CRC

HANDBOOK of CHROMATOGRAPHY

Volume II

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Handbook

of

Chromatography

Volume II

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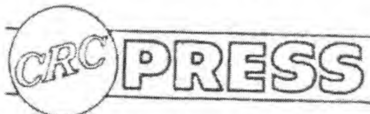

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Section I

PRINCIPLES AND TECHNIQUES

Joseph Sherma

I.I. Introduction

A. HISTORY

A *definition* of *chromatography* similar to one originally stated by Strain is as follows: Chromatography is a separation method in which a mixture is applied as a narrow initial zone to a stationary, porous sorbent and the components are caused to undergo differential migration by the flow of the mobile phase, a liquid or a gas.

The originator of chromatography as it is practiced today was Michael Tswett (1872–1919). In 1906, Tswett, a Russian botanist, published a paper (which has been translated into English and evaluated (*1*)) describing the separation and isolation of green and yellow chloroplast pigments by column adsorption chromatography.

In his original experiments, Tswett tamped a fine powder (such as sucrose) into a glass tube to produce a column of the desired height. After extracting the pigments from leaves and transferring them to petroleum ether, he poured a small volume of the solution onto the column. When the solution had percolated and formed a narrow initial zone beneath the top of the adsorbent, fresh solvent (e.g., petroleum ether) was added and pressure applied to the top of the column. As the solvent flowed through the column the individual pigments moved at different rates and eventually separated from each other. Figure 1 illustrates the development of a chromatogram by Tswett's chromatographic method with a solvent employed by Strain in his extensive studies of chloroplast pigments.

The key features of Tswett's technique were the application of the mixture as a narrow initial zone and the development of the chromatogram by application of fresh solvent. Other early workers had employed procedures based on the phenomena of adsorption or partition, but these lacked Tswett's critical development step and therefore did not yield extensive resolution of the mixtures.

Tswett's original column adsorption chromatographic method has been modified in many ways resulting in the different types of chromatography described in the sections below. The *history* of *chromatography* is outlined by the following chronological listing of some of the key contributions to the development of these modifications:

1848	Way and Thompson	Recognized the phenomenon of ion exchange in solids.
1850–1900	Runge, Schoenbein, and Goeppelsroeder	Studied capillary analysis on paper.
1876	Lemberg	Illustrated the reversibility and stoichiometry of ion exchange in aluminum silicate minerals.
1892	Reed	First recorded column separation: tubes of kaolin used for separation of $FeCl_3$ from $CuSO_4$.
1903–1906	Tswett	Invented chromatography with use of pure solvent to develop the chromatogram; devised nomenclature; used mild adsorbents to resolve chloroplast pigments.
1930–1932	Karrer, Kuhn, and Strain	Used activated lime, alumina and magnesia adsorbents.
1935	Holmes and Adams	Synthesized synthetic organic ion-exchange resins.
1938	Reichstein	Introduced the liquid or flowing chromatogram, thus extending application of chromatography to colorless substances.
1938	Izmailov and Schraiber	Discussed the use of a thin layer of unbound alumina spread on a glass plate.
1939	Brown	First use of circular paper chromatography.

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1940–1943	Tiselius	Devised frontal analysis and method of displacement development.
1941	Martin and Synge	Introduced column partition chromatography.
1944	Consden, Gordon, and Martin	First described paper partition chromatography.
1947–1950	Boyd, Tompkins, Spedding, Rieman, and others	Ion-exchange chromatography applied to various analytical problems.
1948	M. Lederer and Linstead	Applied paper chromatography to inorganic compounds.
1951	Kirchner	Introduced thin-layer chromatography as it is practiced today.
1952	James and Martin	Developed gas chromatography.
1956	Sober and Peterson	Prepared first ion-exchange celluloses.
1956	Lathe and Ruthvan	Used natural and modified starch molecular sieves for molecular weight estimation.
1959	Porath and Flodin	Introduced cross-linked dextran for molecular sieving.
1964	J. C. Moore	Gel permeation chromatography developed as a practical method.

B. NOMENCLATURE

Tswett is responsible for much of the nomenclature that is used by most chromatographers today. A glass or metal *tube* (or column) is filled with an active solid (*adsorbent*) to form a chromatographic *column*. The mixture to be separated is applied in an *initial zone* and it is washed with the *solvent*, *wash liquid*, or *developer*. The resultant series of zones is the *chromatogram*, and the washing of the initial zone to form the chromatogram is the *formation* or *development* of the chromatogram.

If the separated zones are colorless they must be detected in some way. (See Reference (*2*) for a general discussion of detection methods.) If the chromatogram is treated with a chemical reagent to form colored derivatives, the chromatogram is sometimes referred to as having been *developed*. It is best, however, to reserve this term for the formation of the chromatogram by washing with solvent.

The combination of the solvent, the mixture and the sorbent is termed the *chromatographic system*. Each chromatographic system is composed, then, of a *mobile phase* (the solvent) and a stationary phase (e.g., the column). The generalized term *sorbent* may be used in place of adsorbent, ion-exchange resin, paper sheet, etc., when referring to the stationary phase.

If the components of the mixture are analyzed quantitatively as well as separated, the term *evaluation*, *quantification* or *quantitation* is used. If the separated solute is removed from the sorbent by washing (either in a chromatographic fashion or not) before this analysis, it is said to be *eluted* and the solution to be analyzed is the *eluate*. In ion-exchange and gel permeation chromatography, many workers use this term *elution* for the development of the chromatogram, and *eluant* for the solvent or wash liquid; the liquid emerging from the bottom of the column is then the *elutrient* or the *effluent*.

C. CLASSIFICATION OF CHROMATOGRAPHY

The subject of chromatography may be divided and subdivided as follows to include the great variety of methods which have evolved from Tswett's original chromatographic method.

The two major classifications of chromatography are solution or liquid chromatography (LC) and gas chromatography (GC). In the former a liquid carries the dissolved solutes through the sorbent, which can be a column, paper or thin layer. In the latter an inert wash gas (carrier gas) carries the gaseous mixture through the sorption column.

Within each of these major divisions, subdivisions based on the stationary phase are designated. Thus gas-solid chromatography (GSC) involves a column packed with an adsorbent, and gas-liquid chromatography (GLC) involves a solid coated with a stationary liquid as the sorbent.

Liquid chromatography in columns can be liquid-solid chromatography (LSC) or liquid-liquid (partition) chromatography (LLC). If the solid stationary phase is an adsorbent, the process is called liquid adsorption chromatography. If it is an ion-exchange material, either organic or inorganic, it is termed ion-exchange chromatography (IXC). If it is a nonionic polymeric gel (e.g., polystyrene or Sephadex) the term gel permeation chromatography (GPC), gel filtration chromatography or molecular exclusion chromatography is used.

Other subdivisions of non-column liquid chromatography are paper chromatography (PC) and thin-layer chromatography (TLC). These subdivisions include separations on all kinds of paper and thin layers whether the mechanism of separation is adsorption, partition or ion exchange.

Liquid-liquid chromatography in columns or on paper may be further subdivided into normal-phase partition chromatography (fixed polar liquids) and reversed-phase partition chromatography (fixed non-polar liquids).

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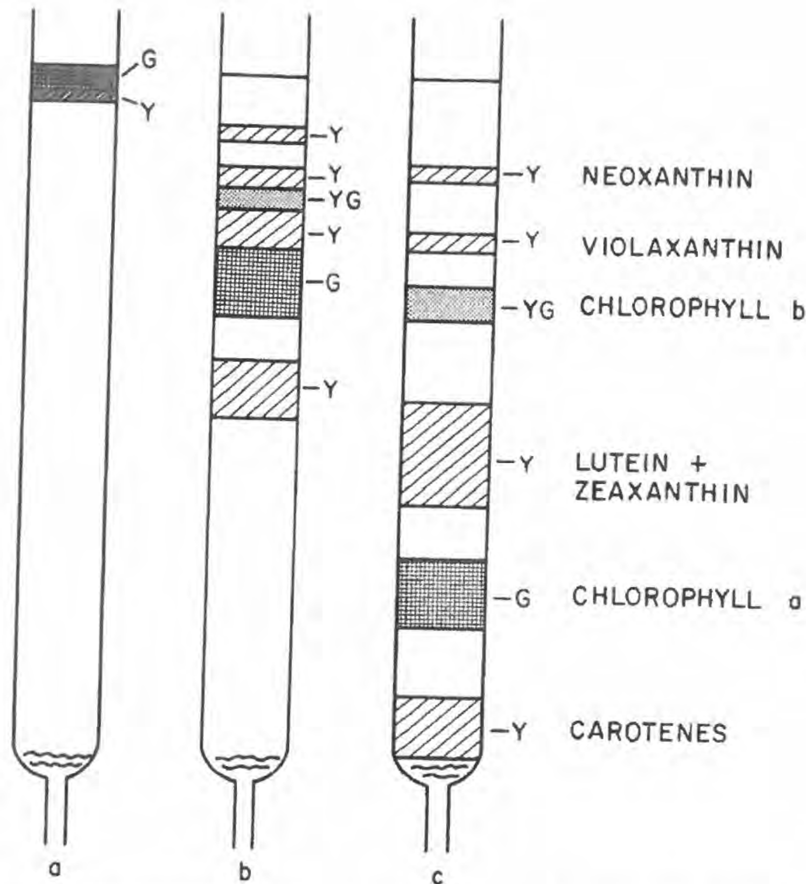

Figure 1. Successive steps in the formation of a chromatogram. Chloroplast pigments of flowering plants, ferns, mosses and lichens and many green algae dissolved in petroleum ether, adsorbed in a column of powdered sugar (a) and washed with petroleum ether plus 0.5 per cent *n*-propanol (b, c). Y = yellow; G = green.

