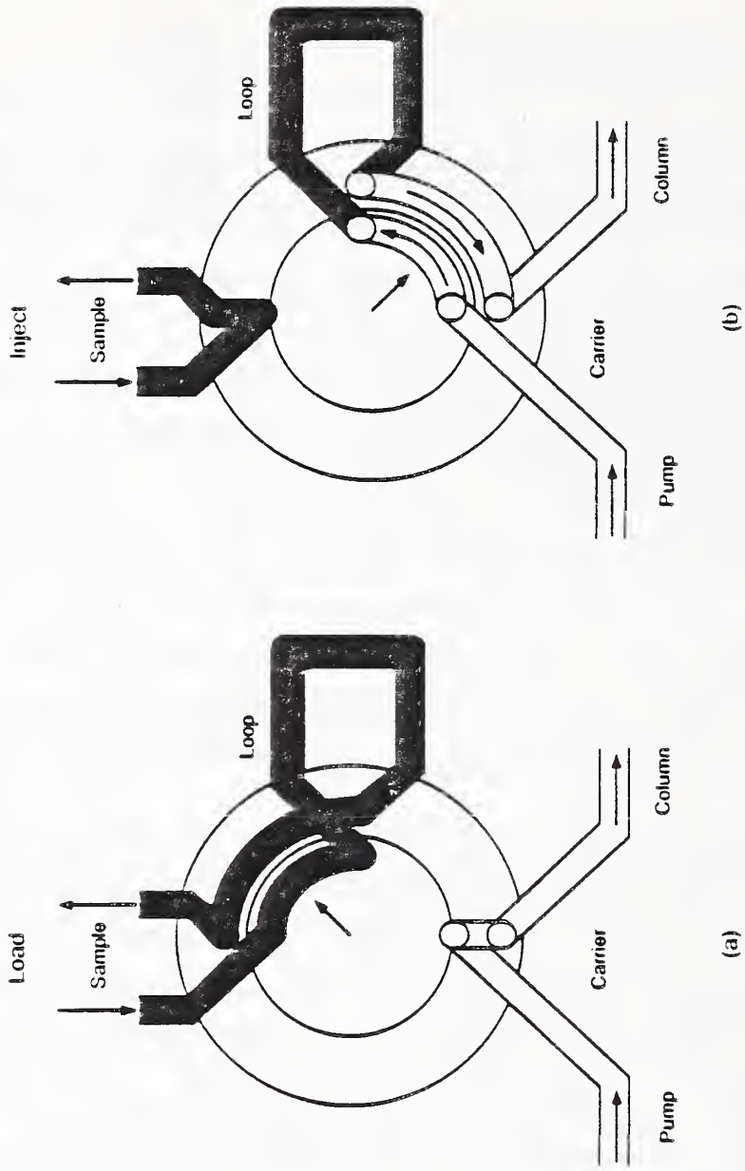


Figure 3.5 A schematic diagram of a sampling valve used for HPLC shown in the fill position (a) and the inject position (b).

(such as carbonyl, multiple carbon–carbon bonds, aryl groups) will absorb energy in the uv region, corresponding to a jump in electronic energy level. Chemical systems consisting of long, conjugated multiple bonds tend to absorb radiation in the visible

region by a similar mechanism as the uv–active functionalities. It is possible to measure this absorption of the radiation using a photometer, and to select and measure its frequency using a spectrophotometer. Ultraviolet detectors vary in sophistication from a very simple photometer to a modified diode–array spectrophotometer capable of recording the "on–the–fly" uv spectra of the eluted peaks. This is useful for qualitative analysis (since one can use uv spectroscopic correlation data) and quantitative analysis (since the optimum frequency can be chosen).

A schematic diagram of a typical uv detector and cell is shown in Figure 3.6. A typical flow cell will have a total volume of approximately 3 to 8 μL. Fixed wavelength detectors excite electronic transitions using (usually) the 254 nm line of a low pressure mercury discharge lamp, with the other frequencies (of lower intensity) being filtered out. Multiple wavelength detectors, using a medium–pressure mercury lamp as the source, provide excitation frequencies of 254, 280, 313, 334, and 365 nm. Variable wavelength detectors, using both a deuterium lamp (for the uv region) and a tungsten–halogen lamp (for the visible region) in combination with grating or prism optics, can provide frequency selection from 190 to 800 nm. The instrument responds to the decrease in intensity of the radiation which occurs as a result of absorption by the sample.

The sensitivity of the ultraviolet detector is in large measure a function of the absorptivity of the sample. A sample that strongly absorbs radiation will have a lower minimum detectable concentration. Typically, one can detect uv–active compounds down to the 1 ng level. The detector is relatively

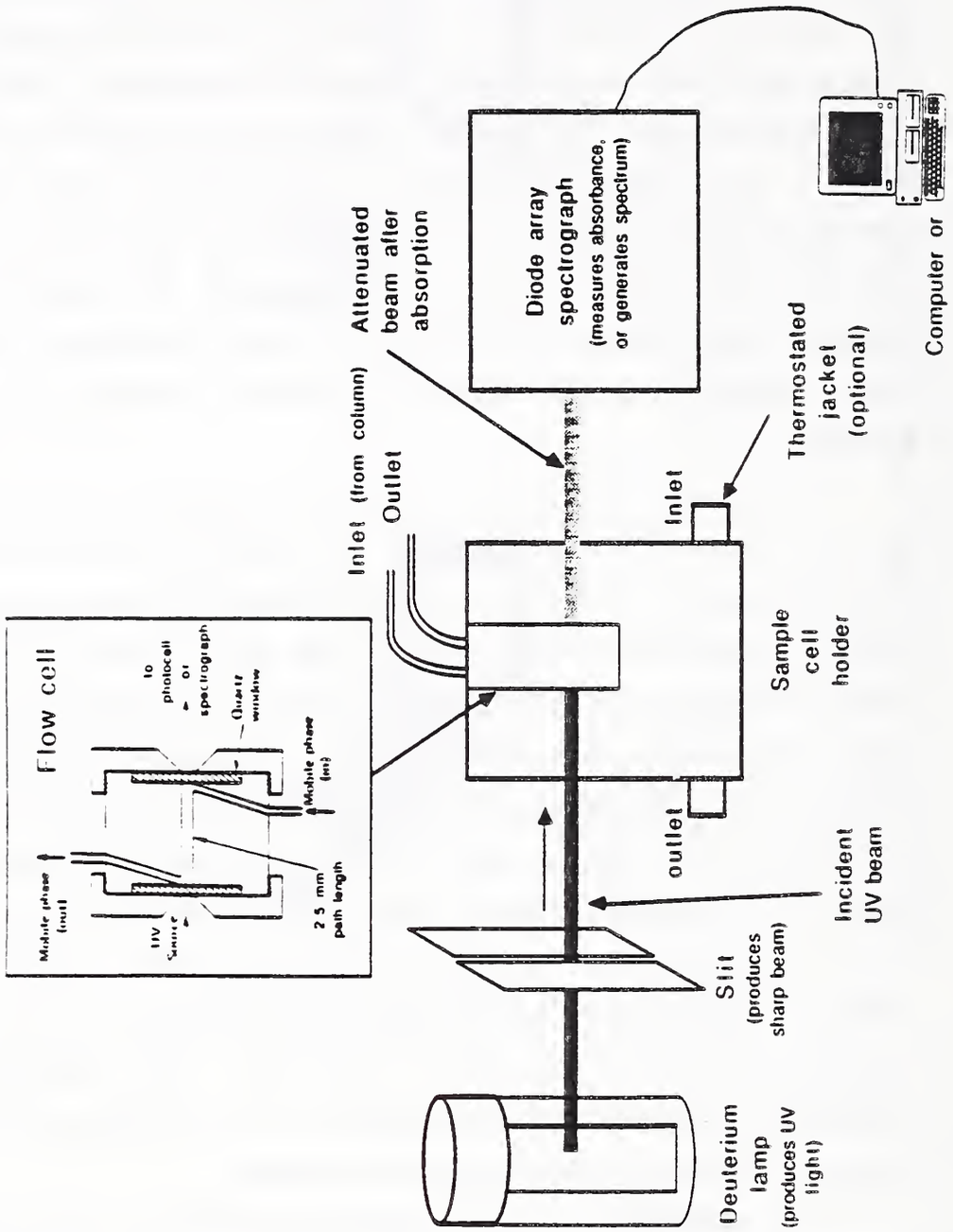


Figure 3.6. A schematic diagram of an ultraviolet detector used for HPLC.

insensitive to temperature and flowrate variations, and is linear over four or five decades. Since it depends upon the measurement of a solute property rather than a bulk property, it is ideal for gradient elution methods. It is not affected by a changing mobile phase composition unless, of course, the mobile phase contains a chromophore. Chromophoric mobile phases are generally not usable with the uv detector. The device is susceptible to misinterpretation of refractive index changes of the eluting stream as a peak. This can sometimes appear to be an absorption, due to the increase in scattering out of the beam path. The presence of air bubbles in the cell can also sometimes appear similar to absorption, but this more often causes the appearance of sharp noise spikes. Along with these minor problems, the major disadvantage of this detector is its selective response only to compounds which contain chromophoric groups. It is nonresponsive to such important classes of compounds as alcohols and ethers, whose uv absorption is in the very high frequency range.

Refractive index detectors (or more precisely, differential refractometers) are the second most widely used detectors in HPLC. They are universal in application since they respond to the bulk property of refractive index of the eluting stream. The response is provided by the change in total solution refractive index caused by the separated solute in the carrier stream. The refractive index detector is far less sensitive than the uv detector, having a minimum detectable concentration in the 1 to 3 ppm range. The ultimate sensitivity of the device is dependent on the magnitude of the refractive index difference between the mobile phase and solute component.

While there are several different designs of refractive index detector, the most popular approach is shown schematically in Figure 3.7. The sample cell, which is illuminated by a collimated, focused light source, consists of a glass chamber which is divided by a diagonal glass plate. A plane mirror is positioned behind the cell. The reference side of the cell is filled with pure mobile phase before starting an analysis, and the reflected light beams from the cell combining at the detector to determine the baseline signal. When the refractive index in the sample compartment changes with respect to the reference, the position of the emergent beam will shift. The detector measures this shift, which is then displayed on the recorder, translated in the form of chromatographic peaks.

The refractive index detector is very sensitive to changes in operating temperature and must usually be thermostatted to provide optimal performance. It is also sensitive to changes in mobile phase flowrate since flow changes will disrupt the temperature control. It is not practical to use this detector with gradient elution methods, since the mobile phase refractive index is continually changing. Although the detector can resolve refractive index differences as small as 10×10^{-8}, it is still two orders of magnitude less sensitive than the uv detector.

There are other detectors available which are useful in many special situations. The fluorimetric detector is a very high sensitivity device applicable to fluorescent solutes, or to solutes which can be rendered fluorescent by chemical derivatization. This detector is often used in tandem with the ultraviolet detector. Infrared absorption can be used to detect compounds not amenable to detection by uv or refractometric methods. It

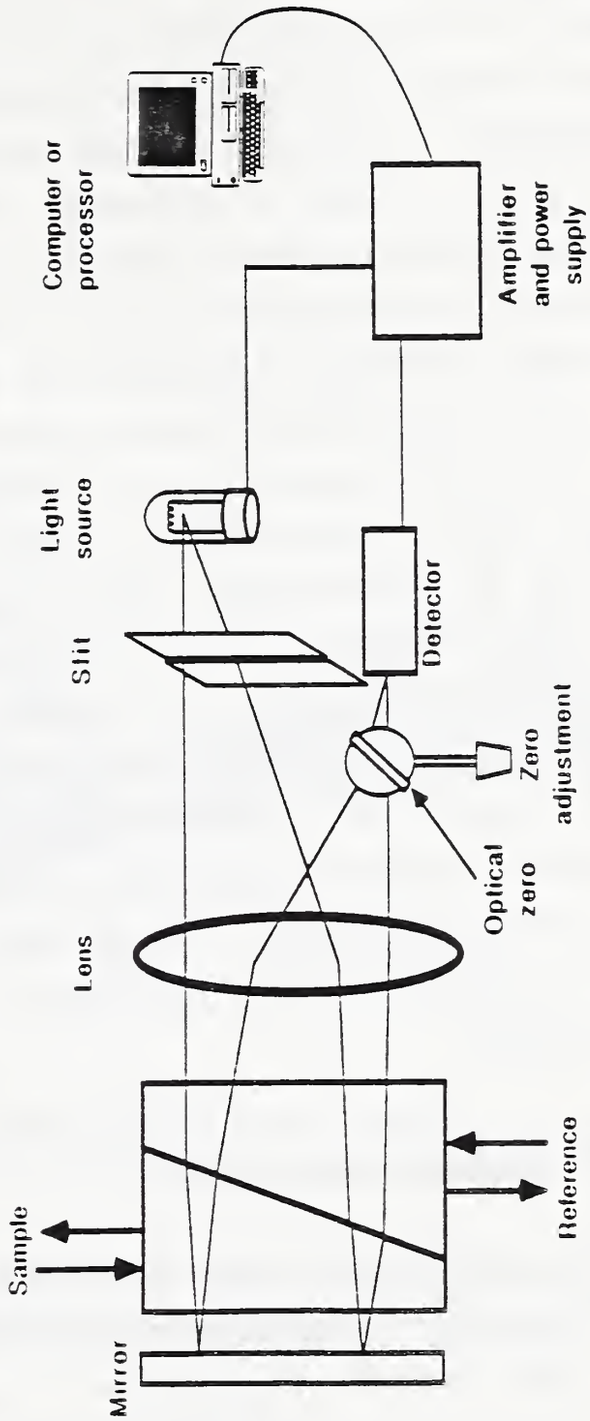


Figure 3.7 A schematic diagram of a differential refractometric detector used for HPLC.

is analogous in concept to the uv absorption detector except for the use of the vibrationally–active bands of the solute molecule. The use of Fourier transform infrared spectrophotometry offers the possibility of obtaining complete infrared spectra of the eluted zones. This is extremely valuable for sample identification, since the infrared spectrum is rich with structural information, and the fingerprint zone of the spectrum contains peaks which are usually characteristic of particular individual compounds. Electrochemical detectors can provide sensitivities higher than those obtained using uv absorption, and are useful for solutes which are easily oxidized, in high conductivity mobile phases such as buffer solutions. They are generally less costly than a typical uv detector, but are difficult to use and optimize. Mass spectrometry has been used with HPLC for a few years, and some successful commercial systems based on a thermospray interface (Figure 3.8) have been introduced. The mass spectrometer is usually a quadrupolar mass filter, similar to that used for gas chromatography. This approach, while providing a wealth of information about the solute, is the most costly detection system.

3.6.4 Miscellaneous Hardware

Most of the detectors used in HPLC are nondestructive. This allows the use of secondary methods to be applied to eluted fractions from the last detector exit. Automatic fraction collectors can be used to collect the column eluent in a series of vials, each of which accepts a specified quantity of eluent before the next vial is positioned for collection. Fraction collectors

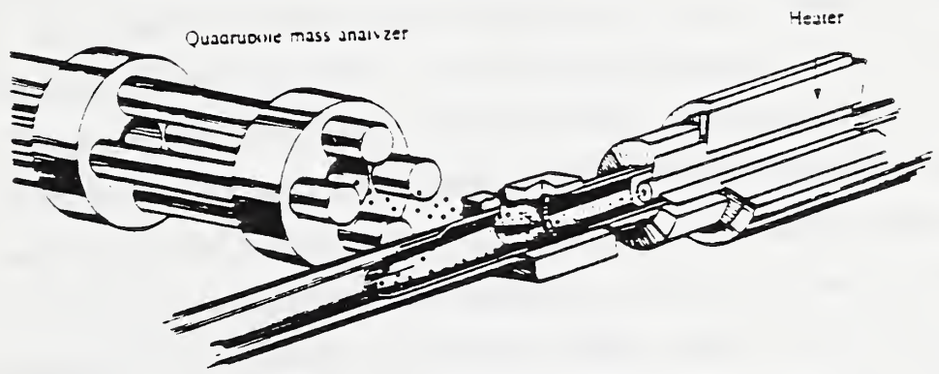


Figure 3.8 A thermospray interface from a HPLC into a quadrupolar mass spectrometer. (Courtesy of VG Instrument Corporation.)

have been used for many years as part of size exclusion methods, especially in biochemical applications involving protein macromolecules.

An automatic sampling device can be added to an injection system to provide unattended analyses. These accessories are most useful for repetitive analyses of a large number of very

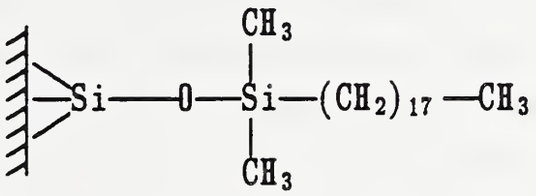
similar samples. They provide the advantage of highly reproducible injection volumes, which can be important with certain types of calibration methods. They tend to be expensive, and are usually not used in labs which do significant amounts of methods development work.

3.7 STATIONARY PHASES FOR HPLC

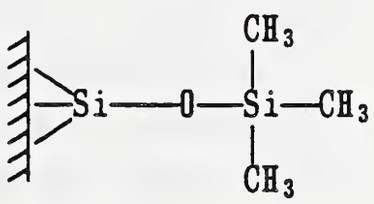
The majority of HPLC work is done today using the liquid–liquid partition mode, and we will devote a good deal of attention to the appropriate phases. We can divide LLC into two categories: normal phase and reverse phase chromatography. Normal phase HPLC involves the use of a relatively polar stationary phase, and a relatively nonpolar stationary phase. Elution from the column begins with nonpolar compounds, followed by the more polar compounds in order of increasing polarity. Reverse phase LLC employs a nonpolar stationary phase, and a relatively polar mobile phase. Elution in reverse phase LLC thus begins with the most polar compounds, followed by the least polar compounds, in order of decreasing polarity. For several reasons that we will discuss shortly, reverse phase HPLC has become the more useful of the two approaches.

Virtually no work is done today using the mechanically held liquids on supports, as mentioned in the introduction, and we will not discuss this approach. The development of chemically bonded phases, especially for use with reverse phase chromatography, is responsible for revolutionizing the method. A bonded phase is one in which a chemical functionality is

covalently attached to the surface of a silica support. An example is the octadecylsilane (ODS, n–C16) phase, the most widely used packing in HPLC:



This phase is usually made by reacting octadecyltrichloro–silane with the surface silanol (Si–OH) groups present on the silica. The surface is first modified with an hydroxylating reagent to maximize the number of surface silanols available for reaction. The silica used for bonded phase preparation is of uniform particle size, usually 3, 5, or 10 μm. The particles can be pellicular (having a porous surface, with a solid nonporous core) or microporous (in which the entire particle is porous). The microporous particles are far more useful for bonded phases since higher efficiencies can be obtained. Because of steric hindrance (the physical interference of one molecule in close proximity to another), it is not possible to cap each surface silanol with an ODS group. Since the silanol group is very polar and would interfere in the partition process, the surface is further treated with trimethylchlorosilane to form an end cap:



The surface formed by this process has a brush–like structure of hydrophobic, nonpolar hydrocarbon species. Solutes which are hydrophobic will selectively partition into the stationary phase brush, while more polar solutes will prefer the relatively more hydrophilic environment of the mobile phase solvent. The more polar the solvent, the weaker an eluent it will be in reverse phase mode. Since the bonded phase is sensitive to strongly acidic and strongly basic conditions, the solvent pH should be maintained between 2 and 7. The separation mechanism is the effect of the solvent "forcing" the solute into the hydrocarbon–like stationary phase.

While the ODS bonded phase has been of great value for general use, it is especially useful for solutes of low polarity which are soluble in aliphatic hydrocarbon. More specialized bonded phases have been developed in an effort to finely tune HPLC separations. Bonded octylsilane (n–C8, eight–membered carbon chain) and methysilane (C–1) materials are useful for moderate and higher polarity solutes, respectively. A chart providing general guidelines for the use of these materials with various solvent systems is shown in Figure 3.9. In addition to these carbonaceous bonded phases, phenyl, diol, cyano, nitro, and cynaopropyl functionalities can be bonded to a high performance silica surface. These more specialized bonded phases are useful for providing additional selectivity for certain classes of compounds. Some of these phases, such as the phenyl, can be used in both reverse and normal phase solvent systems.

An important recent development in HPLC stationary phases has been the introduction of the chiral phases. These phases are used to separate optical isomers (stereoisomers), or

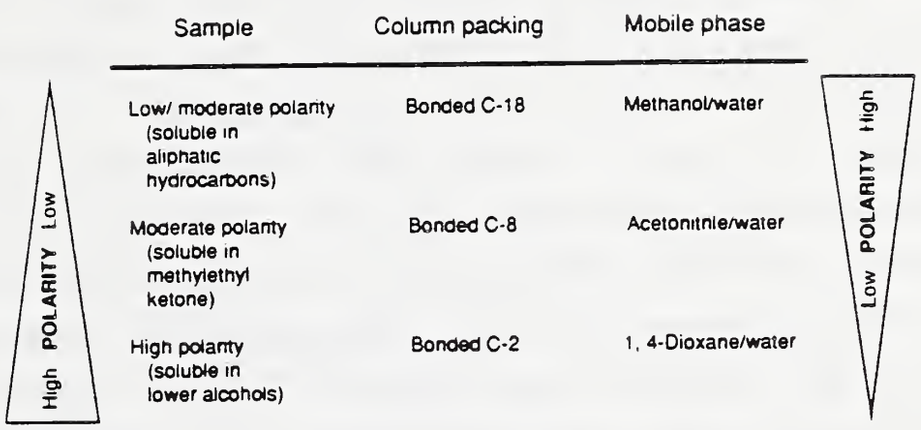


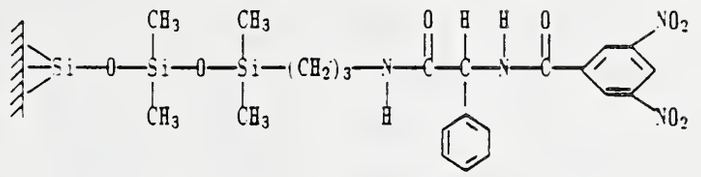
Figure 3.9 Common reverse phase packing materials and useful solvent characteristics. (Reproduced with permission of Wadsworth Publishing Company.)

so—called chiral compounds. These are compounds that have structures which are not superimposable upon their mirror images, as we discussed in Chapter 1. Separation of chiral compounds has become increasingly important in the pharmaceutical industry and with the emergence of

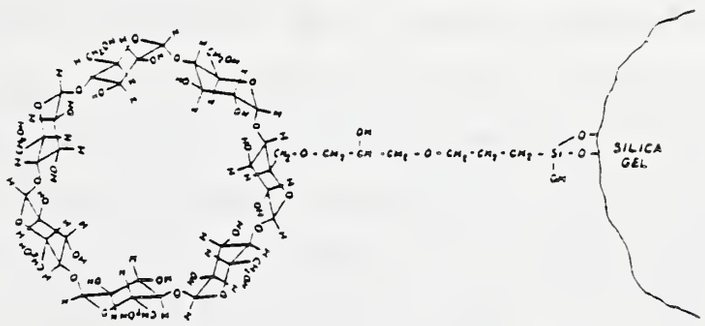
biotechnology and bioprocessing. It is not infrequent that two enantiomers (optical isomers) will show drastically different biological activity. One enantiomer may be a useful drug, while the other may be poisonous or addictive. Although some adsorbed chiral liquids on silica are effective in performing chiral separations, the most useful chiral stationary phases are the bonded Pirkle phases and the β–cyclodextrins. Examples of these are shown in Figure 3.10.

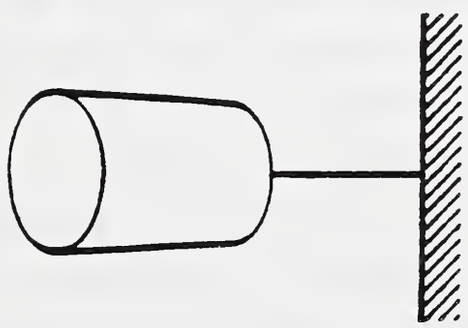
Stationary phases for liquid–solid chromatography (LSC) usually consist of silica or alumina microporous particles having a high concentration of surface silanol groups. The great majority of work in modern LSC involves silica phases. Separation of nonpolar and moderately polar solutes can be obtained with high efficiency (for example, columns having >40,000 plates/m are available). The most efficient columns are made from particles of 5 μm diameter. Pellicular packings are available, usually in the 40 μm diameter range. These packings produce columns of lower efficiency and lower pressure drop. The surface of the silica is highly acidic, and will cause tailing in peaks of basic solutes such as amines.

Stationary phases for ion exchange chromatography consist of a support matrix and an organic ion exchange functional group. Several types of packings have been developed. The first we will consider is a pellicular bead having a 30 to 40 μm diameter, coated with the exchanger film of 1 to 2 μm in thickness. These packings generally have low ion capacities (defined as the number of exchange sites per unit mass of packing), but are useful for large samples. Porous surface–coated exchangers consist of a solid core coated with a



a)





b)

Figure 3.10 The structures of some useful chiral stationary phases for HPLC. Figure 3.10a Pirkle phase. Figure 3.10b β—cyclodextrin, both the chemical structure and the overall shape. (Reproduced with permission of D. W. Armstrong).

layer of porous microparticles that hold the exchanger. The solid cores of these and the pellicular packings can be either silica or a polymer. The third type of ion exchange packing is the porous resin, based on styrene–divinylbenzene with the exchanger on the surface. These particles can have large channels throughout their volume in addition to the usual micropores which we have discussed earlier. Packings having the large channels are termed macroreticular, and have better dimensional stability than classical microreticular beads. Macroreticular packings that have particle sizes of 5 to 10 μm provide higher efficiency than the larger pellicular beads. In general, cation exchange columns have a higher temperature stability (sometimes as high as 150° C) than anion exchange columns, which have operating maxima of between 40 and 80° C. Pellicular packings can be used only at ambient temperature.

Exclusion chromatography has been used for many years to separate high molecular weight solutes such as proteins. Most of these classical methods have used long glass columns containing xerogel materials such as cross–linked dextran. More details on gel materials can be found in the discussion of electrophoresis presented in Chapter 5. These materials are generally too soft for use in HPLC. The packings used in HPLC consist of either semirigid cross–linked polymer gels or rigid, porous glasses and silicas (called aerogels). The semirigid packings are usually based on a styrene–divinylbenzene, and the pore sizes available allow fractionation of solutes from 100 to 8×10^5 g/mol in molecular weight. The particles are 5 or 10 μm in diameter, and can be used at pressures of 41 MPa (6000 psi). The aerogel packings consist of glass or silica containing a

solvent. Thus, it is not a gel in the strict sense, since it does not collapse upon removal of the solvent. These packings are used at a pH below 10, and can be used with organic or aqueous solvents. A wide range of pore sizes are available and separations of molecular masses of 1000 to 1.5 million g/mol can be done using a series of columns. Hybrid phases incorporating properties of both a xerogel and an aerogel are also available.

3.8 SOLVENTS FOR HPLC

The solvents used in HPLC must be given careful consideration. The use of high chemical purity materials is a given. The presence of particulates must be addressed using milliporous filtration. Bacteria removal may also be a consideration when using aqueous solvent systems such as buffers. We have already discussed the effect of dissolved gases on pumps and detectors. Dissolved oxygen can cause a specific problem when using a uv detector at 210 nm, since the absorbance level can change in a nonreproducible way. The solvents must be compatible with the stationary phase, and vice versa. Acetone, for example, will react in the presence of silica to form diacetone alcohol. High basicity solvents can dissolve silica and will destroy a packing.

When choosing solvents, the primary consideration must be given to solubility. The solvent or solvent system (in a gradient elution method) must completely dissolve the sample, and not allow precipitation in the column. This may seem like a silly point to emphasize, but many samples such as lipids are soluble only in very polar solvents, and the separation selectivity

that is obtained during an analysis may be poor. Protein samples in aqueous solutions at a pH near the proteins' isoelectric point can also present special problems in this regard. Proteins tend to be least soluble at this pH (see Chapter 5).

The next factor one must consider is solvent polarity, for it is the polarity (or solvent strength) which will provide the separation with selectivity on a given column. As we mentioned earlier, obtaining the optimal solvent system may require several survey analyses. The eluotropic series (Table 3.1), developed for LSC, is a scale of solvents arranged according to their energies of adsorption on alumina and silica. These energies are called the solvent strength parameters, represented by ϵ°. The solvent energy of adsorption on the stationary phase is important because the solute and solvent act in a competitive equilibrium to occupy sites on the solid surface. Solvents which have a high value of ϵ° will tend to displace solute molecules from the surface of the stationary phase. The stronger solvent will thus cause the solute to elute faster, since the solute will be bound by the stationary phase for a relatively shorter time. The eluotropic series thus provides guidance in choosing the necessary solvent strength. The scale can be used for reverse phase applications with octadecylsilane, octylsilane, and tetramethylsilane as well, but the order of solvent strength is essentially reversed, with a few exceptions.

When one is choosing between solvents of very similar polarity which are suitable for a particular application, the next variable to consider is viscosity. Solvents having a lower viscosity will cause a lower pressure drop through the column, and result in higher permeability through the stationary phase.

TABLE 3.1a ELUOTROPIC SERIES FOR HPLC SOLVENTS

Solvent	ϵ° (SiO_2)	ϵ° (Al_2O_3)
Pentane	0.00	0.00
Hexane		0.00
Cyclohexane	—0.05	0.04
Carbon disulfide	0.14	0.15
Carbon tetrachloride	0.14	0.18
1—Chlorobutane		0.26
Diisopropyl ether		0.28
2—Chloropropane		0.29
Benzene	0.25	0.32
Diethyl ether	0.38	0.38
Chloroform	0.26	0.40
Methylene dichloride		0.42
Methyl isobutyl ketone		0.43
Tetrahydrofuran		0.45
Acetone	0.47	0.56
1,4—Dioxane	0.49	0.56
Ethyl acetate	0.38	0.58
1—Pentanol		0.61
Acetonitrile	0.50	0.65
1—Propanol		0.82
Methanol		0.95
Water		Large

TABLE 3.1b RELATIVE ELUOTROPIC VALUES FOR HPLC SOLVENTS ON OCTADECYLSILANE (ODS)

Solvent	Eluotropic Value
Acetone	8.8
Acetonitrile	3.1
Dioxane	11.7
Methanol	1.0
Propanol—2	8.3
Tetrahydrofuran	3.7

Lower viscosity solvents will thus allow for faster separations. Last but not least is the matter of economics. Since it is not unusual to require in the range of hundreds of milliliters of solvent for a separation, the most economical solvent system should be chosen, as long as the important analytical questions can be answered to the desired degree of precision. It is always advisable to keep things as simple as possible, and this includes the mobile phase. Don't use a ternary system when a binary will do; don't use a binary when a single solvent will do; don't use a gradient elution method when an isocratic nongradient method will do!

3.9 COMPARISON WITH GC

In general, the initial capital outlay and cost of operation of HPLC instrumentation is significantly higher than for high resolution GC. A cylinder of high purity carrier gas used for GC is far less expensive than the high purity solvent and filtration system needed for HPLC. In addition, the detectors available for GC work are usually much more sensitive, linear, and information–rich than those used in HPLC. Separations usually require more time to optimize in HPLC than in GC, since there are more variables to consider and control. While many analyses require HPLC techniques, sometimes a choice between HPLC and GC is possible for a particular analysis. In keeping with the overall philosophy of this volume (keep things as simple as possible, while still obtaining the required information), it is usually best to choose GC methods.

SUGGESTED READING

Introductory Treatments

R. J. Hamilton and P. A. Sewell, Introduction to High Performance Chromatography, 2nd Ed., Chapman and Hall, London, 1982.

A. Braithwaite and F. J. Smith, Chromatographic Methods, 4th Ed., Chapman and Hall, London, 1985.

J. M. Miller, Chromatography: Concepts and Contrasts, John Wiley and Sons (Wiley Interscience), New York, 1988.

Detailed Treatments

C. F. Poole and S. A. Schuette, Contemporary Practice of Chromatography, Elsevier, Amsterdam, 1984.

L. R. Snyder, J. J. Kirkland, Introduction to Modern Liquid Chromatography, 2nd Ed., 1979.

A. M. Krstulovic and P. R. Brown, Reversed–Phase High Performance Liquid Chromatography, John Wiley and Sons (Wiley Interscience), 1982.

A. Pryde and M. T. Gilbert, Applications of High Performance Liquid Chromatography, Chapman and Hall, London, 1980.

Special Topics of Interest

D. J. Runser, Maintaining and Troubleshooting HPLC Systems; A Users Guide, John Wiley and Sons (Wiley Interscience), New York, 1981.

J. C. Berridge, Techniques for the Optimization of HPLC Separations, John Wiley and Sons (Wiley Interscience), Chichester, 1985.

CHAPTER FOUR

SUPERCRITICAL FLUID CHROMATOGRAPHY

4.1 INTRODUCTION

One will often hear that supercritical fluid chromatography (SFC) is something of an intermediary technique between gas and liquid chromatography. While this is true to some extent, SFC has a character all its own, with a somewhat higher level of complexity than either GC or HPLC. This stems from the more rigorous operating conditions involved, and the large number of parameters which must be understood and properly controlled.

In a nutshell, SFC is a chromatographic technique in which the mobile phase is a supercritical fluid, that is, a fluid maintained above its critical point. This region of fluid behavior is illustrated in the P–T (pressure–temperature) phase diagram provided for pure carbon dioxide in Figure 4.1. In the supercritical region, indicated by the shaded area on the figure,

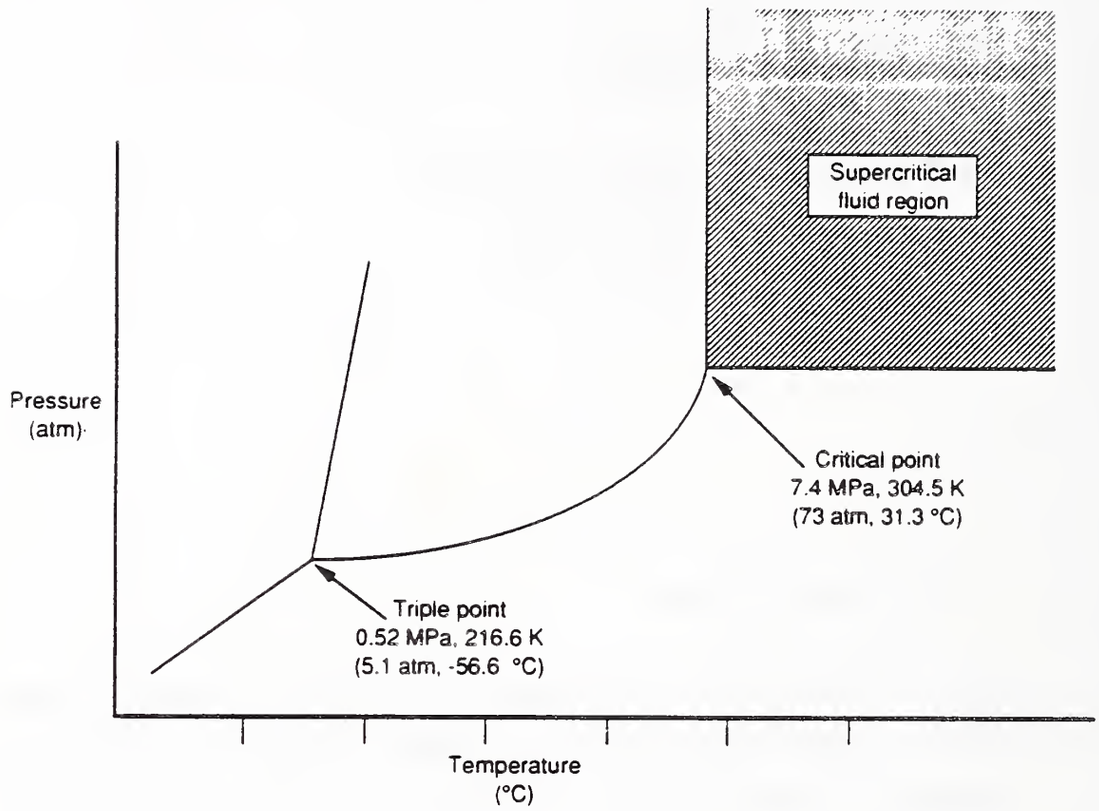


Figure 4.1 A pressure–temperature phase diagram showing the positions of the triple point and critical point, and the supercritical region for carbon dioxide.

the vapor and liquid merge into a uniform phase. In this region, no amount of applied pressure will cause the condensation of a liquid phase.[2] The great usefulness of fluids in the supercritical

[2]This is not completely true. If a suitable interface (surface) is present, a fluid at a supercritical temeprature and pressure can be made to condense.

state results from their relevant thermophysical properties, especially density, viscosity and diffusivity. A supercritical fluid has a density which is very similar to that of a typical liquid, about 0.8 to 1.0 g/mL. This gives the fluid the potential of being a very effective solvent, since a relatively large number of solvent molecules are in contact with a potential solute molecule. The polarity of the supercritical solvent then largely determines the extent of solubility of the surrounded solute molecule, just as in the case of dissolution by a liquid. The increasing solubility of solutes in supercritical fluids at increasing densities is shown in Figure 4.2. Here, a plot of the mole fraction of naphthalene (solute) in carbon dioxide is provided as a function of temperature. As one increases the pressure from 7 to 30 mPa (1015 to 4350 psi) at constant temperature, the increased fluid density is able to accommodate much higher concentrations of naphthalene.

The viscosity of the supercritical fluid is very similar to that of a gas. This is advantageous, since a low viscosity fluid moves more easily through a chromatographic system (consisting of small diameter transfer lines, tubing, valves, etc.) without causing a large pressure drop. The low viscosity of the supercritical fluid also makes for faster analyses as compared with liquid carriers. The diffusivity of a solute is a mass transport property that describes its tendency to diffuse into another species. The diffusivity of solutes in supercritical fluids is intermediate between that in gases and liquids. The magnitude of this mass transport property gives SFC the potential of producing shorter elution times than HPLC methods. The behavior of the binary diffusion coefficient, D_{12}

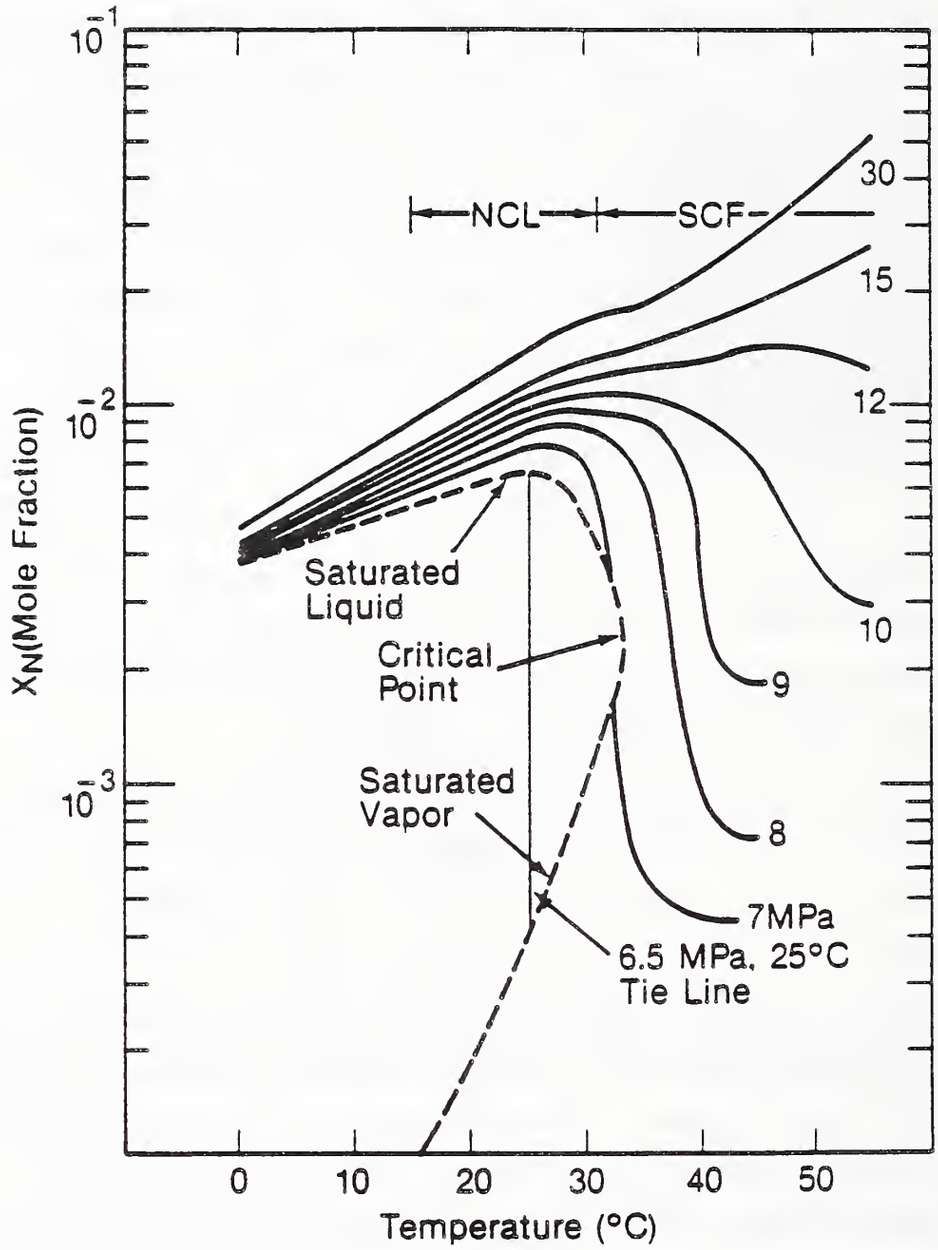


Figure 4.2 A solubility diagram for naphthalene in carbon dioxide showing the enhanced solubility in the supercritical fluid region.