D. MECHANISM OF CHROMATOGRAPHY

The movement of substances during chromatography is the result of two opposing forces, the driving force of the mobile phase and the resistive or retarding action of the sorbent. The driving force acts to move the substances from the origin in the direction of the mobile-phase flow. The resistive action impedes the movement of the substances by dragging them out of the flowing phase back onto the sorbent. Each molecule alternates between a sorbed and unsorbed condition, following a stop and go path through the sorbent. Although the zone moves constantly ahead, only a fraction of the molecules in the zone is moving at any one time. At the end of development, each zone has migrated a certain mean distance and has spread because of the fluctuations in the movement of individual molecules in the zone. The distance travelled by each solute zone in a given time is the resultant of the driving and resistive forces. Substances which move slowly are attracted more strongly to the stationary phase, while those that move quickly spend a smaller fraction of their time in the stationary phase because of less solubility in or affinity for that phase.

The ability to achieve differential migration (i.e., separations) among the mixture components is the result of the selectivity of the chromatographic system. The flow of the mobile phase is nonselective in that it affects all unsorbed solutes equally. As part of the chromatographic system, however, the mobile phase may be selective (e.g., in liquid chromatography) if it helps determine the relative sorbability of the solutes. The sorbent is also part of the chromatographic system and its resistive action (ion exchange, adsorption, etc.) is a selective force. To put this another way, all eluted or nonsorbed components spend equal time in the mobile phase. If there is differential migration, the components spend different amounts of time on the sorbent as determined by the interactions of the chromatographic system.

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VOLUME TWO
SECTION I

PRINCIPLES AND TECHNIQUES

I.II Gas Chromatography

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E. R VALUES

The R value or retention ratio of a substance indicates its migration relative to that of the mobile phase:

$$R = \frac{\text{solute velocity}}{\text{mobile-phase velocity}}. \qquad [1]$$

The R value indicates the fraction of time the solute molecules spend in the mobile phase relative to the time in the sorbent.

In paper and thin-layer chromatography it is customary to use migration distances rather than velocities, and to calculate R_F values:

$$R_F = \frac{\text{distance travelled by the center of the solute zone}}{\text{distance travelled by the solvent front}} \times 100. \qquad [2]$$

This ratio is similar in idea to R as defined above, but R_F values are usually lower than R values because the solvent front moves faster over the dry sorbent than does the bulk solvent. The R_F equation also assumes a constant velocity during the run which it does not actually obtain in these techniques.

R and R_F values are not chromatographic constants but vary with the experimental conditions employed during the run. All conditions (such as the nature and preparation of the solvent and sorbent, the arrangement and conditions for the development, the sample size, the temperature, etc.) should be stated when recording R values, so that they will have maximum significance. Even then, one should not expect to be able to reproduce exactly R values reported by others. The major practical use of R values is to indicate relative sorbability and to show which systems may be useful for obtaining the separation of a certain mixture.

The dependence of R values on experimental conditions can be lessened if solute migration is described relative to a reference standard which is naturally present in the sample or is added to it. This is especially useful for biological samples containing various impurities (e.g., lipids, inorganic salts) which might alter migration behavior. These values are called R_X values (or R values relative to X):

$$R_X = \frac{\text{velocity of (or distance travelled by) solute zone}}{\text{velocity of (or distance travelled by) compound } X}. \qquad [3]$$

F. SIGNIFICANCE, USES AND APPLICATIONS OF CHROMATOGRAPHY

Chromatography is primarily an analytical tool effective for the separation of mixtures and the qualitative and quantitative analysis of the separated substances. Ideally, each component of the mixture will be completely separated from the other components (it will, of course, be mixed with the mobile phase) and each substance will yield a single, well-defined, regularly-shaped zone. In some cases, however, results are anomalous leading to irregularly shaped zones or multiple zonation (a single substance yields more than one zone).

Chromatography is useful for the comparison of substances, for providing clues as to the structure of organic substances, and for the detection of structural changes produced by various chemical reagents or nuclear and biological processes. Chromatography, combined with conventional chemical and instrumental analytical methods, serves to identify chemical species. Chromatography alone is not adequate for positive qualitative identification even when a sample, prepared by mixing the unknown with an authentic standard it is suspected of being, is found to be inseparable under various dissimilar conditions (this procedure is called co-chromatography). In such a case, the unknown and the standard might be either identical or very similar (e.g., isotopes or isomers). If two substances separate chromatographically, this is positive proof that they are not identical.

Chromatographic methods have a nearly unlimited range of applicability. They can be used to separate the smallest molecules (H_2, D_2) as well as the biggest (proteins, nucleic acids). Isotopes can be separated by gas chromatography and less readily by solution chromatography. Because systems making use of all kinds of physico-chemical interactions can be employed, any substance which is either present in a gaseous state at ambient temperature, vaporizable, or soluble is amenable to chromatographic separation under some obtainable conditions. Quantities in the picogram range can be separated and detected by gas chromatography combined with mass spectrometry, while at the other end of the weight range, multigram quantities can be separated and isolated by preparative column chromatographic methods.

Despite significant recent advances in chromatographic theory [see Reference (*3*), for example] which have provided much information about the mechanism of separations and zone migration, the selectivity of chromatographic systems is still incompletely understood so that the conditions required to separate a given mixture cannot usually be theoretically predicted. For most workers, the experimental approach in chromatography is largely an empirical one, based upon analogy, controlled trial and error, and intuition and experience. Therefore, collations of data such as those presented in this Handbook are extremely useful, because the worker who is interested in applying chromatography to a particular problem can benefit from the experiences of others who have worked with the same or similar compounds. By adopting or adapting previously successful systems, effective separation conditions will usually be found. The more successful users of chromatography strive to understand the physico-chemical causes of successful separations and

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the reasons for the choice of particular operating conditions, and are thereby better able to determine parameters which may lead to, and improve, separations.

The sections below are designed to introduce the various kinds of chromatography to those who may not be entirely familiar with them and their basic methods. All these modifications are related, because they involve the application of a narrow initial zone of mixture, the presence of a mobile and stationary phase (i.e., a driving force and a resistive action), and the achievement of separations due to differential migration of the components of the mixture.

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Section I.II

GAS CHROMATOGRAPHY

In GC the mobile phase is an inert gas and the stationary phase is either a solid or a fixed liquid packed into a column. The solid in gas-solid chromatography (GSC) is commonly alumina, silica gel, charcoal or a molecular sieve, and selective sorption on these solids allows the separation of certain permanent gases and low molecular weight hydrocarbons. Aliphatic and aromatic hydrocarbons have also been separated on salt-modified adsorbents.

Gas-liquid chromatography (GLC) is much more important and will be discussed for the rest of this section, although much of what is written applies to both techniques. The carrier gas drives the mixture through the column wherein the solutes partition between the gas and the stationary liquid. In addition to partition, adsorption of the solutes on the solid support and at the gas-liquid interface is also a factor in many cases. Differential migration results if the distribution coefficients of the solutes are different enough to allow the sorbent to selectively retard them. In gas chromatography, unlike liquid chromatography, the mobile phase is always nonselective. There is, however, the added temperature variable which aids in achieving resolution in GC.

A. BASIC APPARATUS FOR GC

A schematic drawing of the basic apparatus necessary for GC is shown in Figure 2. The parts include:

1. A high pressure cylinder of carrier gas. The gas chosen must be suitable for the detector employed, and beyond this should be inert, pure, inexpensive and as heavy (high molecular weight) as possible so as to minimize solute diffusion. If speed is important but highest column efficiency is not, a low-molecular-weight gas such as helium or hydrogen may be used.

2. Pressure regulators and flow-control valves are used to obtain a uniform rate of gas flow, which is measured by a flow meter of the float, capillary or soap bubble type. The latter type is placed at the outlet of the column. Typical flow rates are 25–125 ml/min depending upon the size of the column. The optimum flow rate is chosen by making a van Deemter plot and noting the minimum value of HETP (see "Theory" below).

3. Samples are injected quickly onto the column through a self-sealing septum at the injection port. Gas-tight syringes are used for gas samples and liquid syringes for liquids and dissolved solids. Typical sample sizes range from 0.1–10 μl for gases and 0.004–0.5 μl for liquids on $\frac{1}{16}$ in. capillary columns, to 0.05–5 l for gases and 0.02–2 ml for liquids on 1 in. preparative columns. Regular analytical columns ($\frac{1}{8}$ and $\frac{1}{4}$ in. O.D.) usually receive 0.1–50 ml of gases and 0.04–20 μl of liquids. The sample size (column capacity) depends upon the amount of the liquid phase as well as the column size. Small samples are often applied by injecting 1–2 μl of sample and delivering only part of this to the column via a sample splitter.