(solute, 1, diffusing into solvent, 2) is calculated in Figure 4.3 as a function of temperature, using the Stokes–Einstein equation. The Stokes–Einstein equation is a mathematical relation between D_{12} and the solvent viscosity, temperature and solute size (see Glossary). We can see that the diffusion process will, in general, decrease with increasing density and increase with increasing temperature.

A glance at Figure 4.1 tells us that SFC will involve high pressures and temperatures, usually above room temperature. While this operating regime is somewhat reminiscent of HPLC, the behavior of a supercritical fluid can be far different from that of a liquid. The density of a liquid is relatively insensitive to small changes in pressure. A supercritical fluid, on the other hand, can show a very strong density response to both pressure and temperature. This is especially pronounced just above the critical point (the so–called near–critical region) and can be seen in Figure 4.4, in which a pressure–density plot (for several isotherms), in reduced units, is presented for carbon dioxide. Reduced units means that each value of pressure and density is divided by the critical pressure and critical density, respectively. There is a flattening on the plot as one approaches the critical isotherm. This will be an area of concern when we discuss the applications of equations of state later in the chapter.

The near critical region is an area of mathematical singularity, and of instability and fluctuation in terms of the thermodynamic properties of the fluid. This makes control of density and other variables rather more difficult than in the case of a liquid carrier. The near critical region is, however, also an area of great interest to both laboratory and industrial scale

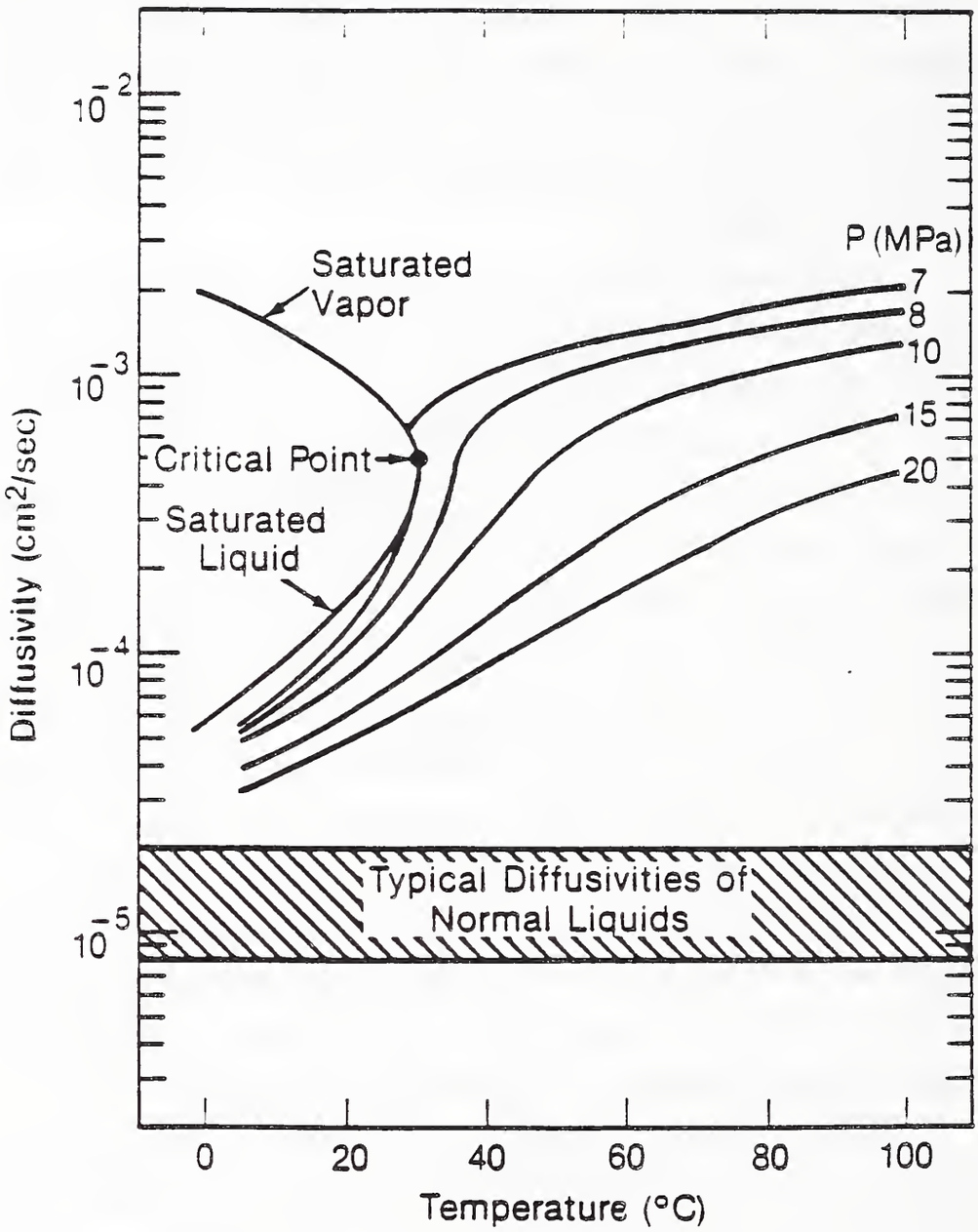


Figure 4.3 A diagram showing typical diffusivities of solutes in fluids.

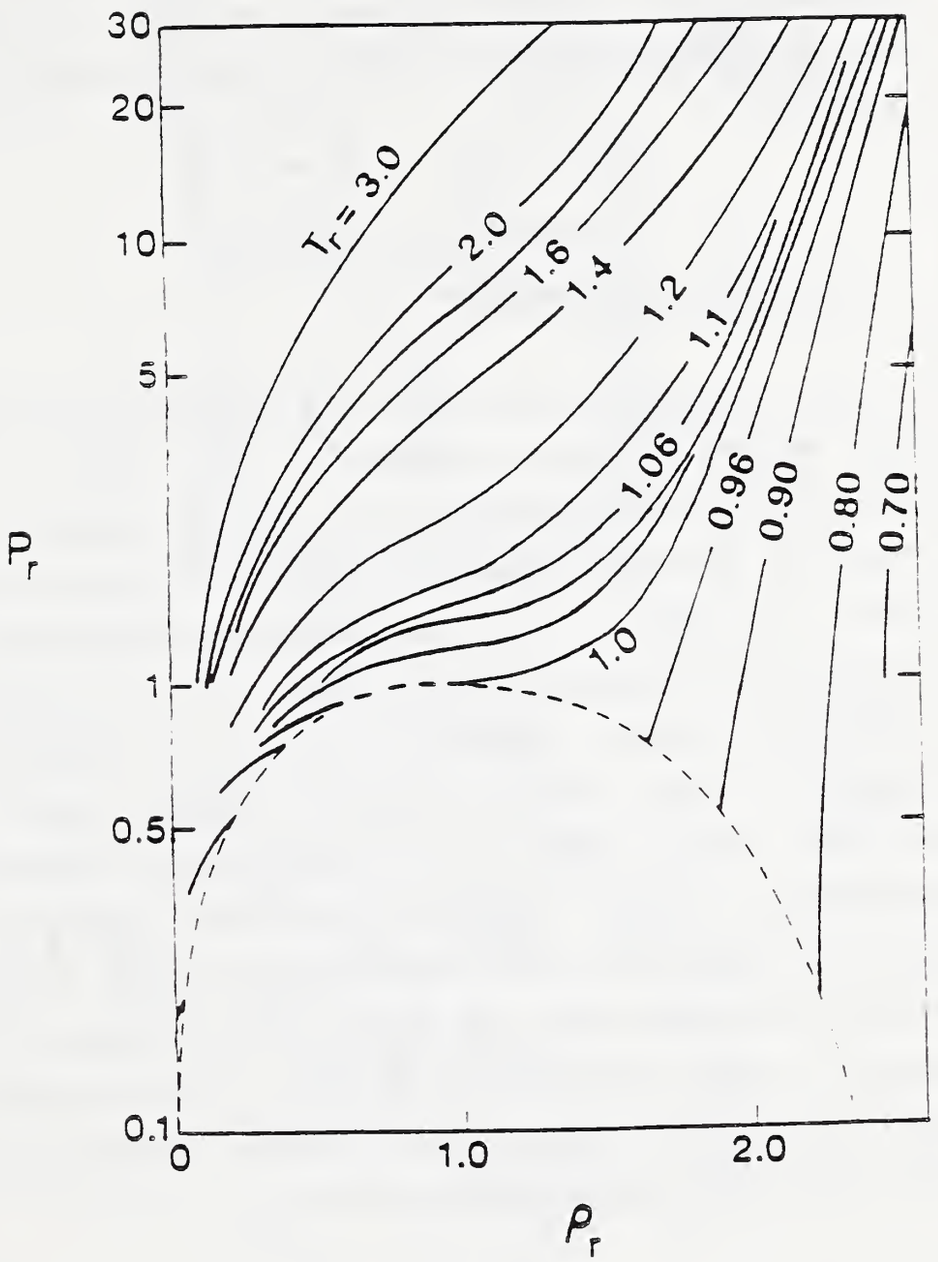


Figure 4.4 A pressure—density phase diagram in reduced units showing the characteristic flattening of the isotherms near the critical point.

separations. The main reason for this is that both operation costs and capital equipment costs increase very rapidly as the temperature and pressure requirements escalate. It is therefore economically advantageous to work as close to the critical region as possible.

4.2 HARDWARE FOR SFC

In many respects, the instrumentation required for supercritical fluid chromatography is similar to both GC and HPLC hardware. The main components are shown schematically in Figure 4.5. A typical apparatus consists of the solvent delivery system, the injector, the column and column thermostat, the detector, and the data collection system. It is also possible to incorporate a fraction collector at the exit of a nondestructive detector, to allow off–line analysis of selected zones. The detection device may be a sophisticated spectroscopic instrument, which is capable of providing qualitative and structural information about the sample, as well as a calibrated response for quantitation. The data collection system for SFC is similar to those used for GC and HPLC. It may consist of a dedicated electronic integrator, or a personal computer equipped with appropriate chromatographic software.

4.2.1 Solvent Delivery Systems

The mobile phase solvents used in SFC can be divided into two categories from an instrumental point of view, those which

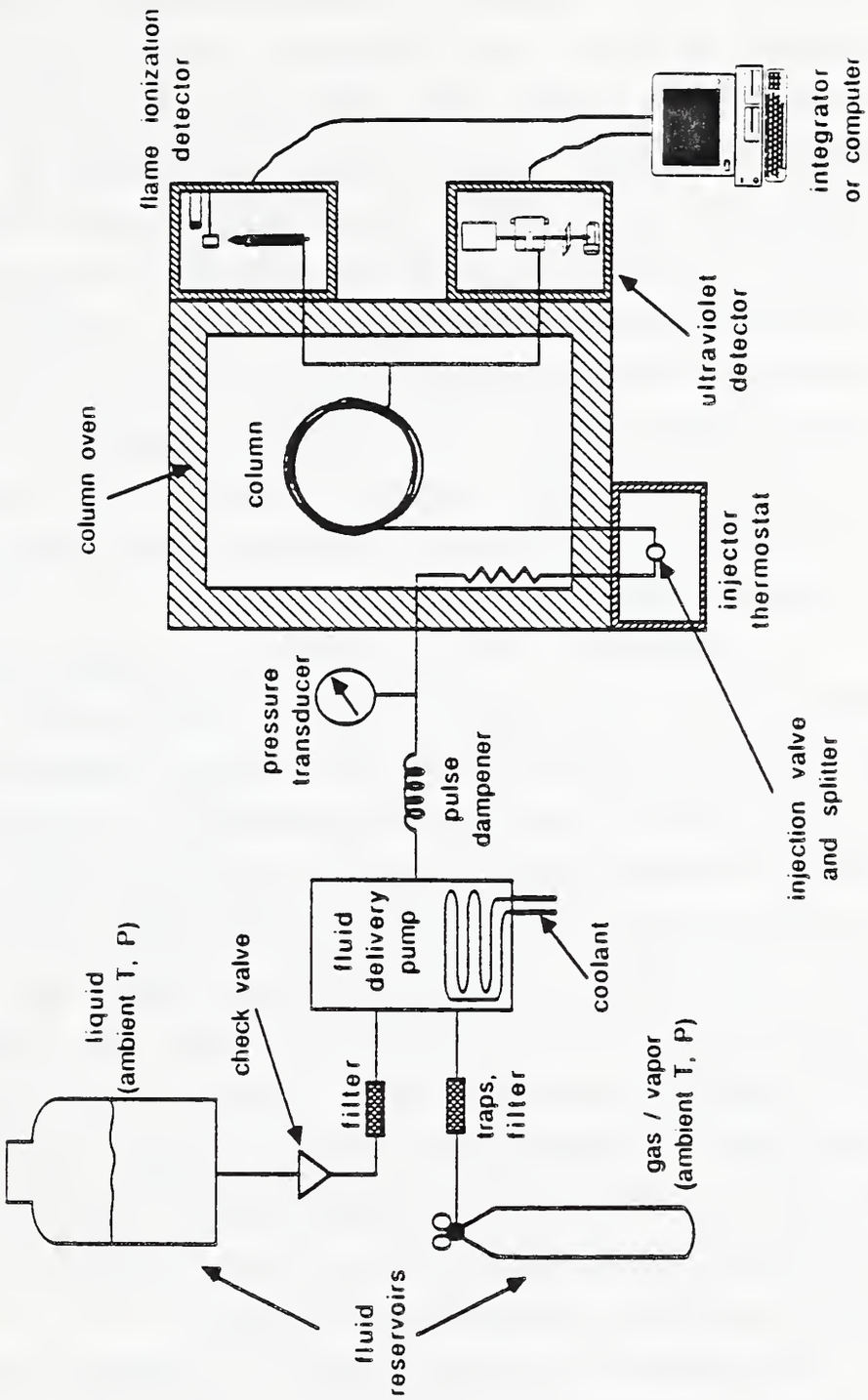


Figure 4.5 A schematic diagram showing the essential components of a typical supercritical fluid chromatograph.

are liquid and those which are vapors or gases at room temperature and pressure. The solvents that are liquids at ambient conditions can be delivered easily using certain modified HPLC pumps. The mobile phases that are gaseous at ambient conditions (carbon dioxide, for example) are usually condensed to the liquid phase before being pumped. This can be done by circulating a chilled liquid (such as a glycol–water mixture) around the pump head, or by using a Ranque–Hilsch vortex tube to cool the wetted pump components using an air stream chilled to –40° C. A low operating temperature will also help prevent cavitation (bubble formation) in the pump head, but may hasten wear of pump components such as seals, which may become brittle.

The desirability of a pulse–free carrier stream, which we covered in our discussion of HPLC, also pertains to SFC. Although the pulsation problem can be slightly less serious than in HPLC (due to the higher compressibility of a supercritical fluid), a syringe pump or a pulse compensated piston pump (followed by a pulse dampener) is usually chosen for SFC. The pumps for SFC are pressure–controlled rather than flow–controlled, as they are in HPLC. The flow rates required in SFC are much lower than in HPLC, usually on the order of a few μL/min. This is especially true for capillary column SFC. The pump must be capable of delivering this rather low flow rate precisely and reliably over long periods of operation, and it is only recently that this has become possible.

Since density is the main operative parameter in SFC, it is desirable to measure and control the density of the mobile

phase. The pressure, which is controlled by the pump through a pressure transducer, is related to the density through the P–V–T surface of the fluid. The P–V–T surface is a three–dimensional plot describing how the pressure, volume and temperature of the fluid are related. The reduced pressure–density plot presented in Figure 4.4 may be considered a projection of the P–V–T surface in two dimensions. A mathematical model (called an equation of state) may be fitted to the surface to allow estimation of the density, given the desired temperature and pressure. Equations of state vary in complexity from the van der Waals equation (which is the least sophisticated two parameter fit of experimental data) to the Schmidt–Wagner equation (which contains thirty–two adjustable parameters). The parameters for such an equation are determined using nonlinear curve–fitting routines on large quantities of reliable, critically evaluated experimental P–V–T measurements. The accuracy of the resulting fit depends greatly upon the quality of the experimental data used for the fit.

Although the more complex equations can precisely correlate the P–V–T behavior of a fluid, most are somewhat inaccurate in the near–critical region. If we refer back to Figure 4.4, we can observe the characteristic flattening of the isotherms near the critical point. It is this feature of the surface which is difficult to model mathematically, since the equation must become non–analytic in this region. While a discussion of the complexities of equation–of–state correlation and testing is outside the scope of chemical analysis, the results and consequences are of great importance to the practice of SFC.

In GC, we use temperature programming to enhance the elution of less volatile solute components, while in SFC we use a program of increasing density. A computer program incorporating an equation of state for the mobile phase adjusts the pump pressure (at constant temperature) to produce the desired density program. The increasing density over time increases the solubility of the less volatile solutes, and they are drawn into the supercritical mobile phase by the difference in chemical potential of the solute in each phase. The column temperature can also be programmed in SFC, to increase the volatility of the solute. It must be remembered that increasing the temperature at constant pressure will result in a decrease in density (or, alternatively, an increase in specific volume). This will partially offset the advantage of the increased solute volatility. The best approach would be to program both density and temperature, which allows control over the solute volatility and solute solubility in the mobile phase. The reader will note the increased level of complexity already apparent in the operation of a SFC instrument. The separation design and control process is thus more exacting than either GC or HPLC.

The pressure is maintained through the column by a flow restrictor placed at the column exit. This restrictor is usually a length of fused silica tubing of small internal diameter. Both the length and diameter will determine the back pressure obtainable. Another type of restrictor employs a glass frit held in a fused silica tube. Back–pressure regulators, used in early SFC instruments, are rarely installed in current instruments, although a new restrictor–back pressure regulator combination offers some exit flowrate control.

4.2.2 Injectors

SFC can be performed using both capillary columns and packed columns containing chemically bonded phases similar to those used in HPLC. The choice of column type will determine the type of injection system that will be needed. All injection in SFC is now done using variations of HPLC–type sampling valves incorporating low volume sample loops or internal loop valve rotors. Injection into a packed column can often be done directly, while injection into a capillary column will require the use of a flow splitter following the sampling valve because of the low capacity of these columns. The flow splitters are not as sophisticated as those used in GC, which incorporate complex pneumatics to control the split ratio. For SFC, splitters usually consist of a branched transfer line equipped with a restrictor to provide the flow path for the vented stream. No satisfactory variable–flow restrictor has been introduced commercially to provide the operator with continuous control of the split ratio. A recently developed combination of restrictor and back–pressure regulator appears to have good potential, however.

Whether a packed column or a capillary column is used, the sample is deposited into the loop of a high pressure injection valve in one of several ways. Delivery can be done very simply by using a syringe (of the type used for HPLC valve loading), with the sample dissolved in a suitable solvent. Alternatively, the sample may be delivered to the loop from an on–line supercritical extraction cell (Figure 4.6). In the latter case, the sample arrives at the injection valve already dissolved in the supercritical mobile phase. This is useful for trace analysis as

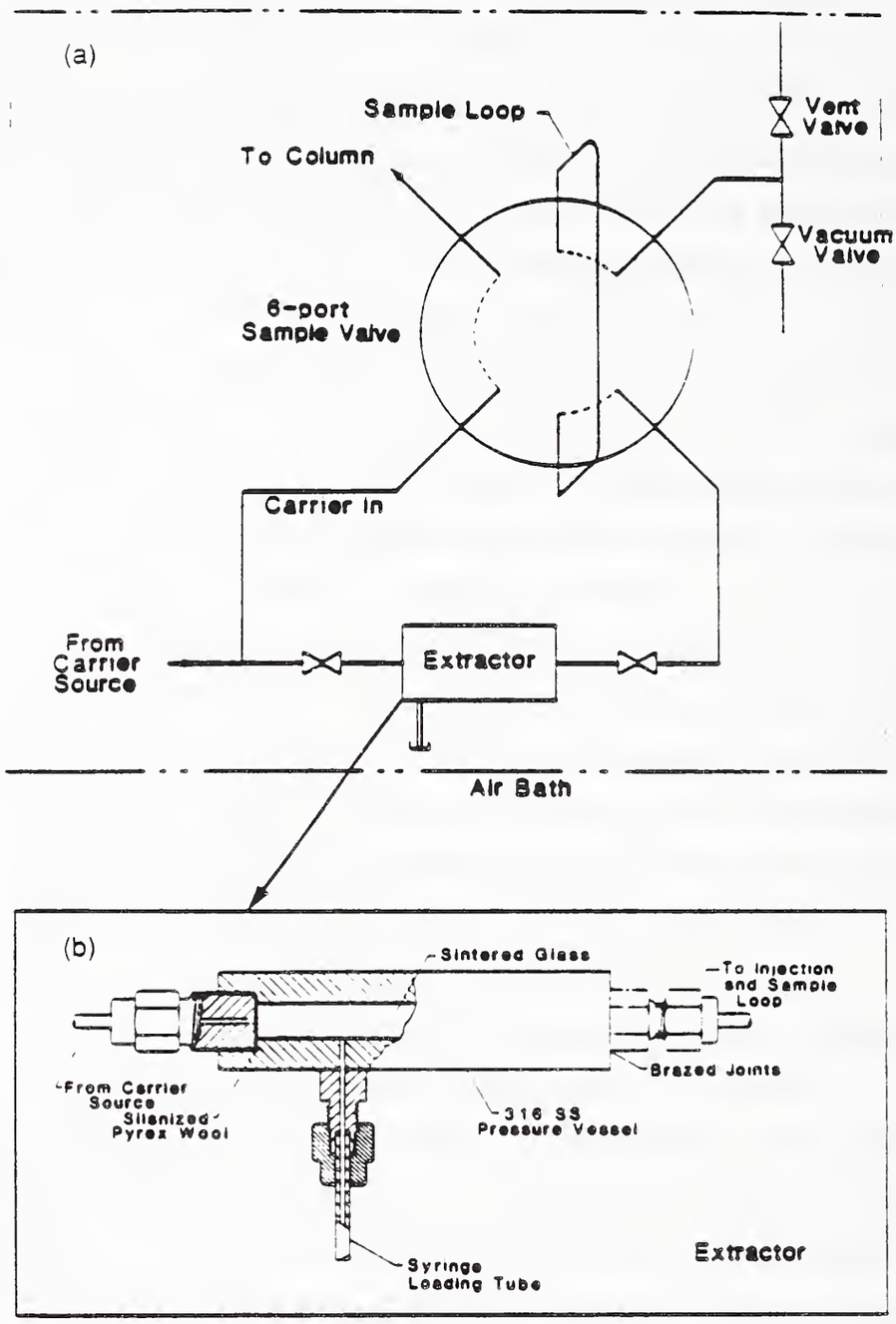


Figure 4.6 A schematic diagram of an extractor–based injection system, showing a possible manifold (a) and an extractor cell (b).

well as physicochemical studies, where the presence of a solvent in addition to the supercritical eluent is undesirable. The availability of on–line supercritical fluid extraction as a sample preparation technique is a very attractive feature of SFC. Other injection techniques are also reported in the literature, but these two methods are the most generally useful.

4.2.3 Detectors

Many detectors originally designed for use in HPLC and GC have been modified for use in SFC. The two most popular are the uv spectrometric detector and the flame ionization detector. These detectors are described in detail in the HPLC and GC chapters, respectively, and only the salient points of modifications for SFC will be described here.

The optics and electronics of the uv spectrometric detector are essentially identical to those used for HPLC. The main difference is in the design of the flow cell, which must accommodate much higher pressures. This is achieved by minimizing the size of the wetted or pressurized components, thus decreasing the loading (force per unit area) to a small level. Flow cells usually have an optical path length of 2 to 5 mm, with the larger size more suitable for routine analysis. The smaller path length is needed when closely eluting components are being analyzed, since the smaller cell will lower the detector contribution to peak dispersion. It is not uncommon to experience cell sealing difficulties, especially when the cell is first used or after several weeks of down time. The cells usually contain Teflon gaskets which tend to cold flow under load and

must absorb some of the supercritical carrier and swell slightly in order to produce a tight seal. In general, this detector provides good sensitivity and stability, but can sometimes suffer from baseline drift during density programming. Naturally, the ultimate sensitivity is dependent upon the absorptivity of the sample, and the detector is only applicable to the analysis of compounds which contain chromophoric groups. Quantitation is dependent upon calibration involving the Beer–Lambert law, so care must be taken to ensure that all the requisite assumptions of that relationship are satisfied.

The flame ionization detector can be modified for SFC by allowing the exit restrictor to vent at the base of the hydrogen flame (Figure 4.7). This is usually done using a fused quartz capillary, since an electrically conducting transfer line would disturb the operation of the FID. The detector is operated at a high temperature (approximately 300° C), since baseline spiking can occur if the mobile phase cools significantly upon decompression. For packed column operation, the FID can be fitted with additional exit tubes to vent off excess mobile phase. An FID for packed column SFC use was introduced commercially several years ago, with mixed results. The detector is very sensitive and linear, and is not adversely affected by a slightly pulsing profile in the mobile phase flow. The main problem with the FID is that it cannot be used with flammable mobile phase fluids. This includes nonflammable fluids modified with small (1 to 2 percent) quantities of flammable fluids, such as the very useful carbon dioxide + methanol or carbon dioxide + methanol systems.

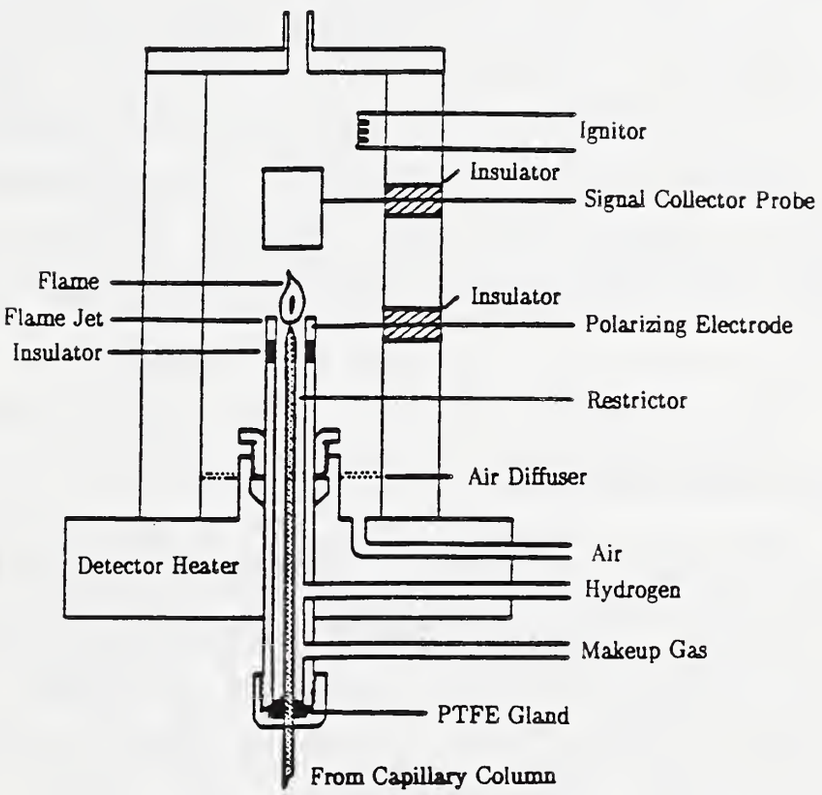


Figure 4.7 A schematic diagram of a flame ionization detector modified for use in SFC.

Mass spectrometers can be used as detectors for capillary column SFC, since the flow into the ion source is relatively low (0.5 to 5 L/min) and will not cause a harmful overpressure. The instrumental details, are, in general, very similar to the application of this detector in capillary GC. Using a mass spectrometer with packed columns requires a more carefully designed interface, and most often chemical ionization rather than electron impact methods are used. Other possible detectors include Fourier transform infrared spectrometric methods, which allow "on–the–fly" infrared spectra of solute components to be recorded. This is especially valuable, since the infrared spectrum is rich with structural information useful for qualitative analysis. Fluorescence, flame photometric and thermionic detectors have been applied to SFC, but to a much smaller extent than the other detectors which we have discussed.

4.3 MOBILE PHASES

By far the most popular mobile phase for SFC is carbon dioxide, for a variety of important reasons. The critical temperature of carbon dioxide is 31.3° C, which is convenient from an operational point of view. It can be liquefied at just a few degrees below room temperature, and flashed into the supercritical state just a few degrees above. It is nontoxic and economical, readily available at an adequate purity level, and further purification is relatively easy if it is required. Carbon dioxide is also nonflammable and nonexplosive, and will not give a background response in a flame ionization detector.

Other common fluids for SFC are pentane, hexane, sulfur hexafluoride, isopropanol and methanol. Methanol is especially useful as a mobile phase modifier. It is added to carbon dioxide in small amounts (0.5 to 2 percent) to increase the overall polarity of the mobile phase. Other fluids either used or suggested as mobile phases for SFC are listed in Table 4.1, along with their relevant critical properties.

The critical temperature of a fluid being considered for a SFC analysis is an important methods development parameter, since the mobile phase temperature must be maintained above this value. The fluid must be stable at the required operating temperatures, while in contact with the construction materials of the chromatograph. These include silica and several varieties of stainless steels. This is an important consideration, since, for example, methanol decomposes near its critical point when in contact with stainless steels of the 300 series (AISI designation), and forms hydrogen, carbon monoxide, and formaldehyde.

The critical pressure of a potential carrier fluid is useful as an approximation of the solvent strength of the fluid. The critical pressure is usually higher for more polar fluids, although there are some exceptions. Thus, in general, the higher the critical pressure, the higher the solvent strength of the mobile phase. The critical density is also a good indicator of solvent strength, since solvent strength generally increases with increasing critical density.

When a homologous series of hydrocarbons is examined for potential usefulness as mobile phases, some definite chromatographic effects and trends can be observed. The lower hydrocarbons (such as propane) provide high resolution

TABLE 4.1 SOME USEFUL FLUIDS FOR SUPERCRITICAL FLUID CHROMATOGRAPHY

Fluid	T_c(°C)	ρ_c(g/mL)	P_c(MPa)
Carbon dioxide	31.413	0.460	7.4
Ammonia	132.5	0.235	11.4
Nitrous oxide	36.5	0.450	7.3
Sulfur dioxide	157.8	0.520	7.9
Water	374.1	0.40	22.1
Methanol	239.5	0.272	8.0
Isopropanol	235.3	0.273	4.8
n—Pentane	196.6	0.232	3.4
n—Hexane	234.2	0.234	3.0
Dichloro—fluoromethane	178.5	0.522	5.2
Trichloro—fluoromethane	198.0	0.554	4.4
Chloro—trifluoromethane	28.9	0.578	3.9
Dichloro—tetrafluoroethane	145.7	0.582	3.6
Dichloro—difluoroemethane	111.5	0.558	4.0
Benzene	288.9	0.304	4.9
Xenon	16.6	1.155	5.9
Toluene	320.8	0.29	4.2

separations at a relatively low supercritical temperature. The higher members (such as pentane) provide fast analyses at a somewhat lower resolution, since they have a somewhat higher solvent strength.

Unfortunately, the most operationally useful fluids for SFC (carbon dioxide and n–pentane) are nearly nonpolar. For example, carbon dioxide has about the same polarity as n–hexane. The lack of more polar eluents than these is one of the major limitations of SFC. Many investigators have used polar modifiers (sometimes also called entrainers, especially by the engineering community), added in small amounts to the nonpolar fluids to increase the effective polarity of the mobile phase. The most common additive, methanol, was mentioned earlier. The gradient elution method has also been used with carbon dioxide and 1,4–dioxane. The use of a mixed mobile phase introduces great complexity into the density prediction problem which we discussed earlier.

While it is possible (although in many cases difficult) to develop equations of state for nonpolar mixtures, there is no reliable model for mixtures having a polar component. Most equations of state for mixtures depend upon mathematically combining the properties of the pure components, using a set of equations called mixing rules. In the absence of reliable data on the properties of pure modifiers under the conditions of interest to SFC, the development of even a marginal equation of state becomes very difficult. Even the most sophisticated approaches, such as those which employ an extension of the principle of corresponding states, have been only marginally successful. These methods make use of a well–characterized reference fluid

with which to scale the properties of a fluid of interest, using reduced parameters.

The use of supercritical ammonia has been suggested as a possible experimental solution to this difficulty, since it has a relatively high polarity. There are still problems of materials compatibility and safety with this fluid, however.

4.4 STATIONARY PHASES

As we mentioned earlier, SFC can employ either capillary or packed column methods. While the basic chromatographic theory will not change from column to column, the techniques will change significantly. Before we discuss individual phases, it would be advisable to first discuss the two approaches in general terms. The choice between capillary and packed columns will primarily depend on the solute mixture to be analyzed, but this decision will have important instrumental consequences. A few of these considerations have already been mentioned in our discussion of injectors and detectors.

The important physical characteristics of capillary columns are the column length, the stationary phase film thickness, and the internal diameter of the column. As in GC, capillary columns can provide high efficiency (in terms of total theoretical plates per column) for the separation of complex mixtures containing many components. They can provide 250 to 1250 more plates per centimeter than packed columns. The stationary phase film should be as thick as possible without itself becoming an unfavorable contributor to peak dispersion.

The length of the column should be maintained as short as possible, to allow for faster analyses. (The speed of an analysis cannot, in general, be increased by increasing the carrier velocity.) The pressure drop through the capillary is relatively low, making these columns very suitable for analyses which can be done at lower mobile phase densities. Capillary methods are quite amenable to detection by FID and MS methods. Lower flow rates are used for capillary column methods, which require higher accuracy in solvent delivery and control. Flow splitters are needed for operation with the smaller diameter columns.

The important physical characteristics of packed columns are the column length, column diameter, packing particle size, and particle surface area. There are more independently variable parameters in packed column SFC, and therefore more flexibility and also more complexity. The packings are usually modified silicas or polymers. The pressure drop through the column is very high, so packed columns are somewhat more suitable for use with higher density mobile phases. They are suitable for separations requiring low or moderate efficiency, such as relatively simple mixtures having about ten peaks. They are also useful in situations in which complete separation of the mixture is not critical. Analysis is much faster on packed columns than on capillary columns. Mobile phase modifiers have a somewhat different and more critical role with packed columns. Since the silica surface always contains unreacted silanol sites, the polar modifier is very often needed to "tie up" these sites, and to prevent excessive solute peak tailing. This often requires the use of higher operating temperatures for packed columns, since the mobile phase mixture must be

maintained in the supercritical state. The use of the FID with packed columns is possible; however the presence of flammable modifier will very likely impair performance. If the modifier concentration must exceed approximately 1 percent, the FID probably cannot be used at all. The MS can also be used, especially in chemical ionization mode, provided that the interface can handle the higher volume of eluent. The ultraviolet and infrared detectors can be used in regions where the mobile phase does not show a strong absorption. The recent appearance of packed capillary columns has excited much interest, since they offer a useful compromise between packed and capillary columns.

The most common stationary phases used with capillary columns are the cross–linked polymethyl siloxanes, favored for their inertness and homogeneity. Many variations based on the siloxane backbone have been synthesized, including liquid crystalline polymers. For packed column work, most of the phases have been based on silica, either bare or chemically bonded. The bonded phases which are most commonly used are the octadecylsilane and octylsilane materials used in HPLC, although the more polar cyano, amino and diol packings have also been used.

As with GC, SFC can be used to perform physicochemical measurements. Using an uncoated, long capillary column, the binary interaction diffusion coefficient, D_{12}, can be measured using the method of Taylor and Aris. The diffusion coefficient represents the tendency of the solute to be transported into the supercritical fluid phase, and is therefore useful in analytical methods development in work on the design of supercritical fluid

extraction processes. Other physicochemical parameters experimentally accessible using SFC are partial molal volumes near the critical point, partition coefficients, and the polydispersivity of polymeric materials.

4.5 COMPARISON OF SFC WITH GC AND HPLC

As we have observed from the above discussion, the theory and practice of SFC can, in general, be much more complex than either GC or HPLC. The cost of an SFC system is also very high, even when compared to HPLC. There are, however, instances in which SFC will provide an elegant solution to problems not readily solved using GC or HPLC. One important example is in the analysis of thermally labile compounds. These materials cannot withstand the elevated injector temperatures which are often needed to volatize the sample. Compounds such as natural products, biological compounds, and pharmaceuticals often fall into this category. Another example is higher molecular weight compounds such as polymers. These materials often cannot be analyzed by GC due to their very low vapor pressure. Analysis by HPLC is often not possible due to sample insolubility. In the past, one usually had to pyrolize the sample and analyze the products by GC. The enhanced solubility afforded by supercritical fluids can often provide the key to these difficult analyses.

Despite the unique advantages of SFC, there will be significant areas of overlap for the application of this technique with GC and HPLC. In these instances, it will usually be best

to choose the simplest approach which will provide the desired answers to a particular analytical problem. In most cases, this will mean GC if possible, followed by HPLC. If an analysis can be done using gas or high performance liquid chromatography rather than supercritical fluid chromatography, it would be foolish to choose the more costly and complex SFC.

SUGGESTED READING

R. M. Smith, Ed., Supercritical Fluid Chromatography, Royal Society of Chemistry, Burlington House, London, 1988.

B. A. Charpentier and M. R. Sevenants, Supercritical Fluid Extraction and Chromatography, American Chemical Society, Washington, DC, 1988.

S. Ahuja, Ed., Ultrahigh Resolution Chromatography, Chapters 10 and 11, American Chemical Society, Washington, DC, 1984.

CHAPTER FIVE

ELECTROPHORESIS

5.1 INTRODUCTION

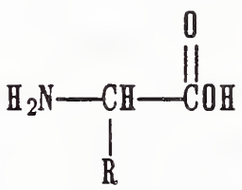
Electrophoresis is a technique used for the separation of ionized, (often) colloidal–sized molecules under the influence of an applied electric field. The method has historically been most useful among biochemists and biologists, but the emergence of commercial–scale biotechnology and the increasing industrial applications of bioprocessing has increased the popularity and importance of this technique in the chemical and engineering communities. Although the most common application of electrophoresis is in the analysis and characterization of ionized species such as proteins in aqueous solution of buffers, the method has also been applied on the preparative scale for the purification of larger quantities of such compounds. The primary application of this technique is for the separation of

proteins and polynucleotides. A short discussion of the nature and structure of these compounds would be helpful before we explore how electrophoresis is used in their chemical analyses.

5.2 PROTEINS

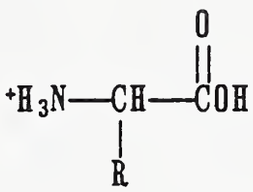
The name "protein" is derived from the Greek word *proteios*, meaning first. Proteins are, in short, the substance of life, since they provide the motive power for all animal species. They are large polymeric molecules which comprise the structure of skin, muscle, nerve, blood, antibodies, enzymes and hormones found in any living cell.