The recommended sample injection technique for liquids in gas chromatography is the solvent flush method (*1*). Pure solvent is drawn into the syringe barrel followed by an air pocket, then the sample solution, and finally another air pocket. The sample volume is read, and then the sample is injected. The flush solvent behind the sample assures that the entire sample is flushed into the column without hang-up.

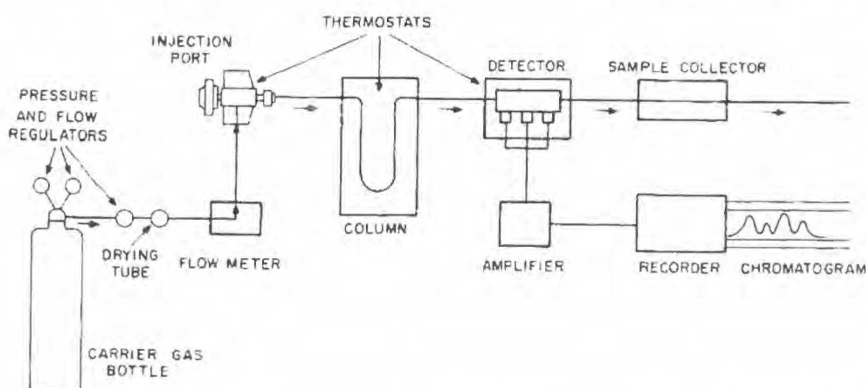

Figure 2. Schematic drawing of apparatus for gas chromatography (not to scale). Arrows show path of gas flow.

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4. Columns are made from copper, stainless steel or glass tubing and are straight, bent or coiled. Except for glass, the columns are packed while straight and then bent. General analytical columns are 6–20 ft in length and $\frac{1}{8}$–$\frac{1}{4}$ in. O.D. Preparative columns can measure up to 2 in. I.D. but are most often $\frac{3}{8}$–$\frac{1}{2}$ in. and 20 ft in length. Capillary columns 0.01–0.03 in. I.D. and 100–1000 ft in length are usual. The nature of the column is discussed further below. Column efficiency generally improves with decreasing column diameter.

5. The detector indicates and measures the solutes in the carrier gas stream. A good detector is highly sensitive, has a linear response over a large concentration range and is relatively insensitive to flow and temperature variations. Either universality or selectivity of response can be advantageous. A further discussion of detectors is found below.

6. Pure samples may be collected from the column effluent in cooled capillary tubing, or in glass collection bottles for larger samples.

7. The signal from the detector must be amplified and fed to a recorder where the chromatogram is drawn on a strip chart. A 1 mv, 1 sec (full scale) recorder is generally useful for gas chromatographs.

8. The temperature must be controlled in three places (Figure 2). The injection port is kept hot enough to vaporize the sample rapidly but not so hot as to decompos[illegible] A temperature 10–50° above that of the column is often recommended. The port need not be heated for gas samples. The temperature of the column is kept as low as possible consistent with good resolution but high enough to obtain reasonably fast separations. Isothermal operation is often adequate for mixtures with a narrow boiling range. For mixtures with a wide boiling range, temperature programming is recommended (see below). The temperature of the detector must be high enough to resist condensation of the sample or the liquid phase or any products formed in the detector if ionization is involved. Temperature control of the column within ±2 °C is usually adequate; close temperature control for some detectors (thermal conductivity) is critical but for others (flame ionization) is not.

B. THEORY

The theory of gas chromatography will be given in some detail in this section. The theory of the other chromatographic methods is essentially the same although there are some differences in terms and actual differences due to variations in the techniques.

The separation of solute peaks in chromatography depends upon the separation of the peak centers and the degree of spreading of the peaks. Obviously, complete separation of narrow peaks can be obtained with the peak centers closer together than if the peaks are spread (Figure 3).

The location of the peak centers depends upon the solute's sorptive equilibrium as determined by the respective distribution (partition) coefficients and the temperature. The interactions between the solutes and the sorbent leading to dissimilar partition coefficients are hydrogen bonds, Debye forces, Van der Waals' forces and specific chemical interactions (the partition coefficient K = the amount of solute per unit volume of liquid phase/the amount of solute per unit volume of gas phase). [4]

Peak width or spread is determined by mass transport and kinetic processes and is related to the column efficiency. Column efficiency is measured by the number of theoretical plates in the column or better by the resolution of the column. The longer a peak is in a column, the broader it becomes (under isothermal conditions).

The number of theoretical plates (N) is measured from the actual chromatogram. Tangents are drawn to the peak at the points of inflection (about two-thirds of the height). Then, referring to Figure 4, N for the first peak is given by

$$N = 16\left(\frac{X}{W_1}\right)^2, \qquad [5]$$

where X is the distance from the point of injection to the peak maximum and W is the length of base line enclosed by the two tangents. Both the numerator and denominator must be in the same units, most conveniently in cm measured along the chart paper.

HETP or height equivalent to a theoretical plate is the column length divided by N. A good column has a large number of theoretical plates and a small HETP (less than one mm for GC). The HETP may be different for each solute on a given column. It should be emphasized that HETP is a function not only of the width of the peak but of its retention time. Therefore a wide peak with a high retention time can represent the same HETP as a narrow peak which is eluted earlier.

The separation of two adjacent peaks is measured by the resolution (R) (see Figure 4):

$$R = \frac{2d}{W_1 + W_2}. \qquad [6]$$

When recording resolution, the specific compounds being considered should be stated. As defined in equation [6], an R of 1 indicates "perfect" (near base-line) separation.

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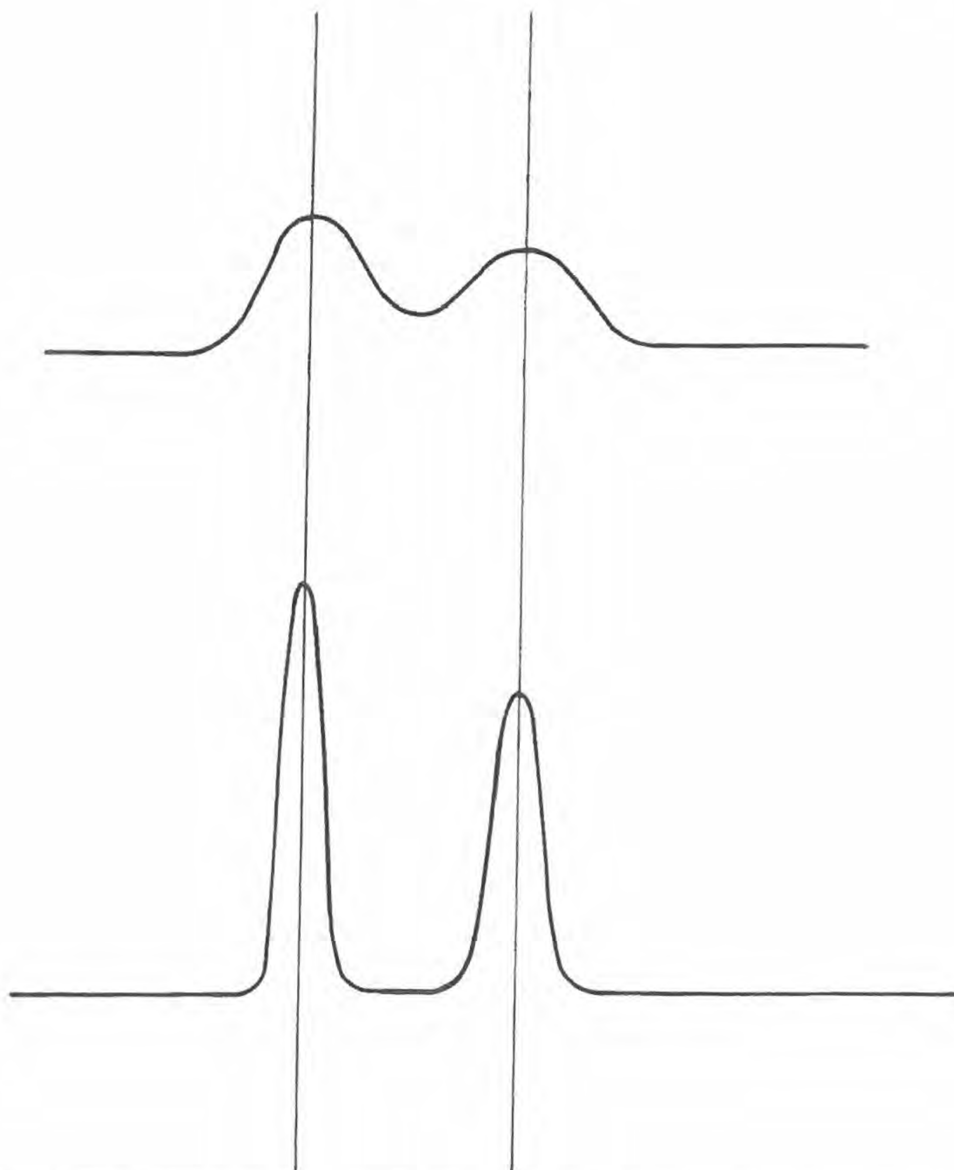

Figure 3. Two chromatograms showing the effect of increased column efficiency on the degree of separation obtained.