Protein polymers are made up of monomeric units of α–amino carboxylic acids (actually, the L–, or levorotary stereoisomer), or more simply, amino acids. The general structure of an amino acid can be represented as:

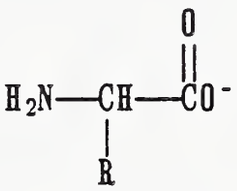


with the amine and the carboxyl groups both bound to the same carbon in what is called the alpha configuration. The "R" group represents a chemical moiety or side chain which is responsible for the differences among the amino acids. The amino acids are crystalline at room temperature and decompose upon melting when subjected to elevated temperatures. They are relatively

insoluble in nonpolar solvents such as the alkanes, but are more soluble in polar solvents and can be very soluble in water, forming solutions having properties similar to salt solutions. The extent of solubility depends upon the nature of the group represented by "R." Amino acids are dipolar ions, and can have either a positive or a negative charge:



(a)



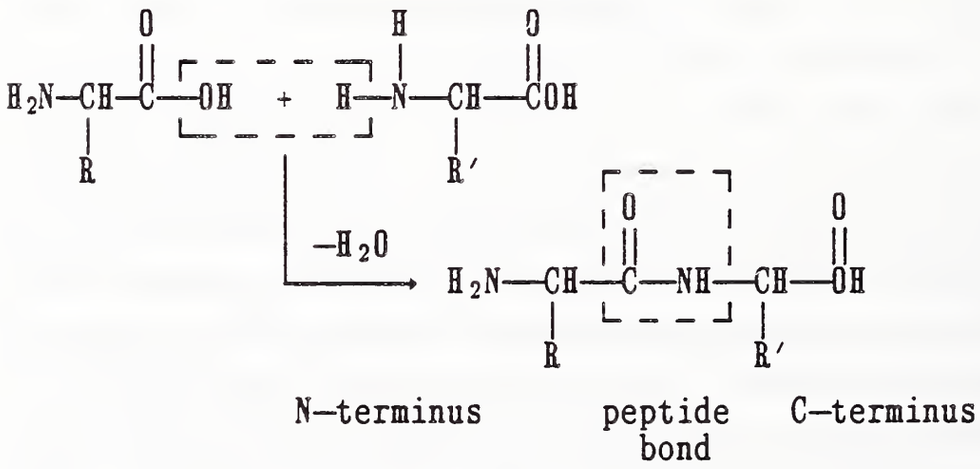
(b)

The pH of the solution will determine the charge that the ion carries. At a relatively low pH level, the carboxylic acid groups will not be ionized, and the amine group will be protonated due to the relative excess of hydronium ions (H_3O^+) in the solution. This will leave the protein with a net positive charge. At a high pH, the carboxyl group will be ionized due to the interaction with the relative excess of hydroxyl ions, and the amine group will not be protonated. This will leave the amino acid ion with a net negative charge. At intermediate pH levels, some amine groups will be protonated and some will not. This depends on the acidic strength of the particular molecule. There will be one particular pH value at which the amino acid will have no net charge. This point is called the isoelectric point, at which the amino acid exists as a dipolar ion, having an ionized carboxyl group and a protonated amine group. The amino acid

will usually be least soluble at this pH level, with the solubility increasing if the solution is made more acidic or alkaline.

Amino acids have interesting geometric features. Every amino acid (except the simplest, glycine) has at least one chiral center. As we discussed in Chapter 1, the presence of chirality (nonsuperimposability of a molecule upon its mirror image) leads to the phenomenon of optical activity, the rotation of the plane of polarized light which is passed though a solution. In fact, all amino acids which occur as part of biological systems are levorotatory (L, or "–" preceeds the name of the amino acid) and rotate the plane of polarized light to the left. Organisms are quite fussy about the optical purity of the amino acids which make up the biological structure, and one of the most important functions of the liver is the destruction of dextrorotatory (D, or "+" precedes the name) amino acids and their polymers.

Proteins are polymers built up from amino acid monomers. The polymerizations result from the reaction of the amino group of one amino acid with the carboxyl group of another, forming what is called the peptide bond, occurring upon release of water:



The amino and carboxyl groups of the individual amino acids are used in the formation of the peptide bonds. This leaves only the terminal amino groups (the N–terminus) and the terminal carboxyl groups (the C–terminus) intact. These terminal groups on the protein are subject to ionization in the same way as on amino acid units, with a strong dependence upon solution pH. The individual amino acids making up the polymer are called residues. The polymer is classified by the number of residues which comprise the polymer. Thus, if a polymer consists of three amino acid residues, we refer to it as a tripeptide. Polymers of molecular weights up to 10,000 g/mol are termed polypeptides, and those above this figure are what we call the proteins. While the peptization reaction is the principle means of protein formation, some amino acid residues will allow additional covalent bonds to be formed. Cysteine, which contains a thiol (–SH) group, is important due to its ability to form disulfide bridges in the protein with other cysteine units. This forms very stable cross–linked proteins. These proteins are often found in areas outside of living cells such as the bloodstream or digestive tract of complex organisms, where the chemical environment is relatively severe.

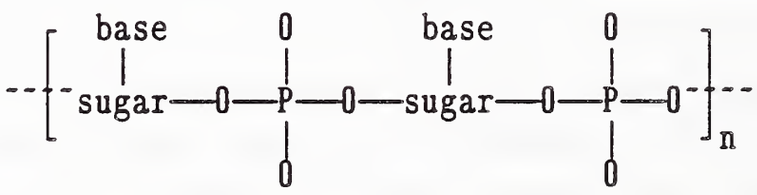
Proteins are divided into two broad classes, fibrous and globular. Fibrous proteins are long, thread–like molecules often containing many intermolecular hydrogen bonds. They tend to be sparingly soluble or insoluble in water. Globular proteins are quasi–spherical in shape and tend to contain intramolecular hydrogen bonds; they are generally more soluble in water. Because of the large size of the protein molecules, the solutions are often very similar to colloidal suspensions. The two general

categories are further subdivided according to solubility and reactivity of the protein. For example, albumins are water soluble proteins which are denatured (irreversibly precipitated or coagulated) by heat. Globulins are proteins which are insoluble in pure water, but soluble in dilute salt solutions.

5.3 POLYNUCLEOTIDES

Polynucleotides are similar in concept to proteins, although they are quite different from both a chemical and a functional point of view. Research into nucleic acids and polynucleotides is one of the most active areas of scientific pursuit, since these compounds are the basis of heredity.

Polynucleotides are polymeric molecules which contain a long chain backbone that differs only in length from one polynucleotide to another. In proteins, the polymer backbone was the polyamide chain. In polynucleotides, a polyester chain forms the backbone. The ester is derived from phosphoric acid and the alcohol portion of a sugar. A heterocyclic base is attached to the first carbon of each sugar. The sugar–base combination is called a nucleoside, and the base–sugar–phosphoric acid combination is called a nucleotide. The general structure of a polynucleotide is made up of these repeating units:



The two most well–known polynucleotides are RNA and DNA. In RNA, the sugar is D–ribose, while in DNA the sugar is D–2–deoxyribose (Figure 5.1a). The bases found in RNA are adenine, guanine, cytosine, and uricil, while those in DNA are adenine, guanine, cytosine, thymine and 5–methylcytosine (Figure 5.1b). Polynucleotides differ from one another in the relative proportion of these bases and their sequence of occurrence on the backbone. RNA consists of a single polymeric strand, while DNA exists as a double strand in the well–known "double helix" structure, stabilized by hydrogen bonding.

Polynucleotides are similar to proteins in that they have characteristic ionization behavior. When polynucleotides are dissolved in aqueous solution, they will always form the anion. The single stranded polynucleotides are soluble only in neutral or basic solutions, and therefore the phosphate groups are never completely protonated. The double stranded polynucleotides shield the potentially ionizable base molecules by the steric (space–geometry) hindrance and the involvement in hydrogen bonding. DNA molecules are also only soluble in basic solution, and exist in aqueous solution as the negatively charged specie.

5.4 ELECTROPHORETIC SEPARATION

Electrophoresis is a technique which uses an electric field applied to a solvent matrix or medium to cause the separation of charged colloidal particles by the mechanism of differential migration. The method was first introduced by the Swedish chemist Arne Tiselius in the doctoral thesis he presented in

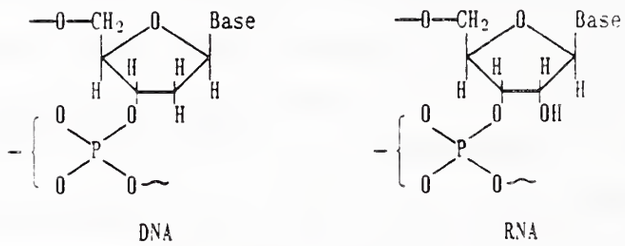


Figure 1a

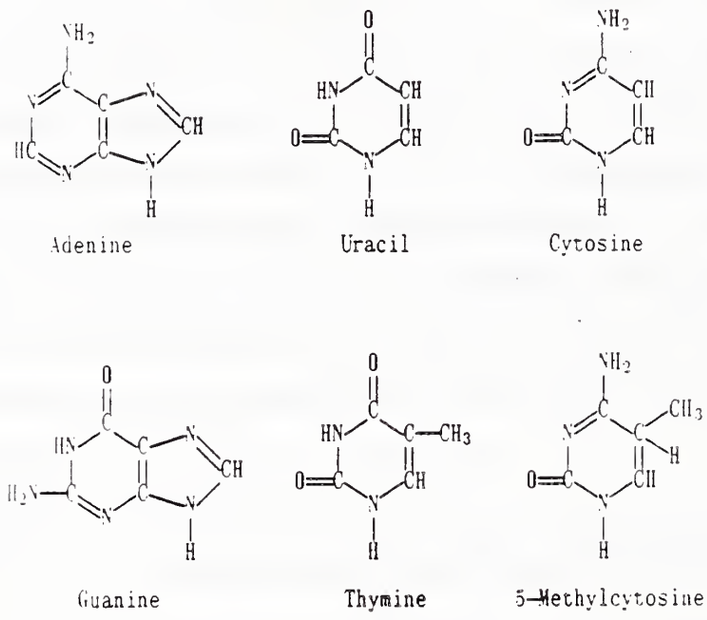


Figure 1b

Figure 5.1 The chemical structures of DNA and RNA (a) and the chemical structures of the nucleotide bases (b).

1930. This work brought him a Nobel Prize in 1948. Although the method has been extensively used and studied for over fifty years, a detailed and quantitative theory remains somewhat elusive. A highly simplified view of the process is shown in Figure 5.2:

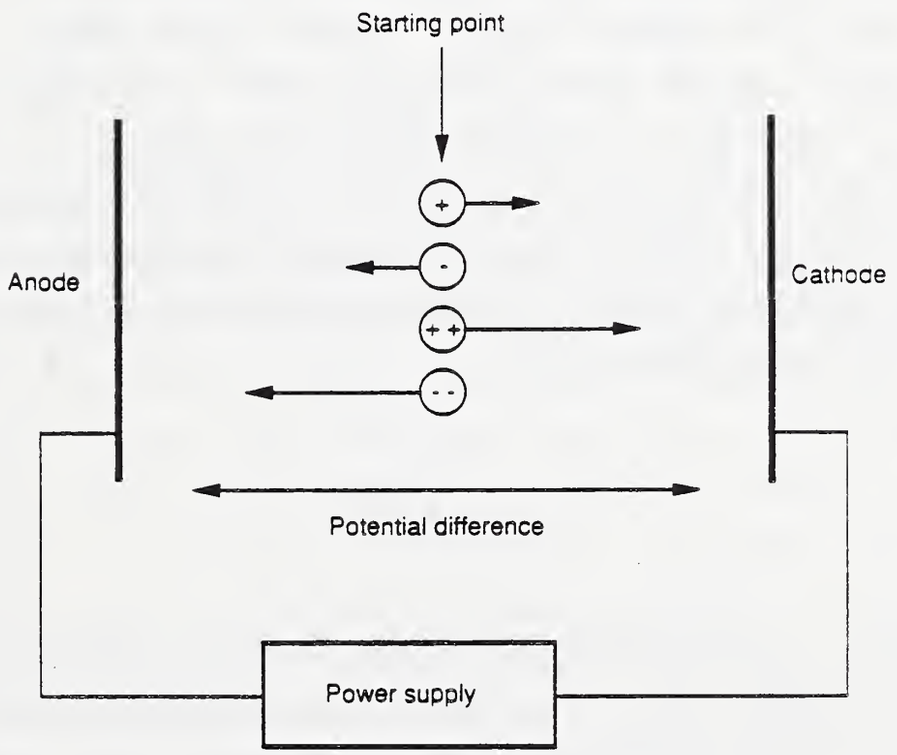


Figure 5.2 A schematic diagram depicting the electrophoretic process.

The length of the arrows on the ions indicate the relative effect of the field on singly and multiply charged ions. The solvent matrix, which is placed between an anode and cathode, is a very important component in the design of an electrophoretic separation, and will be discussed in much greater detail later on. For the present, we will simply assume that it is an electrically conductive material which will dissolve the samples without a significant danger of precipitation. If the sample is applied to the center of the solvent matrix, and the current is turned on, the migration of cations to the anode and anions to the cathode will be observed.

The force of the applied electric field will accelerate the charged molecules until it is balanced by the opposing force of viscous drag produced by the molecules of the matrix or the solution. As you might expect, the effect of the applied field will be greater on a multiply–charged ion than on one which carries a charge of only plus or minus 1. The ultimate velocity at which the molecule then moves toward the electrode is called the "migration velocity." The migration velocity is proportional to the applied electric field:

$$v = uE$$

where v is the migration velocity, E is the strength of the electric field, and u is a constant of proportionality called the electrophoretic mobility (which, by definition, takes the same sign as that of the ion). If many simplifying assumptions and

approximations are made, the viscous drag force of a particle in a medium can be described in terms of Stokes's law:

$$F = 6 \pi r \eta v$$

where r is the radius of the particle (which is assumed to be spherical), and η is the viscosity of the medium. Thus, the magnitude of the viscous drag force is proportional to the viscosity of the medium and the size of the molecule. Conversely, the migration velocity is inversely proportional to the viscosity and molecular size.

We have made many assumptions in the above discussion to simplify the rather complex process involved in electrophoresis. For example, we have assumed that the particles which make up the sample are spherically shaped molecules. In reality, this is never the case, although many molecules such as globular proteins have an approximately spherical shape. The drag forces will increase as the molecular shape becomes less spherical. We have also assumed that the medium exerts no effect other than viscous drag. This is clearly not the case, since the medium is a conductive, ionized system capable of participating in electrostatic interactions with the sample. As the sample moves through the medium, its ions will become surrounded by a shield of solvent ions of opposite sign. This shield will decrease the effect of the applied field, and the sample ion will move through the medium more slowly than we might otherwise expect.

With these considerations in mind, we can summarize the factors which influence the movement of ions in the electrophoretic system. The migration velocity (and therefore, the speed of analysis) will be directly proportional to applied field strength and the net charge on the migrating molecule. The migration velocity will be inversely proportional to the viscosity of the medium, the size of the migrating molecules, and the extent of departure from sphericity of the migrating molecules.

The temperature of the electrophoretic system is also an important consideration, since the viscosity of the medium and buffer solution pH are temperature dependent, as well as the current and voltage across the medium. It is therefore advisable to minimize changes in temperature variations caused by the "Joule heating" by the applied field during an analysis. Other undesirable effects due to heating are sample band distortion and diffusion, buffer solution evaporation, and the appearance of convection currents in the medium. The heat dissipation on the surface of the medium will clearly be different from that in the interior, and some temperature nonuniformity will always be present. Since a higher field strength produces a faster separation, a compromise is needed to obtain an optimally designed experiment. It is generally best to use as high a field strength as possible, but not so high as to incur serious consequences from poor heat dissipation. A high field strength is particularly important in the separation of smaller molecules, since separation may not occur at all at lower levels. The use of a constant current or constant voltage power supply is standard

in modern electrophoresis, and this helps to reduce temperature fluctuations.

Since both the sample species and the buffer solution are ionic, the possibility of undesired interactions is always present, as we mentioned earlier. This interaction usually produces a sample ion which is surrounded by a cage of ions of opposite charge. The larger effective size of sample will lower its electrophoretic mobility. It is therefore desirable to use solution ionic strengths and buffer concentrations which are as low as possible. This will minimize the number of cage–forming counterions. This use of low ionic strength solutions must be considered in the light of sample solubility, however, as we discussed earlier.

5.5 INSTRUMENTATION

The original electrophoretic technique developed by Tiselius was called the moving boundary method, which was used extensively in protein research until about 1950. This was a liquid solution method, in which proteins were differentially concentrated in glass columns below the anode and cathode. Separation was observed by bands of "schlieren," wavy patterns in the solution produced by differences in refractive index of the compounds separated. This technique is rarely used today because it suffers from extremely low band resolution.

The most important and widely used electrophoretic technique used today is the zone method, in which the separated

components are distributed into discreet "zones" in a stabilizing medium. The media, which we will discuss in detail later on, can be a high grade filter paper, a cellulose acetate film, or a gel made of starch, polyacrylamide, or agarose. The process of zone electrophoresis is shown schematically in Figure 5.3, in which sample is applied to the top of the medium, and separation into zones occurs as the current is allowed to flow for up to several hours. Note that on this figure, the zones are all moving toward the anode. This does not always happen, of course, since the migration direction is dependent upon the sample ionization behavior.

A variation of zone electrophoresis is the steady state technique called isoelectric focusing. Recall in our earlier discussion of amino acids, we discussed the formation of the dipolar ion, in which the amine group was protonated, and the carboxyl group is ionized. This dipolar ion is stable only at a specific pH called the isoelectric point. In isoelectric focusing, a stable pH gradient is set up in the medium, such that as one moves along the medium, the pH will either increase or decrease. If a polypeptide or protein is applied to such a medium, and the current is applied, the zones will move along the gradient until the pH corresponding to the isoelectric point of the ion is reached. At this point the zone will move no further. As we noted earlier, a polypeptide or protein may not be very soluble at the isoelectric point, and may even precipitate in the medium, which can sometimes be a major disadvantage. Nonetheless, the method has a high resolving power, and can even be used on the preparative scale.

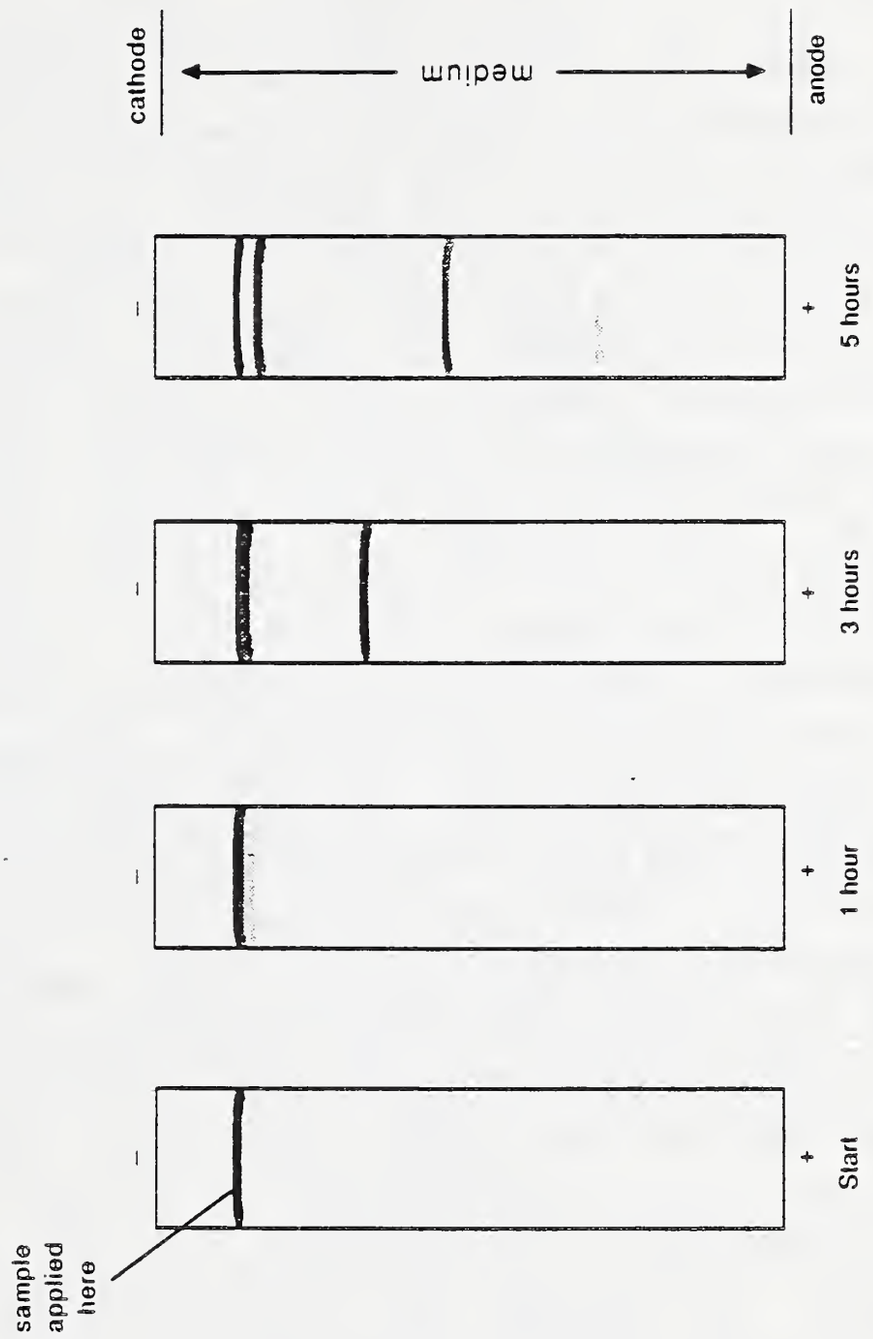


Figure 5.3 A diagram illustrating the progress of a separation by zone electrophoresis.