For describing the performance of a column, both relative retentions and plate numbers are important considerations. That is, one can obtain a good separation of two components whose relative retentions are significantly different even on a column with a low number of plates. However, if the relative retentions are very similar, even a column with many plates may not provide the desired separation.

Another parameter often used to evaluate column performance is the *Separation Factor*, which is the ratio of the adjusted retention times or volumes (Section F3) for two adjacent peaks.

HETP can be theoretically calculated by means of the van Deemter equation. This equation treats chromatography as the flowing system it is, and allows one to see what conditions should be adopted in order to optimize the system. According to this equation

$$\text{HETP} = 2\lambda dp + \frac{2\gamma D_g}{\mu} + \frac{8k'd_f^2}{\pi^2(1 + k')^2 D_l}\mu. \qquad [7]$$

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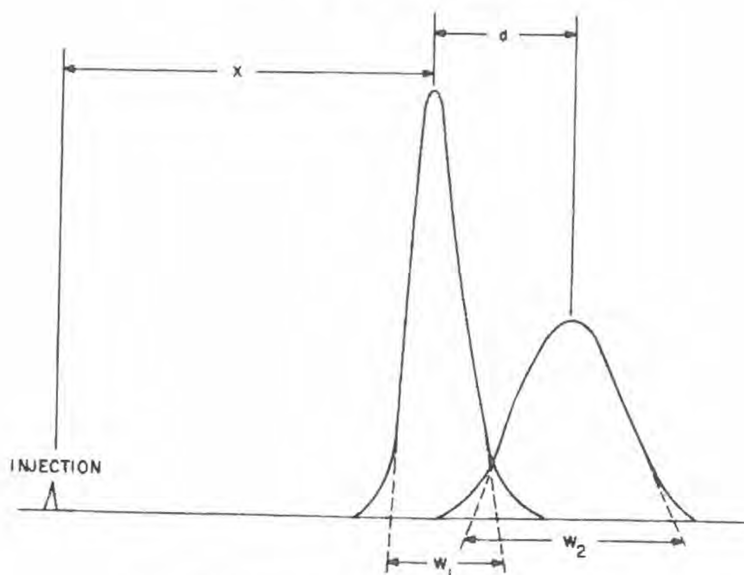

Figure 4. Calculation of N and R from a chromatogram.

where

λ = a constant measuring packing irregularities
dp = the average particle diameter of the support
γ = tortuosity factor
D_g = coefficient of solute diffusion in the gas phase
D_l = coefficient of liquid diffusion
μ = the average linear gas velocity
k' = ratio of the capacity of the liquid phase to that of the gas phase
d_f = average thickness of the liquid film coated on the support particles

Equation [7] can be reduced to

$$\text{HETP} = A + \frac{B}{\mu} + C\mu. \quad [8]$$

The A term is the multiple path or Eddy diffusion term which is independent of flow rate. It describes peak spread due to molecules taking different paths through the packed column. To decrease HETP (and increase column efficiency), one should use particles as small (low dp) and uniform (low λ) as possible, consistent with an adequate gas flow and a low pressure drop. The B term accounts for the normal longitudinal molecular diffusion of the solute in the carrier gas due to the concentration gradient in the zone. Diffusion is inverse to flow rate and decreases when a high-molecular-weight carrier gas is used (low D_g). The C term involves resistance to mass transfer and is proportional to flow rate. To decrease this term, a thin, uniform film of low-viscosity liquid should be used as the stationary phase (low d_f and high D_l). Lowering d_f changes k' and this complicates prediction of the optimum value of this term.

The optimum carrier-gas flow rate is determined by plotting HETP *vs* μ and choosing a value at or slightly above the minimum in the curve. Since the outlet pressure is usually one atmosphere, the flow rate chosen will dictate the inlet pressure to be used.

C. THE COLUMN

The choice of the column packing is the most critical in GC. In GLC, the column can be one of two types, either packed or capillary. Packed columns contain an inert solid support with a thin coating of the liquid phase. As seen above, small, uniform particle sizes (40–60 to 100–120 mesh) give the highest efficiency columns. The support is usually diatomaceous earth (kieselguhr), e.g., the Chromosorbs (see Section II.III, Table 2). In order to reduce tailing and decomposition of certain classes of compounds (steroids), the support surface must be made entirely inert by reacting surface hydroxyl and oxide groups with a compound such as dimethyldichlorosilane.

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The liquid phase (see Section II.III, Table 1) should have a low viscosity and a high and differential solubility (leading to different distribution coefficients) for the mixture components. A loading of 2–10% of the liquid is generally used; less-loaded columns give faster separations at lower temperature but have lower sample capacity and may require inactive solid supports (e.g., VarAport 30 or Teflon). Retention times decrease (at a given temperature) as the loading becomes lighter, and adjacent peaks become narrower with centers which are less separated. The liquid should neither react with the support or the solutes nor "bleed" appreciably from the column during the run. There is a recommended maximum column temperature for each liquid phase.

Liquid phases can be classified according to their polarity: the most polar liquids are capable of forming strong hydrogen bonds (e.g., FFAP, Carbowaxes, Hallcomid, etc.) while the least-polar can interact only by forming weak Van der Waals' bonds (e.g., SE-30, OV-1, squalene, etc.). Solutes can be classified in the same way, ranging from polar (alcohols, acids, phenols) to nonpolar (saturated hydrocarbons). Liquid phases which are similar to the components retard these components compared to liquid phases which are not similar. Separations are best achieved by matching the solute and liquid types; for example, hydrocarbons are best separated on squalene, alcohols on Carbowax, and fatty acid methyl esters on polyesters (see Section II.III, Table 1). For mixtures containing solutes of different polarity, it is best to choose the liquid to match the most polar solutes. It should be recalled that "polarity" is a function of temperature and that dipole interactions become very weak at high temperature.

Some liquid phases react chemically with certain solutes and are very selective for these compounds. Selective liquid phases give large differences in retention times and require fewer plates (shorter columns) to achieve a desired separation.

Porapak porous polymer resins (Section II.III, Table 2) are used for GLC with no liquid phase. The mechanism of separations on Porapak is apparently a combination of partition, adsorption, and sieving based on size.

Capillary (or open tubular) columns originally contained a liquid film of ca. 0.5 μ thickness coated onto the inside wall of a glass or metal tube. Lately, solid-coated capillary columns containing a layer of coated support around the inside tube wall but still having an open center have been employed. These latter columns are superior because more sample can be applied and the optimum flow rate is higher (about 2–5 ml per min rather than 1 ml per min). Because more sample can be applied, analytical sensitivities are greater and the use of a sample splitter (or other special splitless injection techniques) might be avoided. Support-coated capillary columns must be purchased commercially, whereas wall-coated columns can be prepared in the laboratory. Workers often do not specify in the literature the exact percentage composition of the solution they used to coat a capillary column, but instead state only the phase and the solvent. Ten percent coating solutions are generally used for coating open tubular capillary columns by the dynamic coating method, and two percent solutions or less for the static coating method (*2*).

The number of plates per foot with either type of capillary column is similar to packed columns, but the maximum total number of plates is much higher (several hundred thousand) because very long capillary columns can be employed in the absence of a pressure drop. A disadvantage is that the void volume of capillary columns is so large relative to packed columns that the capacity ratios are significantly smaller. As a result, resolution in the two types of columns is not too different.

Capillary columns with a complete loose packing have also been described (*3*), as have sandwiched capillary columns consisting of one or more carbon threads inserted in a glass capillary and coated with the stationary liquid (*4*). The performance of sandwiched capillary columns is between open tubular and classical packed columns. Their advantage lies in the ease of preparation and lower pressure drop corresponding to a minimum plate height.

A new type of stationary phase developed by Halasz consists of organic molecules attached chemically to the surface of an inorganic support. [See Reference (*5*) for a review of these phases.] The stationary phase is then like a brush with organic bristles on the inorganic surface. Such a material may be produced by esterifying the surface hydroxide groups of silica gel with an alcohol. Changing the esterifying agent results in a change in the polarity of the organic bristle. These brushes (available commercially from Waters Associates under the trade name Durapak) are useful in both gas and liquid chromatography and typically provide 4–25 effective plates per second (GC) as compared with 0.1–4 for packed columns and 30–100 for open (capillary) columns. They have highly ordered interfaces leading to unusually high mass transfer, so that separations in columns packed with brushes are about as fast as those with open tubes. They have low sublimation pressure (i.e., bond decomposition, which in this case corresponds to "bleed") and can be employed at temperatures up to 145°C with normal tank N_2 or above 200°C with dried nitrogen. HETP for these columns is independent of temperature, retention time or sample type and load.

Still another new class of stationary phases contains those substances described by Annino and McCrea (*6*) which undergo reversible temperature-dependent compositional changes to produce large variations in selectivity for various classes of solutes. By combining substances differing in melting point and solute selectivity (e.g., stearic acid and 1,9-nonanedioic acid), a mixture is produced which can be used to prepare a column exhibiting varying degrees of specificity through choice of an appropriate column temperature. Positive relative retention shifts of the order of 100% can be achieved with a 20° increase in temperature, facilitating solute identification and resolution.

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D. COLUMN TEMPERATURE

Lowering the temperature usually improves resolution while increasing the temperature will decrease the analysis time. The temperature chosen for an isothermal separation must therefore be a compromise. If the liquid loading is reduced a lower temperature can be used, a condition which could be beneficial for the separation of heat-labile solutes. The column temperature should, of course, never be high enough to either change the sample or to decompose or cause excessive vaporization of the stationary liquid. Because of possible decomposition, isomerization, etc. of the sample, chromatography above 250 °C is seldom practical. As a first approximation, a column temperature around the average boiling point of the major components of the mixture can be tried.

For wide boiling range mixtures, better resolution at the low end and faster elution at the higher end is obtained if temperature programming is employed. This method, which usually involves a controlled linear increase in temperature during the run, sharpens the later peaks and makes the sensitivity of analysis the same for both high- and low-boiling components.

Temperature programming is also advantageous for preparative GC. A large sample can be slowly applied (directly on column, if possible) by repeated application of small increments with boiling-off of the solvent in between. The temperature of the column is then slowly raised and the components migrate down the column in the order of their boiling points. This results in sharp, high-concentration peaks which can be easily trapped.

The program can be started at sub-ambient temperatures by placing a chunk of dry ice in the column compartment. The detector is generally hot throughout the run. It should be recalled that the viscosities of gases increase as the temperature increases, so that flow rate tends to decrease during the temperature program. A differential flow controller is employed to keep the gas flow constant.

Bleeding of the liquid phase is often a problem with programmed temperature GC. Use of a dual-column gas chromatograph, with both columns containing the same liquid but not necessarily of the same length, can be used to mask (not eliminate) the bleeding and maintain a reasonably level base line. This method works with a thermal conductivity detector but not ionization detectors. Alternatively, the bleeding material can be trapped by a column of an appropriate, highly-stable sorbent placed between the analytical column and the detector. Column bleed can be checked by measuring periodically the decrease in retention time of a standard compound.

An alternate technique that can be used to separate a wide boiling-point mixture is flow (pressure) programming GC. This method, in which the flow rate of the carrier gas is changed during the run, is described in detail in Reference (7).

E. DETECTORS

The two most widely-used detectors are the thermal conductivity (TC) detector (katharometer) and the flame ionization detector (FID).

1. *Thermal conductivity detector*—This detector consists of two identical metal cells, each containing a tungsten, rhenium-tungsten or gold-sheathed tungsten wire filament or a thermistor (Figure 5). The column effluent flows through one cell and pure carrier gas with a high thermal conductivity (H_2 or He) through the other. On the sample side the gas flows directly over the filament while on the reference side the gas may pass over the wire or pass near and diffuse to the wire (Figure 5). The wires are heated by an electric current. The temperature of the wires, and their resistance, depends upon the composition of the gas flowing over them. When pure carrier gas flows through both cells the temperatures are the same and the detector is balanced. When a gas with a lower thermal conductivity appears in the effluent cavity, the temperature of the wire will rise (heat will not be so quickly lost to the wall of the cell) and its resistance will rise. The cell is initially arranged as part of a balanced Wheatstone bridge circuit, so that the resistance increase will cause an imbalance in the bridge and gives rise to an electrical signal which is amplified and fed to a strip-chart recorder where it shows up as a peak.

For maximum sensitivity, the filament temperature is increased, the block temperature and flow rate are decreased, and a carrier gas with a high thermal conductivity is chosen (most organic gases have relatively low heat conductivities). Operation at a high temperature shortens the life of the wire filaments, of course. The TC detector is nondestructive and responds to any gas with a TC different from the carrier gas. The response is concentration dependent, but correction must be made depending upon the molecular weight of the gas being detected.

2. *Flame ionization detector*. Components in the column effluent are ionized by burning them in a hydrogen-air (or oxygen, for increased sensitivity) flame. This allows the gas in the detector to conduct an electrical current. Above the flame, a collector electrode, to which a DC potential is applied, measures this conductivity. When pure carrier gas (H_2) is combusted, few ions are formed and the conductivity is low. The combustion of organic compounds increases the conductivity and the resultant current is amplified and fed to a recorder.

This detector is highly sensitive and therefore often used with capillary columns, and its range of linear response to increasing concentration is very wide. It responds to almost all compounds except the inorganic

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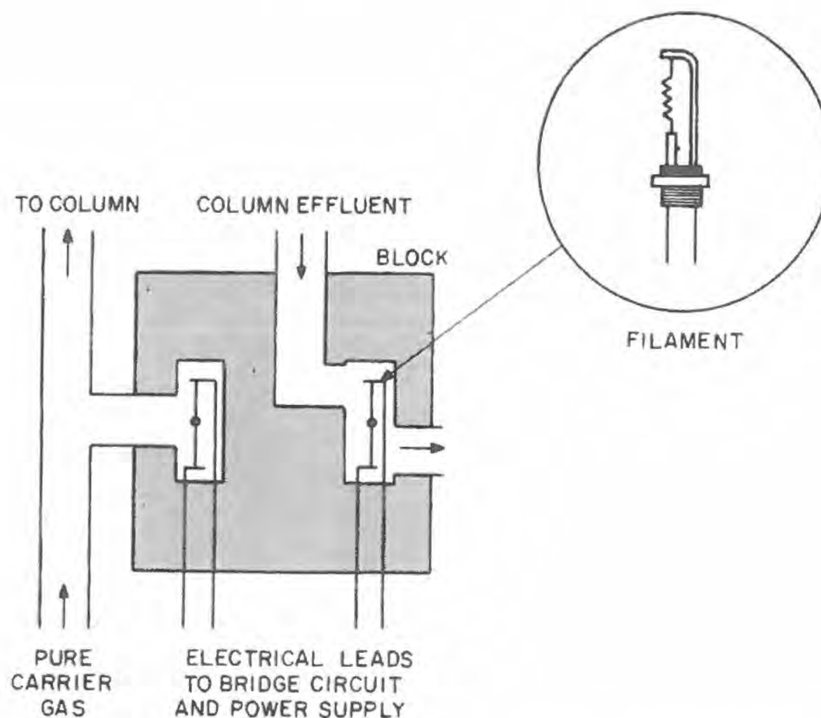

Figure 5. Schematic drawing of a thermal conductivity detector employing thermistor beads (black dots) or wire filaments (insert).

gases, CS_2 and COS. The response is based on the carbon weight percentage in the molecule; as the level of oxidation increases (e.g., from saturated hydrocarbon to alcohol to acid), the response of the detector decreases. A disadvantage is that the detector is destructive; if the concentration is sufficiently high, the effluent stream is often split and part of the solutes collected for confirmatory analysis by other methods.

The *flame ionization detector* responds to mass flow rate, i.e., the peak area is proportional to the total mass of eluted component. The *thermal conductivity detector* responds rather to concentration, and for accurate quantitative analysis with such a detector, constant flow rate is critical. Constant flow rate is therefore not so critical for a flame ionization detector as for a thermal conductivity detector.

3. *Other detectors.* The *electron capture detector* is highly sensitive to halides and is therefore valuable for pesticide analyses. It gives little response to hydrocarbons other than conjugated carbonyls.

The *alkali flame detector* is a flame ionization detector with a KCl or CsBr salt tip placed on the burner jet. It is especially sensitive for phosphorus detection.

The *helium detector* is used for fixed gases separated by GSC. It cannot be used with partition columns because it is so sensitive to organic liquids bleeding from the support.

A *flame photometric detector* (developed by Brody and Chaney) is a flame emission detector in which a photomultiplier tube monitors the chemiluminescent emission from a hydrogen-air flame. If appropriate filters are inserted between the detector and photomultiplier, the response is selective for sulfur (394 nm) or phosphorus (526 nm).

A *reaction coulometer* can be used as an absolute detector for compounds combustible over a platinum catalyst. Oxygen coming from a generator at a constant rate mixes with the carrier-gas stream and passes into a reactor where any combustible materials in the stream are burned. An *oxygen detector* senses an oxygen deficiency caused by the burning and produces a signal which is amplified and fed to the generator, so that its output increases to make up the oxygen deficiency. A recorder or integrator records the extra amount of oxygen needed, from which the amount of each solute is calculated without reference to calibration standards.

The properties of various GC detectors are given in Table I, page 14.