5.6 SUPPORT MEDIA

Filter paper (high quality, 96 percent alpha–cellulose) was the first medium used for zone electrophoresis. It was introduced for this application in the early 1950's, and was popular because it allowed simple and low cost separations to be performed relatively quickly. The paper is saturated with a buffer solution, and then bent over a plastic holder with its ends dipping into buffer solution compartments, one containing the anode and the other the cathode. A bell jar or similar cover is then placed over the system to exclude dust and minimize evaporation. Since the passage of current will result in the production of heat, the apparatus is usually located in a refrigerator or "cold room." A relatively low field strength of about 20 V/cm is used with paper, which is sufficient for larger polypeptides and proteins. The use of paper as the medium is relatively rare now because of some important disadvantages. Thin paper produces sharper bands, but is more prone to tearing and must be handled with extreme care. Thick papers have been used mainly for preparative scale separations, but the resolution is somewhat lower. The chemical structure of the paper itself can also pose a problem, since proteins will interact with the free hydroxyl groups which are always present on its surface. This is analogous with the problems associated with the free silanol groups on the surface of HPLC packings, and the result here is the same. The interaction will cause peak broadening and tailing, ultimately leading to a significant loss in resolution. Another serious problem is the electro–osmotic effect, caused by the tendency of the molecules on the paper surface to take on a

negative charge in the presence of water. It is probable that the small number of carboxylic acid groups on the cellulose structure ionize, forming hydronium ions, H_3O^+. The hydronium ions are very mobile and move toward the cathode under the influence of the applied electric field, thus disturbing the transport of the protein or polypeptide ions of interest.

Cellulose acetate, introduced in the late 1950's, was a significant improvement over cellulose filter paper. The hydroxyl groups of cellulose are treated to form the ester, and are thus unavailable to interact in an unfavorable way with the sample molecules. Cellulose acetate dissolves in many solvents, which makes sample recovery easier than with filter paper. The medium is used in the form of a thin membrane, and the apparatus and methods employed are similar to those involving filter paper. Unfortunately, cellulose acetate will also show an electro–osmotic effect due to the ionization of the carboxyl groups, however some commercially available membranes show only a very small effect due to methylation of the anionic groups. Cellulose acetate is still used as a medium in many clinical procedures, especially those involving small samples. The analysis times are short, with separation occurring in one or two hours, thus partially offsetting the relatively higher cost of the material (as compared to paper).

Most of the modern research–level electrophoresis is done with gel media. A gel is a two–component system (consisting of a disperse phase and a continuous phase) with a semi–solid structure, having some properties similar to colloidal systems. This semi–solid form of the system may be distinguished from its semi–liquid form, which is called a sol. The gels used in

electrophoresis are molded into flat slabs or poured into columns made from glass or plastic tubing. The continuous phase is a three dimensional cross–linked polymer having an essentially random bond repetition pattern, but producing a constant "pore" size. The pore size, which can be controlled within certain limits, can provide a sieving effect which is conceptually similar to the action of molecular sieves in GSC. For a given net ionic charge, smaller molecules will be transported through the gel faster than larger ones. The pore size is controlled by varying the relative amount of polymer in making up the gel; adding additional continuous phase polymer will decrease the average pore size. In preparing polyacrylamide gel slabs, the pore size is controlled by the quantity of cross–linking agent. The disperse phase in an electrophoresis gel is a conductive buffer solution which will dissolve the samples under study.

The principal types of gels used in electrophoresis are made from starch, polyacrylamide, agarose, and a polyacryl–amide—agarose combination. The earliest gel work was done using hydrolyzed potato starch. Although most of the work today is done using other gel media, starch gels are still used in many laboratories because of their low cost, speed and relative ease of preparation and operation. It is still one of the best choices for the analysis of isoenzymes (separate species of protein having the same catalytic activity). The gel is prepared by heating the starch in a solution of the buffer until the resulting colloidal suspension becomes transparent. The mixture is then poured into a mold, in which the gel forms. The pore size of a starch gel is variable only over a very narrow range, by slightly changing the starch concentration. Too much starch

will produce a gel which is too stiff to be poured and molded, while too little will give an excessively soft, "runny" gel. Another disadvantage of starch is due to its chemical structure, which contains negatively charged side chains. These can interact with protein molecules to hinder migration and also cause an electro–osmotic effect similar to that observed with paper and cellulose acetate.

Polyacrylamide gels are the most important and widely used media in electrophoresis. They are prepared by cross–linking acrylamide (in an approximate concentration of 15 percent mass) with a co–monomer, N,N′–methylenebisacryl–amide. Only the highest purity monomers can be used, since the cross–linking reaction is very sensitive to the presence of impurities. Other copolymers can be used to provide special solubility properties. For example, gels can be prepared using N,N′–bis–acrylylcystamine, producing a gel which can be easily dissolved under specific conditions, allowing the sample to be recovered for further analysis. In addition to the co–monomers, an initiator and a catalyst are required. The common chemical initiators are ammonium persulfate and potassium persulfate, although light will initiate the reaction in the presence of riboflavin. The most common catalyst is N,N,N′,N′–tetra–methylethylenediamine (TEMED). The pore size of a polyacrylamide gel is controlled by the ratio of this reagent with the acrylamide monomer. The reaction scheme is shown in Figure 5.4

The gel is either poured as a flat slab (typically 10 cm square) or poured into glass or plastic tubes (7 to 10 cm long, 5 mm in diameter). A schematic diagram of the slab apparatus

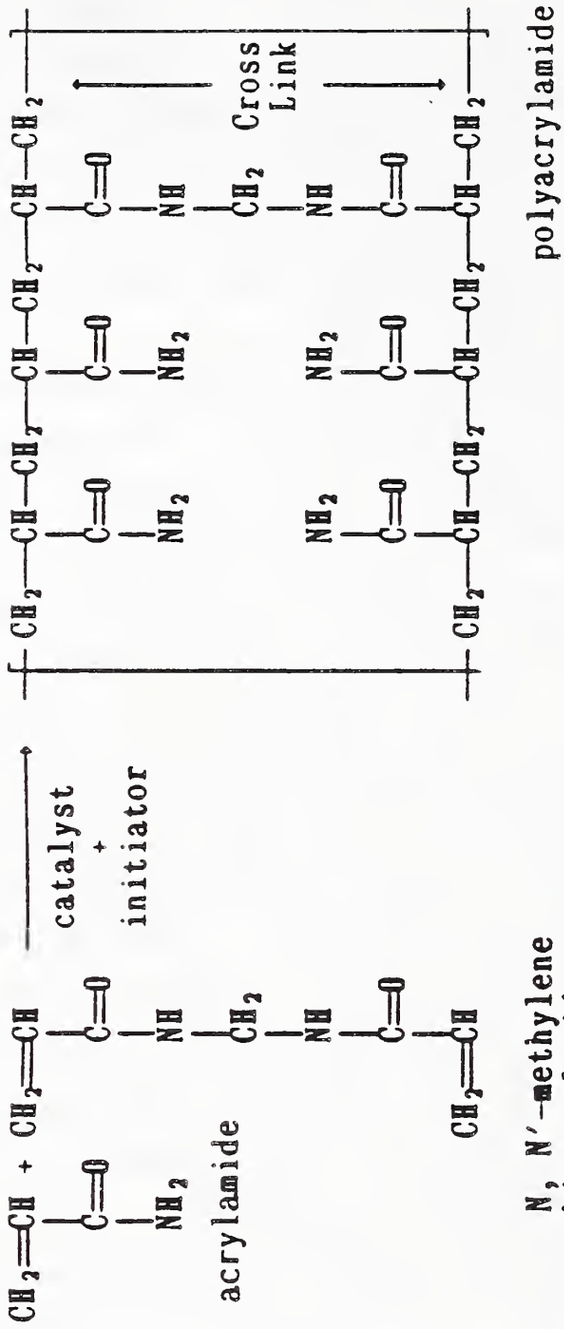


Figure 5.4 Reaction to form polyacrylamide gel medium.

is shown in Figure 5.5. In the case of the slab configuration, a slot former (usually made from Teflon) is incorporated into the gel to form sample cavities, which are then loaded with sample in a buffer solution, usually containing sucrose or glycerol to control density. It is possible to analyze many samples (along with several known standards) under identical conditions using slab gels, and the efficient heat dissipation of the thin medium allows the use of high field strengths. The resolving power is somewhat better than tube gels. The slab gel is more expensive (since it requires more gel) and difficult to prepare than the tube gel, however it has found a great deal of utility in RNA and DNA separations.

Agarose gels are formed from a linear polymer of D–galactose and 3,6–anhydrogalactose, with the structure being maintained by hydrogen bonds. The average pore size, which is controlled by the agarose concentration, is larger than either starch or polyacrylamide, making them suitable for the analysis of larger protein species (1.5×10^8 g/mol). The polymer is soluble in boiling water, with the gel formation occurring upon cooling to about 38° C. The apparatus needed for using agarose gels is the same as for polyacrylamide and starch gels. The problem of heat dissipation is more critical using agarose, since the viscosity shows a strong temperature dependence. The main historical disadvantage with agarose gels, however, is the presence of a small number of ionized sulfate and carboxyl groups. This leads to interaction with sample molecules and the presence of electro–osmotic effects. Hydrolysis with an alkali can minimize these difficulties.

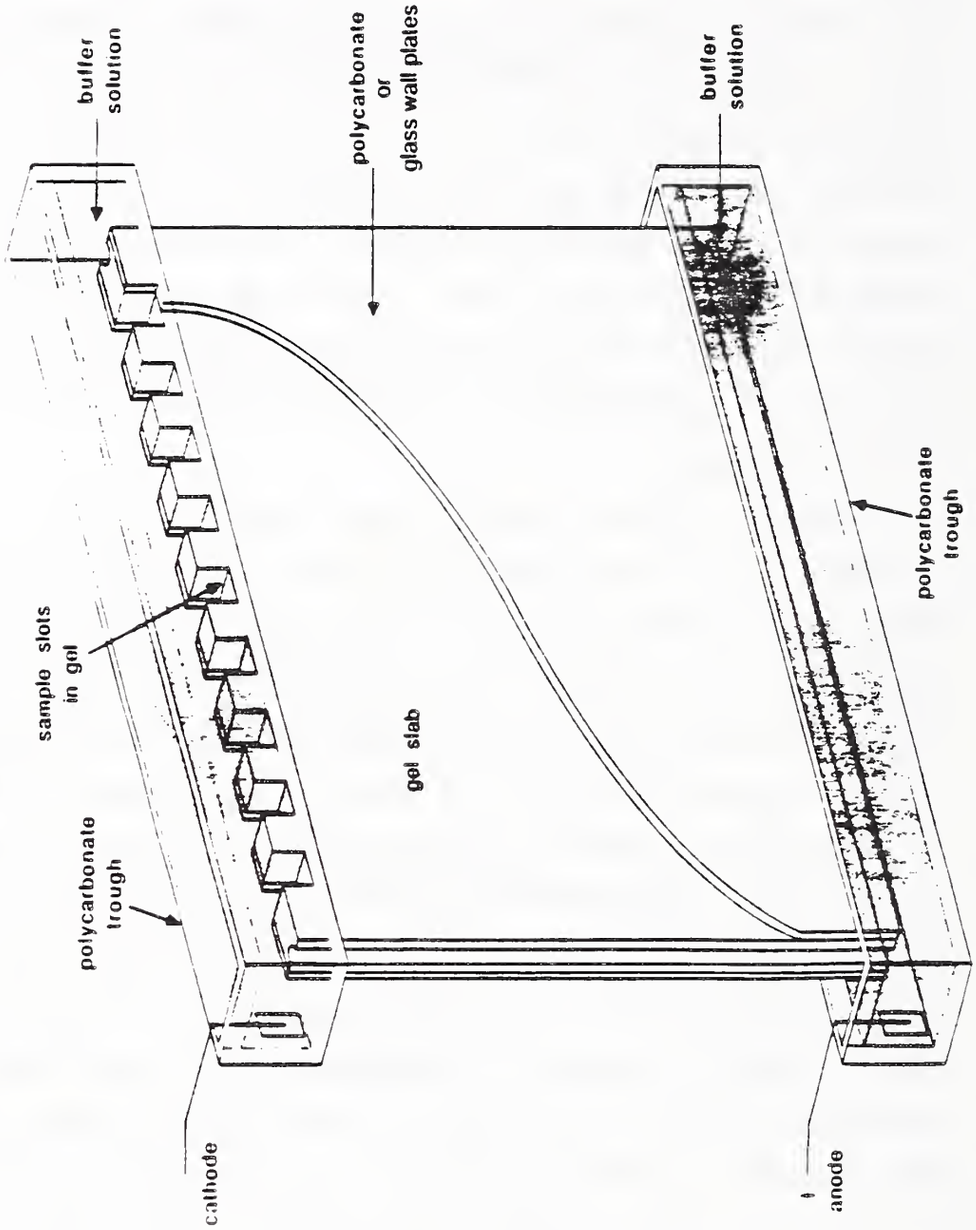


Figure 5.5 An apparatus used for gel slab electrophoresis.

The use of a polyacrylamide–agarose hybrid gel can produce a gel of reasonable mechanical strength, yet with a pore size which is larger than that in polyacrylamide. These mixtures have been useful in nucleic acid work, and in separations of very large proteins.

5.7 DETECTION

Once an electrophoretic separation has been completed, the separated bands must be rendered visible. This will allow a determination of sample purity (by observation of the number of separated zones), qualitative analysis (possibly from a comparison with standards), and recovery of the individual bands for further analysis or study. The usual methods of detection are zone staining, ultraviolet absorption, radiochemical analysis, and biological assay.

5.7.1 Staining

The most widely used method of detection is zone staining, in which the sample is reacted with a chemical which renders the zone opaque (to ultraviolet or visible light) or fluorescent. Stains which render a zone opaque in visible light are termed chromogenic, since they generate a color in the band. Staining reagents can be applied either before electrophoresis or after completion. If the stain is applied before, the zone separation can be observed as it progresses. It is usual to "fix" the developed zones after electrophoresis, in order to stop further

zone diffusion. This is done by drying (the paper or cellulose acetate media) or precipitating the sample (in gel media) using trichloroethanoic acid or ethanoic acid. Once the electrophoretogram is fixed, excess staining reagent is removed by either soaking in a destaining solution, or by electrophoresis at a right angle to the original electrophoretogram. Some of the more common chromogenic stains are ninhydrin, Coomassie brilliant blue, and Ponceau red. Some stains provide quantitative information from the intensity of the colored band. An example is acridene orange, which is used for polynucleotides.

Stains which produce a fluorescent product are usually applied before electrophoresis. The electrophoretogram is scanned using an ultraviolet lamp of appropriate wavelength. The most common fluorescent stain is dansyl chloride, which is also commonly used as a derivatizing reagent in HPLC. Acridene orange, which is used for the quantitation of polynucleotides, can also be used as a fluorescent stain.

5.7.2 Ultraviolet Absorption

It may be possible to detect the separated zones on gels from their absorption of ultraviolet light, without the use of staining compounds. Most proteins have chromophoric groups (the carboxylic carbonyl, for example) which show absorption maxima between 230 and 280 nm using a deuterium lamp as the excitation source. Scanning with uv radiation using a recording densitometer provides a quantitative record of the zone profile along the gel. Polyacrylamide gels present problems because of the presence of unreacted monomer in the gel which will also

absorb in the uv region. This will cause a high background level. Agarose gels are usually dried to produce a thin film in order to decrease light scattering.

5.7.3 Radiochemical Methods

Radiochemical detection methods are based upon prelabeling the sample with a radiotracer, the level of which is measured by liquid scintillation counting or autoradiography. The most common tracer isotopes are ^{125}I (a gamma emitter), ^{32}P (a high energy beta–emitter), ^{14}C, ^{35}S (both medium energy beta emitters) and ^{3}H (a low energy beta emitter). These tracers can be incorporated into the sample somewhere in the preparation sequence, especially using alkylation. The addition of radioactive labels will very rarely change the biological activity of the material, and will not change the ionic charge.

To use liquid scintillation counting, the gel or paper is dried and the zones are cut apart using a scalpel or special sectioning instrument. Samples separated on paper or cellulose acetate can be dissolved directly in a suitable scintillation mixture, but the samples on gels must be solubilized. Some samples can be removed using a buffer solution, while others require an organic reagent such as hyamine hydroxide or piperidine. The gel structure can be disrupted using ultrasound in a suitable liquid (formamide for agarose, 30 percent hydrogen peroxide for polyacrylamide). Alternatively, pre–solubilized gels (as we discussed earlier) may be used.

Although a detailed discussion of scintillation counters is beyond the scope of our treatment of electrophoresis, a brief

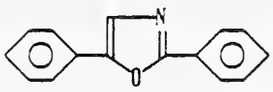
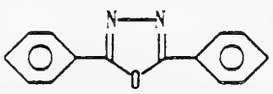
overview is in order. The radioactive label atoms listed above will decay and in the process release beta or gamma particles, which have the potential of electronically exciting absorber molecules. When certain absorber molecules "de–excite," they release a photon which can be detected and amplified using a photomultiplier tube.

Because most of the radiotracers used in electrophoresis have low penetration, the sample is dissolved directly into a solution (called a cocktail) containing absorber molecules in an appropriate solvent. The solvent is usually an aromatic hydrocarbon such as toluene or xylene, since these molecules are readily excited by the radiation, and transfer this energy to the scintillator. The scintillator is an organic molecule which accepts the energy from the solvent, and re–emits it as a photon in the 300 to 400 nm range. This is the photon that is detected and amplified by the counter. Sometimes a secondary scintillator is added to the cocktail, mainly for very high sensitivity work. The secondary scintillator absorbs the photon emitted from the (primary) scintillator, and re–emits a photon at a longer wavelength, near 400 to 500 nm. This is advantageous since the photomultiplier tube is more sensitive in this region. Some typical scintillators are shown in Table 5.1.

The samples are dissolved on an appropriate cocktail and placed in special sealed glass vials. An automatic conveyor system will bring the samples above the counter, where they will be held for a predetermined integration time. Quantitative data can then be obtained by comparison with suitable calibration standards.

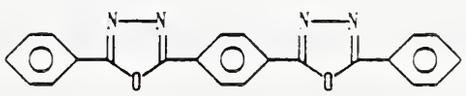
Table I

Scintillator Compounds:

Compound	Structure	Relative Scintillation Efficiency*
p-terphenyl		94
1.4-diphenyloxazole (PPO)		100
1.4-diphenyloxadiazole (POD)		87

*relative to PPO

secondary scintillator:

1.4-di-2-(5-Phenyloxazole) (POPOP)	

Autoradiography is a simple technique especially applicable to soft, large pore agarose gels which cannot easily be sliced for scintillation counting. The gel slab is first dried under vacuum, and placed in a cassette containing a sheet of fine–grained x–ray film, which is backed by a fluorescent intensifying screen. The cassette is then stored at low temperature (below 0° C) for a few days. The tracer species cause the appearance of dark bands on the film corresponding to the labeled zones on the slab.

Biological assay methods are more complex and often much more time consuming than other detection methods we have discussed, but have nonetheless been of great value. The technique is based on observing the biological or biochemical reactions of the separated materials after electrophoresis. The analyses are very sensitive, and detection is selective for specific proteins. The biochemical activity observation is usually enzymatic action (if the species is catalytic) or antigenic (or immunological) action. Usually the gel slab is cut into sections and the activity of each section is recorded. When the enzymatic or antigenic action will produce a visible change (such as a precipitation or color change), the reaction can be done *in situ*, directly on the intact slab. One can also cut a trough parallel to the lane in which the sample migration has occurred. The antibody or antisera is then placed in this trough.

SUGGESTED READING

Introductory Treatments

M. Melvin, Electrophoresis, John Wiley and Sons (on behalf of ACOL), Chichester, 1987.

R. Stock and B. F. Rice, Chromatographic Methods, 3rd Ed., Chapter 3, Chapman and Hall Science Paperbacks, London, 1978.

E. Heftmann, Ed., Chromatography: A Laboratory Handbook of Chromatographic and Electrophoretic Methods, Van Nostrand Reinhold, New York, 1975.

Detailed Treatments

A. T. Andrews, Electrophoresis: Theory, Techniques, and Biochemical and Clinical Applications, Clarendon Press, Oxford, 1986.

Basic Biochemistry

M. Yudkin and R. Offord, Biochemistry, Houghton Mifflin Co., Atlanta, 1975.

L. Stryer, Biochemistry, 2nd. Ed., W. H. Freeman Co., San Francisco, 1975.

M. Malm, Fundamentals of Organic and Biochemistry, Van Nostrand, New York, 1980.

APPENDIX I

CALIBRATION

I.1 INTRODUCTION

In the preceding chapters, we have discussed some of the most important and valuable instrumental techniques available to perform chemical analysis. These techniques can provide both qualitative and quantitative information. Qualitative information includes, for example, the number of components in a natural gas mixture from a wellhead or the identity of the proteins present in an industrial fermentation broth prior to extraction. To obtain quantitative information (for example, how much, what concentration, what level of purity?), one must usually use some sort of standardization or calibration on the instrumental response. This is in contrast to absolute methods, which do not depend directly on the comparison of instrumental response of a sample with that of a standard.

Chemical analysis always seeks to answer some question or series of questions. We referred to such questions above with our hypothetical natural gas mixture and fermentation broth. The personnel who require the "answers" from a chemical analysis are typically plant engineers or managers who may have little or no background in analytical chemistry. It can sometimes be difficult for the analyst to communicate the difference between "quant" and "qual." For example, upon providing a plant manager with a qualitative statement such as "the trouble with extraction 51 is an excess alcohol content" the next question will invariably be, "well, how much excess is there?" The difference between these two levels of information lies in calibration. Calibration can be a simple process, or it can be a long, involved time–consuming task requiring the utmost skill on the part of the chemists or technicians. It is therefore important to thoroughly understand the questions which have been posed for chemical analysis to answer.

I.2 ABSOLUTE VERSUS COMPARATIVE METHODS

There are very few analytical methods which can be considered truly absolute. Even the methods which are usually thought of as being absolute (such as gravimetry, gasometry, coulometry, and titrimetry) use instrumentation which is "standardized" in some way. In the United States, the National Institute of Standards and Technology (NIST, formerly the National Bureau of Standards) under the Department of Commerce is legally charged with the responsibility of standardizing basic weights

and measures. For example, even the most precise and sensitive analytical balances built for use in gravimetry must be standardized using weights traceable to a primary standard weight set available for reference at NIST.

Analytical methods which are not absolute are called comparative methods. These methods require calibration of the instrumental response against the response of known standards under identical experimental conditions. It cannot be emphasized too strongly that without some type of calibration, no quantitative information whatsoever can be obtained from comparative methods. The standards which are employed should be prepared to match the character of the sample as closely as possible. This is done to minimize possible interfering effects of other substances. This process is called matrix matching, and should be practiced as far as is practical with all the calibration methods we discuss. Of course, exact matching of the standard and sample matrix may often be impossible, especially in the case of very complex sample mixtures. In these cases approximate matrix matching may be adequate. We must remember that practicality, expedience and economics also play a role in "real world" analytical problems.

The methods used for calibration range from simple, fast comparisons to detailed and costly designed studies. In keeping with the overall philosophy of this volume, the simplest, fastest, safest and most economical approach that will provide the desired information at the required level of accuracy is the approach which is advocated. Our discussion will begin with the simplest methods, and graduate to the more involved procedures. Treatment of the most sophisticated research–

oriented methods, which often require complex nonlinear analysis software, are beyond the scope of both this book and most of the problems encountered in chemical analysis.

All quantitative information obtained using chemical instrumentation is based on the observation of a change in response of some detection device with an increasing or decreasing concentration of sample. This change in response may be an increase in absorbance indicated by an ultraviolet detector in HPLC, or the decrease in current sensed by an electron capture detector in GC. Many instrumental responses are linear with sample concentration (Figure A1.1) even if only over a small range of concentrations. This linear relationship provides a level of predictability in the response of the detector. It is not absolutely necessary that the relationship be a straight line, but it is necessary that (1) a relationship actually exists, and (2) the relationship and its associated uncertainty must be confidently predictable.

I.3 SINGLE POINT CALIBRATION

The simplest (and least certain) form of instrument calibration is the single point method. While fast and easy, it can only be justified under a strictly defined set of conditions. In applying the method, we assume that the response is in fact a straight line, passing through the origin (Figure A1.2), and that there are no systematic errors in the method. We must also establish that the method gives a zero blank response, that is, the response is in fact zero when no analyte (the substance being

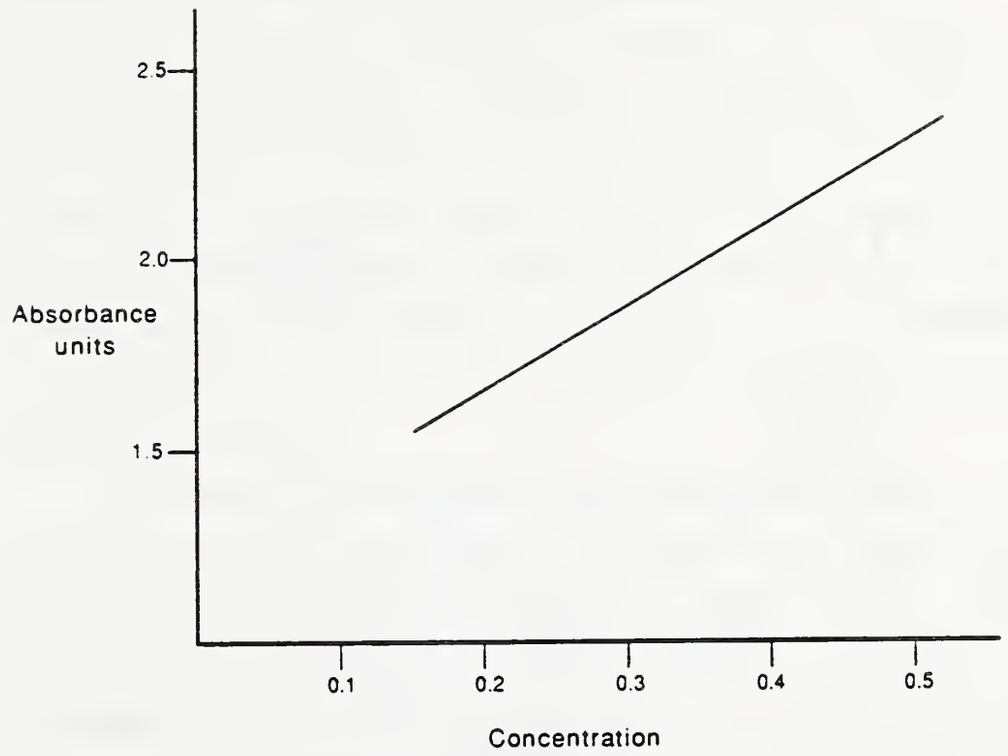


Figure A1.1 A plot showing a linear relationship between the measured ultraviolet absorbance and solute concentration.

analyzed for) is present. That these criteria are indeed met must have been established previously by a more extensive series of analyses. Only then can a single point calibration be used with any certainty for routine work. The measurement of a

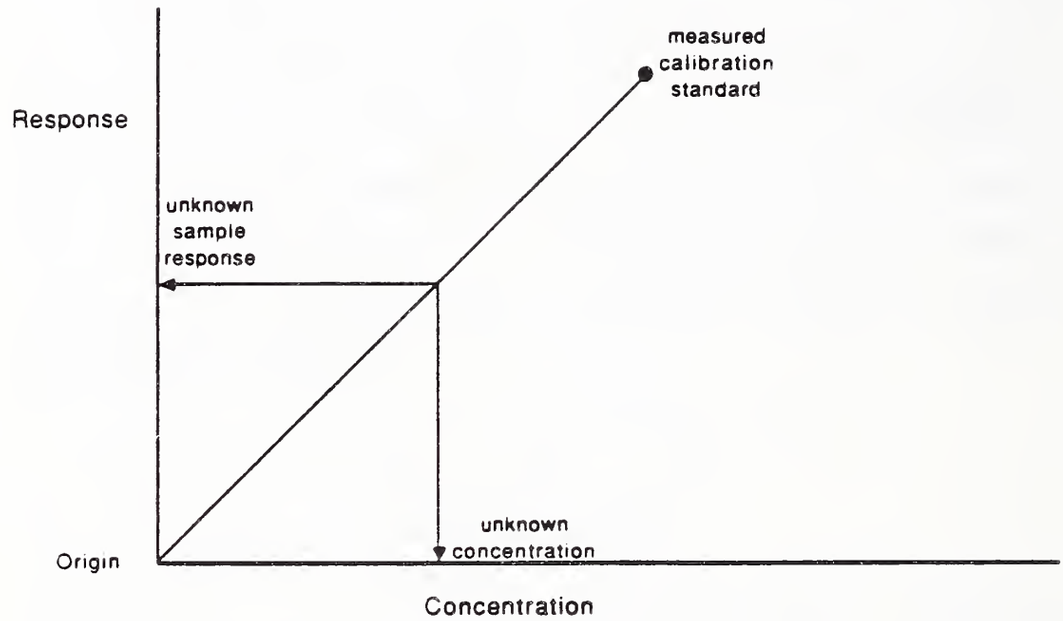


Figure A1.2 A plot illustrating a single point calibration.

standard sample of known concentration then allows the calculation of the unknown concentration by a simple ratio:

$$C_s\,(R_u/R_s) = C_u \qquad \text{(A1.1)}$$

where R_u and R_s are the responses of the unknown and standard, respectively, and C_s and C_u are their associated concentrations. The concentration of the standard is, of course, chosen to be higher than that of the unknown. This serves to bracket the unknown between a known concentration and a previously established zero point response at the origin.

This calibration method is subject to a number of important disadvantages. Mistakes in procedure cannot be detected if only one standard is used, since one must rely on the "known" value as being correct. There is no way to average or smooth the indeterminate or random instrumental error which is always present in any method. In addition, it may be difficult to know of or correct for the presence of interfering chemical species in the sample.

The single point calibration can be improved somewhat by always running a blank solution. This will allow the analyst to correct for the possible presence of impurities, or slight differences in analytical conditions between the time the standard and the unknown are run. Naturally, when an analytical method is known to produce a finite, nonzero blank value, this measurement will be absolutely necessary. The value of the response to the blank is then subtracted from that of the standard and the unknown.

I.4 TWO POINT CALIBRATION

The two point (plus a blank) method offers a refinement which does not significantly add to the complexity or cost of most

analyses. Two standard samples are measured, and the response of a blank solution is subtracted. The concentrations of the two standards are chosen to bracket the expected value of the unknown concentration. It is often useful to make the high concentration standard either near the limit of linearity of the method, or near the highest expected sample concentration. The lower concentration standard should be near the minimum detectable level of the sample using the particular method. This will provide the calibration with a wide operating range. Naturally, our assumption of a straight line behavior with concentration must have been previously established. The unknown concentration can then be determined graphically or by computer.

I.5 MULTI–POINT CALIBRATION CURVE

A logical extension of one and two point calibrations is the determination of a calibration curve using a set of known standards having concentrations varying over the range of interest for a particular analysis. While this is usually done by preparing a set of standard solutions of increasing concentrations, an *in situ* approach is also possible. The *in situ* method is performed by sequentially adding increasing amounts of the standard to a single solution, taking into account the change in solution volume that this process causes. A blank sample should always be run and included in the calibration curve. The blank reading should not, in general be subtracted

from the rest of the standards, since it is subject to the same instrumental errors.

Two examples of calibration plots are shown in Figure A1.3, one being a straight line and the other curved. Although both straight line and curved plots are usable, the linear plot is to be preferred. The main reason for this is that the curved plot requires more standards in order to adequately describe the response, especially in the region of curvature. Statistical analysis software packages which perform linear regression are readily available for use on nearly all personal computers. These programs are capable of fitting the standard plots to equations (often called mathematical models) which account for the pattern or structure of the response. While the most familiar functional form of the fitted equation is a straight line ($Y = mC + b$, where Y is the instrument response, C the concentration, m the slope of the line and b the intercept), other forms can be calculated. Higher order linear polynomial models (such as quadratic and cubic), exponential, logarithmic, and power models are easily calculated with standard software packages available commercially, usually at a modest cost.

An important advantage of the calibration curve modeled to an equation is the ability to generate statistical measures of error, tests of coefficient significance, residual magnitude and pattern, analysis of variation (anova) tables, and probability plots. While we will not treat the calculation or interpretation of these parameters in this discussion, they are very important since they allow the analyst to take full advantage of the available data. Mistake recognition and outlier identification

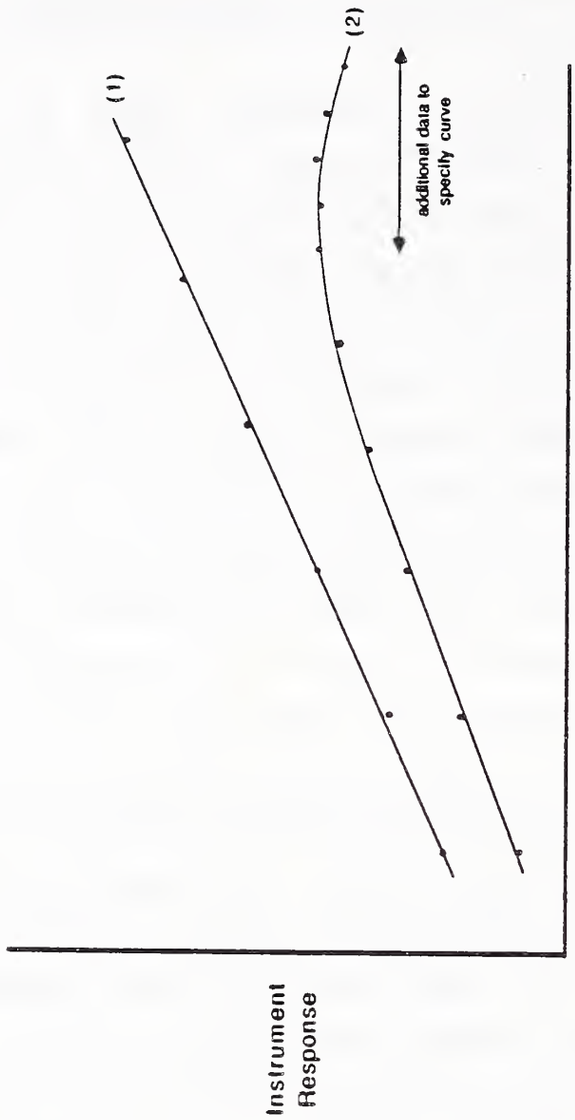


Figure A1.3 A plot showing a linear and a curved calibration curve. More standard measurements are required in the curved region in order to specify the curvature.

are made much easier using these statistics, a feature not shared with the simpler calibration methods discussed earlier. In addition, a much higher degree of precision can be obtained

using a calibration curve. The accuracy, as usual, will depend on the accuracy of the individual standards.

I.6 STANDARD ADDITION METHODS

We have discussed the effects of the sample matrix, and how matrix matching is desirable to prevent interferences from other chemical species present in the sample. When matrix matching is difficult or impossible, standard addition methods are especially useful. The method involves measuring the instrumental response of the sample solution, followed by the addition to this solution of a known quantity of the analyte. The change in the instrument response is related to the amount of the standard added.

The method is applicable only when a known straight line relationship has been established between the instrument response and the analyte concentration. If a determinate or systematic error is present in the measurement technique, it must be constant over the whole range of analyte concentration of interest. This means, in effect, that a single blank value must apply to all analyte concentrations, even though a blank sample may not be available. The sample cannot contain any chemicals which can be mistaken for the analyte, since this will be perceived as being built into the determinate error. The change in volume of the solution upon the addition of the standard must either be negligible or accounted for computationally.

While in some well defined and studied circumstances it may be possible to use a single standard addition, most of the

time it is far better to use multiple standards. Single standard addition suffers from some of the same disadvantages as single point methods which we discussed earlier. There is no built–in error check to guard against mistakes in technique, and no way to assess or average over the experimental errors. Using multiple standard addition, one can apply the mathematical modeling software described earlier. This will provide the analyst with a more reliable error analysis. The number of standards used will have to be decided, based upon consideration of two primary factors. The first factor is the extent of linearity of the measurement. It is undesirable to use measurements outside of this region. The second factor is the required precision of the results.

While the standard addition method is generally rapid and addresses the sample matrix problem, it suffers from some disadvantages. Often, larger quantities of sample may be required as compared to other methods. The most serious shortcoming from a conceptual point of view is that it is an extrapolation method, rather than in interpolation method (as in the case of the calibration curve earlier). This makes the method inherently less precise than using a calibration curve.

I.7 INTERNAL STANDARD METHODS

One of the most useful methods for the calibration of chromatographic analyses is the internal standard method. The method is especially valuable when it is not possible to exactly control the quantity of sample analyzed. An example would be

the GC analysis of a hydrocarbon mixture using syringe injection. Sample evaporation from the syringe can change component concentrations and ruin analyses. Another application would be when using instrumental methods having parameters which are difficult to control, such as the gas flow rates in some types of atomic spectroscopic techniques. In general, the precision of the internal standard method is very high as compared to the calibration curve methods we have discussed earlier.

To implement the method, a standard solution is prepared which contains not only a known quantity of the analyte, but also a known quantity of another compound, called the internal standard. Since this compound must be chosen carefully, we will discuss the important characteristics more fully later on. The internal standard compound is also added to the unknown solution containing the analyte. The instrument response of the standard solution is then measured, and the response factor, R_{12}, determined according to:

$$(S_1/S_2)\,(C_2/C_1) = R_{12} \qquad \text{(A1.2)}$$

where S_1 and S_2 are the instrument responses of the analyte and internal standard, respectively, and C_1 and C_2 are the corresponding concentrations. The instrument responses can be, for example, chromatographic peak area counts (as in chromatography) or absorbance units (as with an ultraviolet spectrophotometric detector). The next step is to measure the instrument response of the sample solution, which contains the

unknown analyte concentration and the known internal standard concentration. The unknown concentration is then determined from:

$$(S_1/S_2)\,(C_2/R_{12}) = C_1 \text{ (sample solution)} \qquad \text{(A1.3)}$$

where, as before, the subscript 1 refers to the analyte and 2 refers to the internal standard. It is possible to use a series of standards, all prepared with a different concentration of the analyte but the same concentration of the internal standard. One then plots a calibration curve of the response ratio, S_1/S_2, versus the analyte concentration C_1 for each solution. The resulting plot should be a straight line having the origin as its intercept and the response factor R_{12} as its slope. The unknown solution is prepared in the same way as the standards, having the known quantity of internal standard added. The concentration can then be determined graphically by interpolation of the S_1/S_2 ratio, or by using an equation fitted to the curve.

As mentioned earlier, the choice of the internal standard is very important. The standard should, of course, not already be present in the sample. It should be similar to the analyte in terms of physical properties such as vapor pressure, polarity, density, and viscosity. Close attention must be paid to any physical property which may affect the detector response. The sample and standard should give a similar magnitude of response to the detector being used, without causing interference with the other's signal. For example, in GC work the two compounds

should produce baseline resolved peaks having integrator area counts within 20 or 30 percent of each other. The standard substance must be miscible with the sample solution, and must not cause a chemical reaction. The standard and the analyte should be easily separable using standard methods such as chromatography, or should give spectroscopic signals which are easily resolvable. The concentration of the internal standard should be close to that of the analyte, to minimize error in the response factor. While it may not always be possible to design a calibration to fit all of these criteria, one can usually obtain a nearly optimum measurement by using an internal standard which is a homologue of the analyte.

The high level of precision of the method is the result of making both standard and analyte measurements on the same solution. In addition, the measurements are done either at the same time or at times very close to one another. The advantage of high precision that this method provides is offset somewhat by the rigorous, time–consuming work required.

I.8 STANDARD REFERENCE MATERIALS

Throughout our discussion of calibration, we have made many references to the process of comparing the instrumental response of an analyte of unknown concentration with the response of a standard under identical conditions. This standard is of a known concentration, and is very often prepared in the laboratory for the particular analysis at hand. There are many cases, however, in which the preparation of a standard

"in–house" is not adequate or desirable. A few examples might be analyses for regulatory agency submission, analyses which will figure in legal proceedings, and analyses used in the generation of archival data compilations. In these instances, materials of reference fill a vital role in chemical analysis.

The National Institute of Standards and Technology (NIST) is the primary source of materials of reference in the United States. There are actually three types of available materials: reference materials (RM's), certified reference materials (CRM's), and standard reference materials (SRM's). A reference material is a "material or substance one or more properties of which are sufficiently well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials." A certified reference material is a "reference material one or more of whose property values are certified by a technically valid procedure accompanied by or traceable to a certificate or other documentation which is issued by a certifying body." Standard reference materials are certified reference materials that are issued by NIST.