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TABLE I
SUMMARY OF DETECTOR PARAMETERS (*8*)

Detector	Principle of operation	Selectivity	Sensitivity (gm/sec)	Response (coul/gm)	Linear range	Minimum detectable quantity (gm)	Stability	Temp limit °C	Carrier gas	Remarks
Thermal conductivity	Measures thermal conductivity of gases	Universal—responds to all compounds	6×10^{-10}	—	10^4	2–5 μg 10^{-5} of CH_4 per ml of detector effluent	good	450	He, H_2, N_2	Non destructive—requires good temp and flow control. Simple, inexpensive and rugged
Gas density balance	Difference of molecular weight of gases	Universal—responds to all compounds whose MW differs from carrier gas	Variable in range of T.C.D.	—	10^3		good	Better sensitivity 150	N_2, CO_2, Ar	Good for analysis of corrosive compounds. Non-destructive
Flame ionization	H_2–O_2 Flame 2000°C Plasma	Responds to organic compounds Not to fixed gases or water	9×10^{-13} for Alkanes	0.01	10^7	2×10^{-11} for Alkanes	excellent	400	H3, N_2	$H_2O + CS_2$ good solvents because no response. Destructive
Electron capture $^3H^{63}$ Ni	$N_2 + \beta \rightarrow e^-$ e^- + sample → loss of signal.	Response to electron adsorbing compounds esp. halogens, nitrates and conjugated carbonyls	2×10^{-14} for CCl_4 5×10^{-14} for CCl_4	— —	5×10^2 50	10^{-13} for lindane 4×10^{-12} for lindane	fair fair	225 350	N_2 or Ar+ 10% CH_4	Detector is easily contaminated and easy to clean. Sensitive to water; carrier gas must be dry. Can be operated in pulsed or D.C. mode. Non-destructive
Alkali Flame P compd.	Alkali modified H_2–O_2 flame 1600°C plasma	Enhanced response to phosphorus cmpds.	4×10^{-14}	—	10^3	2×10^{-13} parathion	fair	300	N_2	Destructive—requires flow controller for hydrogen and air
N compd.		Enhanced response to nitrogen compounds	7×10^{-12}	—	10^3	2×10^{-10} azobenzene	fair	300	He	Destructive—requires flow controller for hydrogen and air, high sensitivity operating in starved O_2 mode.
Helium	$He + \beta \rightarrow He^*$ Sample $\xrightarrow{He^*} I_0$	Universal—responds to all compounds	2×10^{-14} for methane	28	5×10^3	10^{-12} for fixed gases	poor	100	He	High sensitivity to bleed precludes its uses with columns other than active solids. Non-destructive
Cross section	β + sample → I_0	Universal—responds to all compounds	10^{-9}	—	10^4	10^{-5}	good	225	H_2 or He+ 3% CH_4	

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TABLE I (continued)

PERFORMANCE CHARACTERISTICS OF OTHER GAS CHROMATOGRAPHY DETECTORS (*9*)

Detector	Detectability, sample weight	Universal (U), specific (S)	Species detected	Carrier recommended	Complexity factor[a]	Linearity (some estimated)	Other comments
Thermistor	Nanogram	U		He, H_2	1	50 (est)	Best detectability at low temperature Very small detector volume
Argon	Picogram	U		Ar	3	10^5	Uses unpopular Sr^{90} source No sensitivity to methane
Microwave emission	Subnanogram	S	Halides, phosphorus	85% He, 15% Ar	9	10^3 (est)	Low detector pressures recommended Good specificity to all compounds
Photoionization	Picrogram	U		Ar, H_2, N_2	8	10^5	Application: fixed gas analysis
Nonradioactive helium ionization	Subnanogram	U		He	4	10^4 (est)	Application: fixed gas analysis
Radio frequency	Nanogram	U		He, Ar	4	10^3	Application: fixed gas analysis
Radio gc	Subnano-Curie	S	^3H, ^{14}C	N_2, He, Ar	8	10^4	Very high specificity factor to nonradioactive species
Ultrasonic	Nanogram	U		He, H_2, Ar, N_2, O_2, Air	6	10^6	Best for fixed gas analysis Use of any carrier eliminates solvent
Microcoulometric	Nanogram	S	Halides, sulfur, nitrogen	N_2, He, Ar	6	10^4 (est)	High specificity factors to all interferences
Electrolytic conductivity	Nanogram	S	Halides, sulfur, nitrogen	N_2, He, Ar	5	10^3 (est)	High specificity factors to all interferences
Reaction coulometric	High nanogram	U	Organics	N_2, He, Ar	7	10^4	Can be used to detect O or H in sample molecule Self calibrating
Infrared spectrometer	High microgram	S	Ir absorber	He, N_2	10	none	Special interfacing to gc required
Mass spectrometer	High nanogram	U		He, H_2	10	10^6	Special interfacing to gc required
Far ultraviolet spectrometer	High nanogram	S	UV absorber	He	9	None	Application: low molecular weight organics Special interfacing to gc required
Spectrophotofluorometric	Nanogram	S	Fluorescent compounds	N_2	8	10^3	Special interfacing problems

[a] Author's arbitrary rating on scale from 0–10 (higher numbers—more complexity). Complexity factor considers number of parameters to be adjusted and the amount of accessory equipment needed.

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F. QUALITATIVE ANALYSIS

To begin this section some terms will have to be defined (see Figure 6).

1. The *Uncorrected Retention Volume* (V_R) is the volume of gas required to elute the peak of the constituent in question:

$$V_R = t_r F_C \quad [9]$$

where t_r is the retention time from injection and F_C is the flow rate of the carrier gas measured at the exit of the column. Since the chart paper moves at a constant rate, t_r is measured as a linear distance along the paper, and the same distance can be termed V_R (Figure 6). In other words, the abscissa of Figure 6 can be expressed in inches (or cm), time or volume.

2. The *Dead Volume* or *Gas Holdup* (V_M) is the uncorrected retention volume of the nonsorbed air peak. This volume is in the injection port, tubing, detector and column. The dead volume in the column is called the void or interstitial volume by workers in liquid ion-exchange chromatography.

3. The *Adjusted Retention Volume* (V_R') is the uncorrected retention volume minus the dead volume:

$$V_R' = V_R - V_M. \quad [10]$$

4. The *Corrected Retention Volume* (V_R^0) is

$$V_R^0 = jV_R \quad [11]$$

where j corrects for differences in pressure at the column inlet and outlet (the pressure drop):

$$j = \frac{3}{2}\frac{(\text{inlet pressure/outlet pressure})^2 - 1}{(\text{inlet pressure/outlet pressure})^3 - 1} \quad [12]$$

$j \cong 0.65$ for the typical case when the inlet pressure is double the outlet pressure. V_R^0 is in effect based on the average flow through the column. The inlet pressure is measured by inserting a special commercial device for this purpose into the injection-port septum.

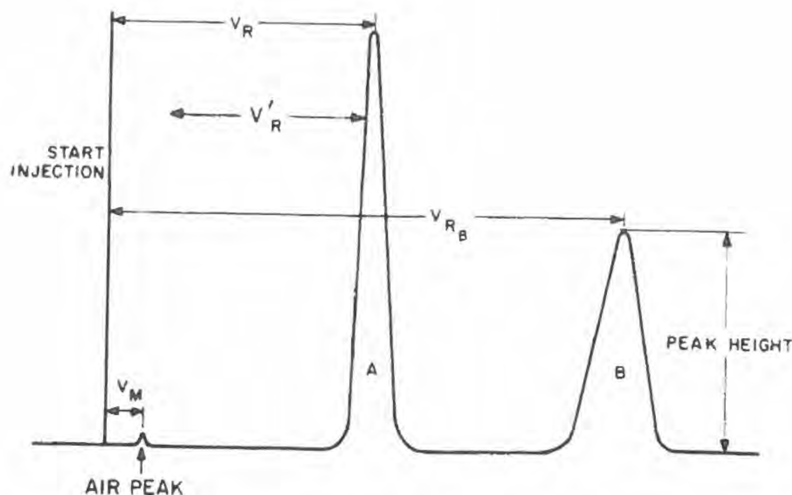

Figure 6. Retention ratio and peak-height calculations from a chromatogram.

5. The *Net Retention Volume* (V_N) is corrected for both pressure differences and dead volume:

$$V_N = jV_R' = j(V_R - V_M). \quad [13]$$

6. The *Specific Retention Volume* (V_g) is the net retention volume at 0 °C with one gram of liquid phase in the column:

$$V_g = \frac{273 V_N}{T W_L} \quad [14]$$

where T is the column temperature in degrees Kelvin and W_L is the weight of the liquid phase.

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7. The *Relative Retention Volume* (r) is the ratio of any of the above retention expressions for a solute relative to a standard component in the same column under the same conditions. If peak B is the standard in Figure 6, r for peak A would be the distance in cm to peak A/distance in cm to peak B, both measured from the air peak, or:

$$r = \frac{V_R}{V_{R_B}}. \qquad [15]$$

Values of r are dependent only on the column temperature and the liquid phase.

The basis of qualitative analysis is that retention volumes reflect the thermodynamic partition coefficients of the solutes. The partition coefficient (K) (defined above in equation [4]) is related to the retention volume as follows:

$$V_R^0 = V_G + KV_L \qquad [16]$$

where $V_G = jV_M$ and V_L is the volume occupied by the liquid phase in the column. Further,

$$V_R^0 - V_G = KV_L = V_N. \qquad [17]$$

Therefore,

$$K = \frac{V_N}{V_L}. \qquad [18]$$

In practice, identification can be made by comparing uncorrected retention volumes for the unknown and standards run at the same time under identical conditions in several systems of very different polarity. It is better to measure adjusted, corrected relative retention volumes. These values should be relatively constant from day to day and lab to lab and can be compared with lists of data compiled earlier for standards to make a tentative identification of unknown peaks.