The materials of reference defined above are developed for three primary purposes. The continuing development of accurate methods of analysis (reference methods) is one vital goal. Another is the calibration of measurement systems used in the exchange of goods (custody transfer) and in quality assurance. The third important purpose is to assure the long term integrity of a wide range of measurement programs. The instrument response values of the NIST standard reference materials are obtained by one of three possible measurement

schemes. The first is the use of a previously validated reference method. The second is through the results of two or more independent, reliable measurement methods, and the third is through measurements done in a network of cooperating, competent and knowledgeable laboratories. In all cases, the measurements are obtained through statistically–designed experiments which are mathematically–planned in advance.

The application of an SRM can be most clearly illustrated by an example. A municipality may wish to set a standard level of several alcohols which may be added to gasoline for the purpose of decreasing carbon monoxide emissions from automobiles. The municipal petroleum inspector will sample the fuel from the supplier's tank, and submit the samples to the laboratory for analysis. Since legal or judicial action may hinge upon the results from such an analysis, it is vital to eliminate as much procedural uncertainty as possible. In such a case, the laboratory will probably perform the analysis according to a standard guideline (possibly one prescribed by the American Society for Testing and Materials), and calibrate the method using a standard alcohol in gasoline reference material obtained from NIST. The use of the standard reference material removes some of the uncertainty associated with the calibration process, since the standard is a uniformly and confidently accepted material. This is further reinforced by the fact that most other laboratories performing analyses of alcohol in gasoline will also use the same standard reference material. An additional benefit is that the SRM provides a baseline of calibration comparability among all of the laboratories engaged in this testing, regardless of location or affiliation.

SUGGESTED READING

Introductory Treatments

B. W. Woodget and D. Cooper, Samples and Standards, John Wiley and Sons (on behalf of ACOL), Chichester, 1987.

D. McCormick and A. Roach, Measurement, Statistics and Computation, John Wiley and Sons (on behalf of ACOL), Chichester, 1987.

J. R. Taylor, An Introduction to Error Analysis, University Science Books, Mill Valley, 1982.

Detailed Treatments

R. L. Anderson, Practical Statistics for Analytical Chemists, Van Nostrand Reinhold, New York, 1987.

J. C. Miller and J. N. Miller, Statistics for Analytical Chemists, John Wiley and Sons (Halsted Press), New York, 1988.

B. Ostle and R. W. Mensing, Statistics in Research, 3rd Ed., The Iowa State University Press, Ames, 1975.

Standard Reference Materials

The Role of Standard Reference Materials in Measurement Systems, National Bureau of Standards Monograph 148, 54 pp., January, 1975.

Standard Reference Material Catalog, 1988—89, National Bureau of Standards Special Publication 260, January, 1988.

GLOSSARY

Accuracy A measure of how close a measured quantity is to the actual or true value of that quantity. In general, the true value is unknown, and measurements are approximations of the true value. Systematic errors will decrease the accuracy of a measurement.

Adsorption Isotherm A graphical and mathematical description of the equilibrium distribution of a solute between two phases, one being a solid surface and the other being a gaseous head space above the solid. The most well known models are the Freundlich and Langmuir isotherms.

Aerogel The dispersion of a gas or relatively small solvent molecule in a solid or liquid structure. It may be considered the opposite of an aerosol. The styrene–divinyl benzene polymeric packings used in size exclusion chromatography columns are loosely called aerogels.

Aliquot A part of a whole which is representative of the whole, such as a sample of a material for analysis or a volume of a reagent used in performing a test.

Beer–Lambert Law The simple relationship between the concentration of a substance in solution which absorbs

electromagnetic radiation, and the extent of the absorption, expressed as:

$$A = \epsilon C \ell$$

where A is the observed absorbance of the solution, C is its concentration, ϵ is the molar extinction coefficient and ℓ is the measurement cell path length. The extinction coefficient is also referred to as the specific absorption and as the Bunsen coefficient. This relation is subject to many assumptions and conditions which, when satisfied, allow the determination of solute concentration from spectrophotometric measurements.

Buffer Solution A (usually) aqueous solution consisting of a weak acid and its salt (called the conjugate base) or a weak base with its salt (called the conjugate acid). The acid or base concentration will determine the pH of the buffer solution. Buffer solutions are useful because they resist changes in pH upon the addition of acid or base.

Certified Reference Material A reference material one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation which is issued by a certifying body.

Chemical Potential A thermodynamic quantity defined as the free energy per mole of a substance. It may be viewed as a thermodynamic driving force, since matter will spontaneously flow from a region of high chemical potential to one of low chemical potential.

Chirality The observation that a molecule is not superimposable upon its mirror image. Such a molecule is called chiral, while one which is superposable upon its mirror image is called achiral. The word is derived from the Greek "cheir," meaning hand, implying handedness.

Chromophore A molecular group or radical which absorbs energy in the ultraviolet or visible region of the electromagnetic spectrum. Common chromophoric groups are the carbonyl ($\rangle C{=}O$), alkeneic ($\rangle C{=}C\langle$) and aromatic groups. They are characterized by a delocalized system of electrons in π orbitals.

Colloid A two phase system consisting of a solvent and a dispersed, gross phase of relatively larger molecules (as compared with the solvent). Colloid dispersions show the expected bulk properties of a solution, but do not show colligative property behavior. Two types of colloidal dispersions are possible, gels and sols.

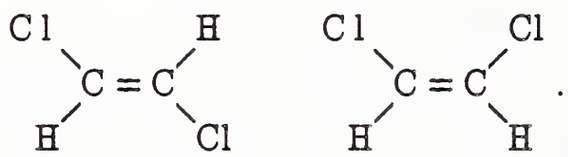
Cross–Linking The formation of bonds in a more or less regular fashion along the backbone of a polymeric molecule. Cross–linking decreases molecular freedom and produces a stronger, harder, and less soluble polymer.

Cryogen A cryogen or cryogenic fluid is usually defined as a liquid which has a boiling point below $-150°C$ at atmospheric pressure. Another (broader) definition considers any fluid having a critical temperature below room temperature to be a

cryogenic fluid. They are used extensively in the laboratory to produce low temperatures. The most commonly used cryogen is liquid nitrogen, which boils at 77 K at atmospheric pressure, and is relatively inexpensive.

Density A fundamental thermophysical property that describes the amount of a material which occupies a given volume at a given temperature and pressure (state). The density of a fluid is described in three dimension by the P–V–T surface, specific volume being the reciprocal of density.

Diastereomers A class of stereoisomers in which the molecules are not mirror images of one another. This includes cis/trans isomers. Diastereomers have different physical properties (such as vapor pressures and melting points), although the chemical properties (reactivity, etc.) will be similar. As an example, one can consider the following:



Diffusivity A transport property which describes the tendency of molecules of a given compound to disperse into molecules of another compound or itself. Kinetic theory and thermodynamics hold that if a gas mixture is of nonuniform concentration, diffusion will take place until the concentration is

uniform and no concentration gradients exist. The diffusivity is both temperature and density dependent.

Dipole Moment A property of a polar molecule, evaluated by multiplying the magnitude of the charges on the atoms (in electrostatic units) of the molecule by the distance separating the charges. A polar molecule is one in which the center of negative charge does not coincide with the center of positive charge.

Electronegativity All atoms which have fewer than eight electrons in their outer electron shell (principal quantum level) have low energy vacancies capable of accommodating an electron from outside the atom, as attracted by the nucleus of the atom. Electronegativity thus describes the relative tendency of the atom to become negatively charged.

Elution The gradual passage of the components of a sample mixture through a chromatographic column as the mixture is carried by the mobile phase through the stationary phase.

Enantiomers A class of stereoisomers in which molecules are not superposable upon their mirror images. They are sometimes called optical antipodes. Enantiomers have the potential of showing optical activity, in which the plane of polarized light can be rotated by passing through a solution of such a compound. Enantiomers have identical physical properties.

Entrainer A mobile phase modifier used for supercritical fluid chromatography or supercritical fluid extraction. This term is more popular in the engineering community.

Explosion–Proof (as in explosion–proof laboratory or instrument) This term refers to designs in accordance with Class A, Group b of the U.S. National Electrical Code. This subsection details the requirements of fixtures, cabinets, switches, and procedures which are designed to minimize the possibility of an explosion being caused by spark sources. It should be noted that the term is not a scientific term, but a specification of a body of code.

Fluorescence One of two principal types of chemical luminescence in which an electron in a ground electronic and vibrational level is excited (by a photon of appropriate energy) to an excited vibrational level of an excited electronic state, resulting in the reemission of the energy. The photon which is reemitted is at a longer wavelength than the exciting photon due to the dissipation of some of the excitation photon energy in the excited vibrational levels. This provides the basis of one of the most sensitive methods of spectroscopic analysis, due to the absence of "noise" from the excitation source which is at the higher frequency.

Fraction Collector A device which collects the liquid eluting from a liquid chromatography column. The instrument functions by placing a series of vials one by one in position to receive the eluent for a specified time interval. It is most

commonly used in exclusion chromatography of biological samples.

Frit A disk or plug of sintered glass or metal, having a controlled pore size, used for filtering or as a carrier for samples in chromatography, and as a restrictor for the column exit in supercritical fluid chromatography.

Gaussian A curve having a bell shape which is described mathematically by:

$$y = \frac{e^{(-(x-u)^2/2\sigma^2)}}{\sigma\sqrt{2\pi}}$$

where y is the ordinate, x is the abcissa, u is the mean value and σ is the standard deviation. It is also called the normal distribution. The shape is an idealization of the geometry of chromatographic peaks, never really attainable in practice.

Gel A two–component colloidal system consisting of a continuous phase and a disperse phase, in a semisolid form. The disperse phase is usually a solvent species, while the continuous phase is a network such as a polymer. Gels are usually transparent, but may become opalescent.

Heterocycle An organic ring compound in which the ring is composed of atoms of more than one element. An example

would be pyridine, which is analogous to benzene with one of the ring carbons replaced by a nitrogen.

Homologous Series A series of chemical compounds in which each member differs from the next by the addition of a constant number of atoms. The series of ethane, propane, butane, pentane, and hexane is an homologous series, since each member differs by the addition of a methylene group, $-CH_2-$. Each member of the series is called a homolog.

Hydrolysis A reaction in which water reacts with a chemical species to form a product, usually involving ionization of the water before the reaction. An example is the conversion of a starch into glucose by water in the presence of a suitable catalyst.

Interatomic Distance The average distance between molecules in a given state of matter at a given temperature and pressure. It can also be thought of as the mean free path (of a fluid phase), the average distance traveled by a molecule before it encounters and collides with another molecule.

Isochoric A process or measurement which is done with the volume of the system held at a constant value. It follows that if the volume is constant, the density will also be constant.

Isomer A compound which has the same chemical formula as another compound, but which differs from the other compound in the way the atoms are arranged (that is, the two compounds

differ in an aspect of molecular geometry). The word is derived from the Greek "isos," meaning equal, and "meros," meaning part.

Isothermal A process which is performed at a constant temperature. An isotherm is a response or measurement which is obtained from a technique maintaining the system at a constant temperature. It also refers to the measured or fitted lines on a P–V–T surface or a phase diagram, in which the represented response is at constant temperature.

Isotope One of two or more forms of an element having the same atomic number (and position on the periodic chart) but a different relative atomic mass. Some isotopes of important common elements are radioactive, and are useful in chemical analysis as tracers.

Labile A system or compound which is unstable and is easily converted to a more stable system or compound by a slight disturbance, such as a temperature increase. Such a system or compound is said to be thermally labile.

Linearity (of a chromatographic detector) The range of sample concentrations over which the response factor remains constant within a specified range, usually plus or minus 20 percent. The response factor is a ratio of signal to sample size. The linearity is often expressed in orders of magnitude (or decades) of concentration.

Mass Spectrum A histogram of ion mass (actually, the ion mass divided by the elementary electronic charge) versus ion abundance produced by a mass spectrometer. In the mass spectrometer, the sample is decomposed into constituent and reconstructed ions (either by an electron beam or by chemical means) which are then separated according to mass.

Modifier A fluid added to the primary mobile phase of a supercritical fluid chromatograph or supercritical fluid extraction system in order to change the polarity, and therefore the solvent strength, of the fluid. The most common modifier is methanol, added to carbon dioxide (the most common mobile phase) in amounts ranging from 1 to 3 percent.

Moiety Although usually defined as one part of approximately two equal parts, a moiety, as the term is used in chemistry, refers to a functional group on a molecule, such as the carboxyl group or moiety.

Permanent Gas An archaic although commonly used term which refers to the gases which are difficult to liquefy, and which were previously though impossible to liquefy. These gases include the noble gases, and hydrogen, nitrogen and oxygen.

Pi Orbital (or pi bond) A theoretical construction describing the chemical bond produced from the overlap of two p–type orbitals. The π–orbital or bond consists of an electron cloud of two parts, one lying above the plane of the atoms and one lying

below. The electron cloud is actually a mathematical description of the probability of finding an electron in this particular volume of space around the molecule.

Polydispersivity A characteristic of polymeric materials in which the molecules occur with a range of molecular weights, rather than a single compound of fixed molecular weight. Such polymer systems are said to be polydisperse.

Precision A measure of the reproducibility of an experimental measurement, due to the effects of random errors on the measurement technique. In general, systematic errors will not be noticed in the precision of a series of measurements.

Preparative–Scale Chromatography The use of chromatographic hardware to achieve separation of relatively large quantities of chemical species, for other than analytical purposes. The most common types of preparative scale chromatography are done using packed column GC, and HPLC. Relatively little is done using SFC, and no such work is done using capillary column GC. Electrophoresis can also be done on the preparative scale.

Quadrupole Mass Filter An electrostatic (rather than magnetic) mass separation device consisting of four rods arranged symmetrically, across which are applied voltages having a dc (direct current) and rf (radio frequency) component. The electrostatic field that results is used to induce controllable trajectories on the ion paths.

Racemic Modification A mixture consisting of equal parts of a given enantiomeric pair. A solution such as this is optically inactive, and will not rotate the plane of polarized light. The mixture is designated by the prefix ± placed before the chemical name.

Ranque–Hilsche Tube See vortex tube.

Reference Material A material or substance one or more properties of which are sufficiently well established to be used for calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.

Refractive Index The ratio of the velocity of light in a vacuum to the velocity of light in a particular medium such as a solid or fluid. The refractive index is dependent upon the wavelength of the light, and the temperature and density of the medium. It is often used in chemical analysis as the basis for qualitative identification (using an Abbe refractometer, for example) or solute detection (using a differential refractometer in HPLC).

Resin A synthetic resin is a man–made high polymer made from a chemical reaction between two or more substances, usually with heat and catalyst. A natural resin is most often a vegetable–derived mixture of carboxylic acids, essential oils and terpenes usually occurring in the bark of many tress and shrubs.

Response Factor The ratio of detector signal output (in units of integrated area) to the size of the sample.

Retention Time The time required for a solute to elute from a chromatographic column. It is related to the retention volume, which is the volume of mobile phase required to elute a solute from a chromatographic column.

Rotary Evaporator A device which rotates a flask (which may be fluted to induce the continuous mixing of its contents) while a vacuum or heat or both is applied to the flask. The rotation serves the dual purpose of preventing bumping or cavitation, and producing a thin layer of solvent on the inside surface of the flask (as it rotates) to hasten evaporation.

Septum A self–sealing flexible gasket that isolates the inside of a chromatographic injector from the outside environment, and through which a syringe is pierced in order to deliver sample to the chromatograph. They are available in different sizes, materials, and temperature ratings. The word is derived form the Latin "saeptum," meaning wall or enclosure.

Septum Bleed Extraneous impurity material released form a septum on a chromatographic injector, eventually entering the detector and causing interference. The impurities may include compounds from previously injected samples, material from the septum itself, or impurities introduced by the operator careless handling a septum during replacement.

Sintered Glass/Metal A solid structure formed by the agglomeration of ceramic (or vitreous) or metallic material at a temperature below the melting point. The energetic driving

force behind the formation of sintered materials is the reduction of the surface area, and the associated reduction of free energy.

Sol A two component colloidal system consisting of relatively large molecules dispersed in a continuous phase solvent.

Solute A compound that is dissolved in a solvent. The name solute implies that the dissolved material is present as a minor constituent, or is relatively dilute in the solution, such as (for example) in the Henry's law region or below.

Solvent Strength In HPLC, the solvent strength is the ability of the solvent or solvent system to provide a desired capacity factor, k'. Stronger solvents provide smaller capacity factors, while weaker solvents provide larger values. A solvent of appropriate strength for a separation provides the necessary capacity factor to achieve separation.

Standard Reference Material A certified reference material issued by the United States National Institute of Standards and Technology (formerly the National Bureau of Standards).

Stereoisomers A class of isomers which differ from one another only by the way the atoms of the molecules are arranged in space. In other respects, the isomers are identical; the chemical formulae are the same, and the same atoms are bonded to one another in the same order.

Steric An effect due to molecular geometry in which atoms on a molecule cause or prevent a reaction or a molecular movement due to what is imagined to be physical interference by the atoms in close proximity to one another. "Steric strain" in a molecule may prevent certain geometric conformations of structure. A large, bulky group of atoms situated near a reactive site on a molecule may prevent a reaction at that site due to "steric hindrance."

Stokes's Law A mathematical relationship which describes the frictional force experienced by a spherical, charged body in a viscous solution as the body moves under the influence of an electric field.

Stokes–Einstein Equation A mathematical relationship derived from hdyrodynamic theory which relates the binary interaction diffusion coefficient of a spherically–shaped solute species with the solvent viscosity, temperature and the size of the solute. It can be expressed as:

$$D_{12} = \frac{RT}{6\pi \eta_\beta r_a}$$

where D_{12} is the binary interaction diffusion coefficient, η_β is the solvent viscosity, T is the thermodynamic temperature, R is the gas constant, and r_a is the radius of the solute molecule.

Theoretical Plate An imaginary section or volume element of a distillation column or chromatographic column in which solute partitioning between phases occurs, and in which equilibrium is established rapidly before the solute moves on to the next "plate."

Thermal Conductivity A transport property which describes the tendency of matter to transfer heat through a temperature gradient which is set up across its volume. Thermal conduction will occur until the temperature gradient is zero. The thermal conductivity of most materials is both temperature and pressure dependent. The primary thermal transport mechanism in solids is conduction, while in fluids, both conduction and convection play a role.

Transport Property A thermophysical property that describes the movement of some physical quantity, such as heat, mass, electrical charge or momentum, from one part of a system (ensemble of molecules) to another. Examples of transport properties are viscosity, thermal conductivity, and electrical conductance.

Ultrasound Radiation just above the region audible to the human ear. Normally, ultrasound that is used in scientific applications is in the 35 kHz region. Ultrasound is used in degassing HPLC solvents, and is the basis of a detector for gas chromatography. There are many industrial applications of ultrasound as well, such as in cleaning, soldering, plating, and in

the acceleration of chemical reactions, especially those occurring through heterogeneous catalytic mechanisms.

Vapor Pressure The pressure exerted by the evaporated vapor or gas that forms above any condensed phase which is held in a closed system. Condensed phases include both liquids and solids.

Viscosity Viscosity is a transport property of fluids that describes the resistance of a fluid to flow. It is the shearing stress per unit area of a fluid undergoing shear (or flow).

Void Volume The total empty volume of a chromatographic system. The void volume establishes the *de facto* minimum retention time, since it represents the time required for an unretained solute to elute from the column.

Vortex Tube A refrigeration device which produces a cold air stream by producing a vortex from a flow of compressed air confined in a pipe. The vortex is forced to decrease in radius at constant angular velocity. Thermodynamics requires that the air stream must lose energy during this process, which produces the cooling effect. A stream of heated air is also produced, which removes the energy given up by the cooling air.

Xerogel A gel–like structure consisting of a solution of linear polymers which has been cross–linked to restrict movement. Alternatively, the movement may be restricted by the presence of intermolecular interactions such as hydrogen bonds.

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This report is intended to provide an introduction to chemical instrumentation to workers whose training lies outside the area of experimental chemistry. It is especially geared toward scientific managers in government and industrial laboratories who must interact on a daily basis with chemical professionals, and who often have highly sophisticated analytical chemistry laboratories under their jurisdiction. This first report deals with instrumental separation methods. After an introduction to the nature of mixtures, the topics of gas chromatography, high performance liquid chromatography, supercritical fluid chromatography, and electrophoresis are discussed. These sections are followed by an appendix covering calibration methods. A glossary is provided in which terms not covered in the body of the report are defined.

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