The Kováts retention index system for reporting retention data is the most precise and repeatable and is becoming widely accepted. This system is based on retentions relative to n-alkanes and the retention index is a logarithmic interpolation between two standards. The equation for the relative retention index is

$$I = 100\left[n\frac{\log R_X - \log R_Z}{\log R_{Z+n} - \log R_Z} + Z\right] \qquad [19]$$

where R_X is the retention time of unknown substance X, R_Z is the retention time of the normal alkane having z carbon atoms, R_{Z+n} is the retention time of the normal alkane having $z + n$ carbon atoms, and n is the difference in the number of carbon atoms for the normal alkanes. The less widely used methylene unit value for characterizing peak positions is obtained by dividing I by 100.

Adjacent members of a homologous series should be about 100 units apart in this system, so knowing the index of any one member gives an idea of the values for all others. Also, the difference in the retention index for a compound on two different columns (polar and nonpolar) gives information concerning its structure. The retention index is temperature dependent. [For details, the reader is referred to Ref. (*10*).]

Another system of indexing GC retention data is the Arithmetic Index of Harbourn (*11*) which is defined by the expression

$$I_A = 100N + 100\left[\frac{R_X - R_N}{R_{N+1} - R_N}\right] \qquad [20]$$

where R_X is the retention time of the unknown peak, R_N and R_{N+1} are the retention times of n-alkane standards and N is the number of carbons in the lower molecular weight standard. This system is mathematically simpler than the Kováts Index and requires only raw retentions rather than adjusted data.

Members of a homologous series can be identified by a plot of log retention time *vs* number of carbon atoms. Once the plot is established with two or three members of the series, other members falling on the line may be identified. Different classes of compounds yield lines of differing slopes.

Information helpful in making identification can also be obtained by splitting the column effluent and passing it through two detectors (e.g., flame ionization and electron capture) sensitive to different compound types. The response ratio is characteristic of different compounds.

The method of reaction GC developed by Beroza for obtaining structural information can be also mentioned. A hot catalyst bed placed at the injection port strips off the functional groups of the solutes and allows the carbon skeleton through. Depending upon the exact conditions, hydrogenation, dehydrogenation or hydrogenolysis may occur. Ozonolysis and subtraction reactions have also been carried out in conjunction with GC (*12,13*). The catalytic hydrogenation method of Franc (*14*) allows a portion of the sample to pass unchanged while the rest is hydrogenated. The difference in retention of the hydrogenated and nonhydrogenated components is a function of the original structure of the material.

Molecular weight and retention data in combination are often adequate for positive qualitative identification of compounds. The technique of mass (molecular weight) chromatography provides this information.

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In this method, a sample is split and analyzed simultaneously with two matched columns and two gas density detectors using different carrier gases. Molecular weight is calculated from

$$MW_X = \frac{[K(A_1/A_2)](MW_{CG_2} - MW_{CG_1})}{K[(A_1/A_2) - 1]} \quad [21]$$

where K is an instrumental constant, A_1 and A_2 are the area responses for the unknown compound in the two detectors, and MW_{CG_1} and MW_{CG_2} are the molecular weights of the two carrier gases. See Reference (*15*) for details of procedures and equipment.

Finally, nonvolatile substances can be pyrolyzed with a heated filament, electric furnace or laser, the pyrolyzate chromatographed, and the resulting chromatogram (pyrogram) used as a fingerprint to identify the substance by comparison to pyrograms of known standards (*16*).

For positive qualitative identification, gas chromatography must usually be combined with other analytical techniques. The column effluent is collected and then analyzed by wet or instrumental methods or it is fed directly to a colorimeter (after reaction with an appropriate reagent in a bubbler), or to an infrared, mass, flame-emission or atomic absorption spectrometer.

G. QUANTITATIVE ANALYSIS

Quantitative analysis is based on a detector response that is linear with respect to concentration (Figure 7), hopefully over a wide range of concentrations. The accuracy of any GC analysis is limited by the overall error encountered, including errors due to the sampling technique, possible sample decomposition (which is checked by comparing IR spectra taken before the injection and on the column effluent), detector and recorder performance, integration, calculations, etc. With good technique (precision), accuracy can be obtained by employing standards properly. Results of an analysis are reported as the average ± the standard deviation of the replicates.

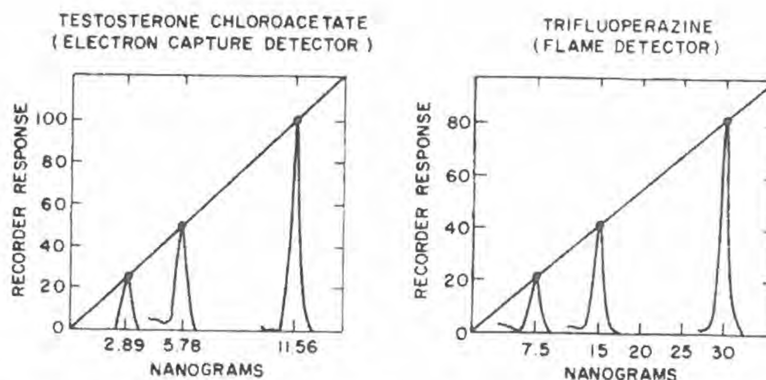

Figure 7. Linear response of the HP Model 402 High-Efficiency GC to nanogram amounts of testosterone chloroacetate and trifluoroperazine (reprinted with permission from Hewlett-Packard, Avondale, Pa.).

Peak areas (or heights) may be employed for analyses in two ways: In the absolute calibration method peak area is plotted *vs* weight for a series of standard solutions, and the weight of the unknown is read from the curve. In this method it is critical that the injections be precise and that the response of the detector remains constant for all solutions. A better method employs internal standardization wherein a calibration

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curve is prepared from known weight blends of the sample and standard and the ratio of areas of the sample and standard peaks are plotted *vs* the weight ratios. A known amount of the standard is also added to the unknown and from the area ratios of the peaks the weight ratio is read from the graph. The sample preparation is most critical in this method.

The percentage composition of the sample is obtained by normalizing the peak areas. For component X among n components:

$$\%X = \frac{A_X F_X}{\Sigma A_n F_n} \quad [22]$$

where A is the area of the peaks and F is the factor to correct for different detector response. The detector must especially be calibrated when working with low molecular weight, dissimilar compounds.

Peak areas are obtained in various ways. Listed in order of increasing precision these are: planimetry, triangulation, height x width at the baseline or at some fraction (usually 0.25 or 0.50) of the peak height, cutting out peaks from chart paper and weighing, disc integration and computer methods.

The recommended methods for quantitative analysis are internal standardization combined with:

(1) Peak height (see Figure 6)—This is useful with sharp peaks as obtained with temperature programmed GC. With good flow and temperature control, 2% relative standard deviation can be obtained, and the method is quite fast.

(2) Disc integration increases precision to better than 1%.

(3) Digital (computer) integration saves much time and increases precision to around 0.5%.

If a fast read-out computer is available, bracketing standards can conceivably be run before and after each unknown. This continual standardization would allow the instrument to be run under less-demanding conditions of flow and temperature control and would permit, in general, cheaper instruments to yield results with good accuracy and precision. The computer can perform all operations on a chromatogram that an analyst can, including measurement of peak locations, heights or areas, and can also correct for base-line shift, noise and other factors. The computer can also be coupled to a gas chromatograph and to a neighboring IR spectrometer or other analytical instrument for rapid, accurate qualitative and quantitative analyses.

Digital recording of data combined with digital control of sample introduction and improved control of flow rate and column temperature can lead to great improvement in the reproducibility of retention times. This allows more meaningful interpretations to be made in the determination of fundamental properties (thermodynamic constants) from GC data as well as aiding qualitative identification and quantitative reproducibility.

A comparison of integration methods for GC has appeared in the literature (*17*).

H. APPLICATIONS

There are presently some 70–80,000 gas chromatographs in use, and they are being used to solve all kinds of scientific problems. GC can separate only gases and volatile substances. However, since only very small samples are required, vapor pressure of the substances need only be a few mm of mercury. Certain columns can be operated in excess of 400 °C at which temperature most compounds, even high-molecular-weight organic compounds, have appreciable vapor pressures. Alternatively, compounds can be made into derivatives with suitable vapor pressures (see Section II.H). Carbohydrates, for example, have been successfully chromatographed as alkyl ethers, acetals and ketals, acetate esters and trimethylsilyl ethers. Hyper-pressure GC (e.g., up to 3100 lb/in.²) allows the satisfactory separation of nonvolatile compounds and compounds which are thermally unstable in some cases (*18*). Figures 8–10 show typical separations achieved by GC.

Many inorganic compounds are readily separated by GC. Various metal chlorides, fluorides, alkyls, chelates, carbides, oxides, sulfides and nitrates have been separated and quantitatively determined.

I. INSTRUMENTS AND COLUMN PACKING

Gas chromatographs and accessories are available in many price ranges from many different manufacturers. Columns can be purchased already packed or can be packed by the worker himself. Once set up, it is best to leave instruments always on with a trickle of gas flowing through. Most instrument manufacturers have active research divisions and are happy to try to answer specific questions such as the choice of a column for a specific separation, the best detector to use, the best temperature and flow rate, etc.

1. *Coating the support.* The support is chosen to be finely divided (2–5 meter ^{2}/g) and should have a uniform particle size within the range of 60–100 mesh. If the limits are closer (e.g., 90–100 mesh), the column will be more efficient.

Liquid is coated on the support as follows: weigh out the required amount of liquid phase relative to the amount of support to be coated and dissolve the phase in a sufficient volume of volatile solvent to cover the support entirely. Mix together the phase, the support and the solvent and shake to form a wet slurry. Evaporate the solvent by carefully warming the slurry, with shaking, under vacuum; a rotary vacuum evaporator is convenient to use for this purpose. After the solvent has evaporated, further dry the coated support in an oven at 90 °C.

Another method for preparing the packing is *solution coating*. This procedure is simple and yields uniform distribution of the stationary phase on the support and is especially recommended if the concentration of the stationary phase is below 5% on the support. The method involves wetting the support with a

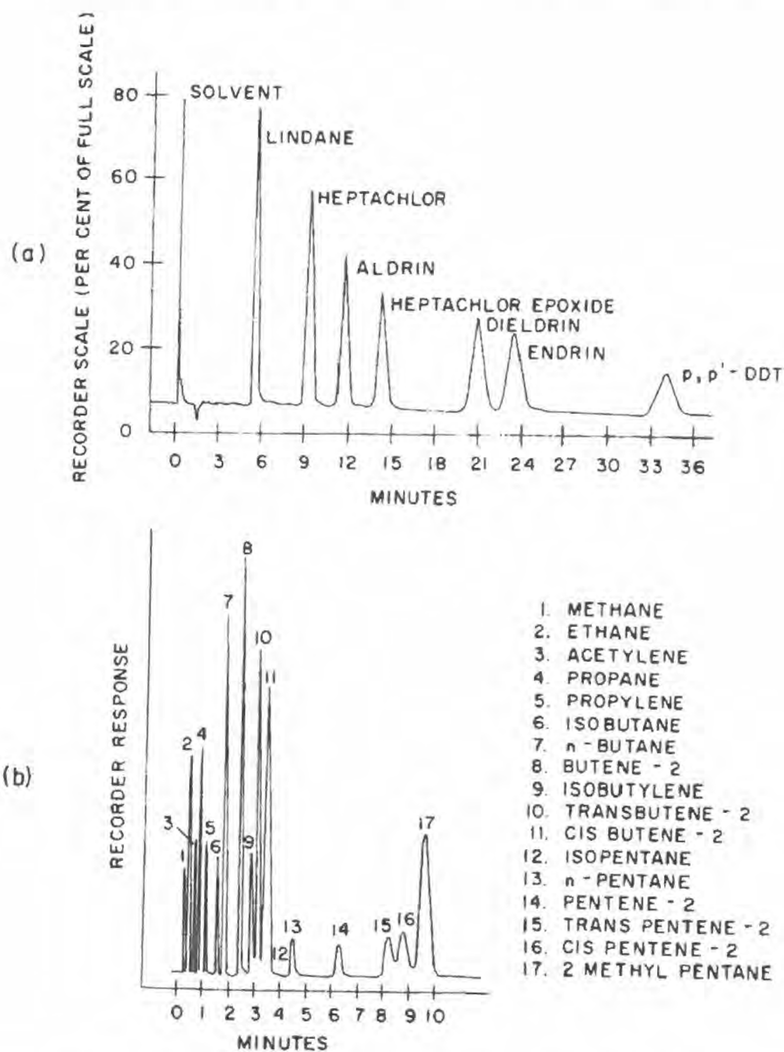

Figure 8. Isothermal GC separation of (a) pesticides on Gas-Chrom Q (silane-treated diatomaceous earth support), 100–120 mesh coated with 10 wt. % D.C. 200. Column: 6 ft × 4 mm I.D. glass U-tube. Detector: Electron Capture. Column temperature: 200 °C. Carrier gas flow rate: 70 ml/min at 25 psig inlet pressure. Sample: 1.0 ng of each component. [Reprinted with permission from Gas-Chrom Newsletter, vol. 9, May, 1968, Applied Science Laboratories, Inc., State College, Pa.]; and (b) C_1-C_5 hydrocarbons on 1.5 m × 0.23 cm column of Durapak *n*-Octane/Porasil C, 120–150 mesh; 25° C; N_2 carrier gas at 25 ml/min; flame ionization detector. [Reprinted with permission from Waters Associates, Framingham, Mass.].

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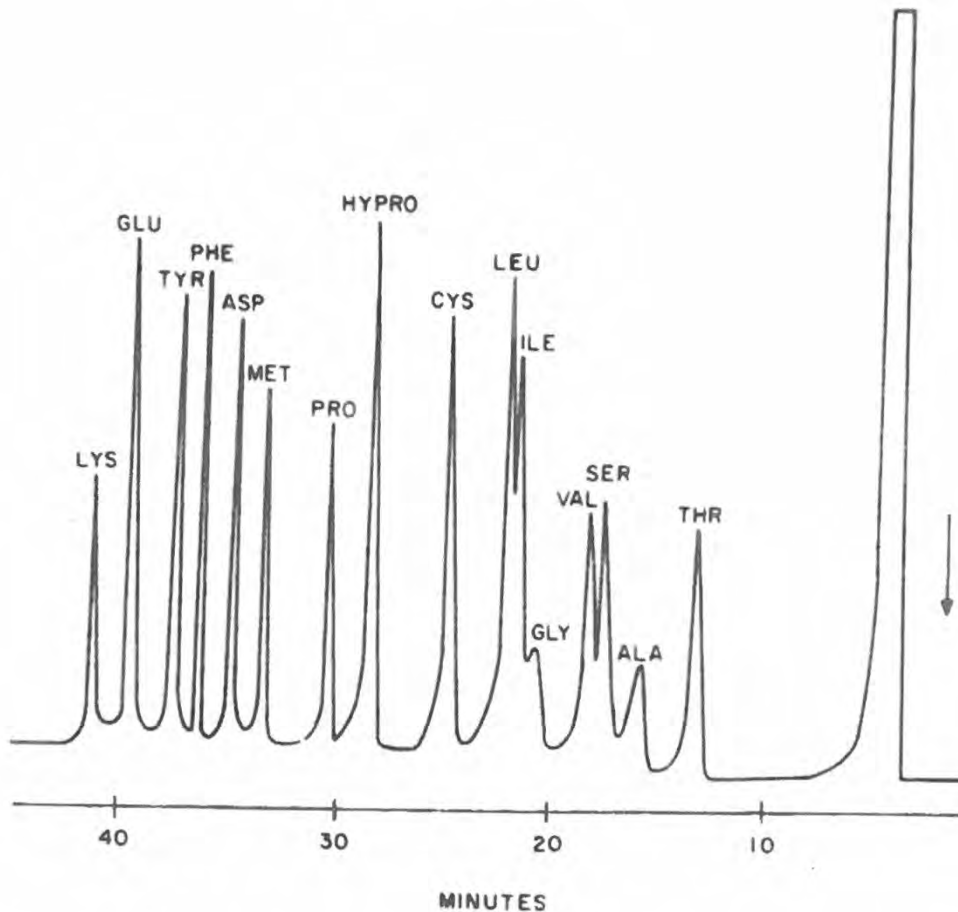

Figure 9. Gas chromatographic separation of amino acid derivatives (N-TFA-O-butyl esters) in a stainless steel capillary column (75 m long by 0.076 cm, I.D.) coated with Polysev [m-bis-m(phenoxyphenoxy)-phenoxy benzene]. LKB 9000 gas chromatograph-mass spectrometer combination. Helium carrier; 1054 kg/cm². Isothermal at 85 °C for 5 minutes, then programmed at 2 °C per minute to 200 °C [after (*19*)]. From Figure 1, Gelpi et al., *J. Chromatogr. Sci.* 7, 605 (1969). Permission to reproduce from Journal of Chromatographic Science, Evanston, Ill.

solution of stationary phase in a volatile solvent. The excess solution is carefully removed with the aid of suction, after which the damp support is air-dried or dried in a commercial packing dryer. The solid is not vigorously mixed with the liquid so that fine particles are not formed.

2. *Packing the column.* To pack the column, a straight piece of metal tubing of the required length is plugged with glass wool at the bottom. A funnel is attached to the top with rubber tubing, and the coated support is added in increments through the funnel. The tubing is agitated and tapped on the floor to aid packing. Finally, the top of the column is plugged with glass wool, and the column is bent or coiled as required. Glass columns are either U-shaped or coiled. The former are packed by introducing coated support through a funnel into one leg of the vertical column and then tapping and vibrating until the solid is well packed. Additional solid is added and the process continued until the column is completely packed, after which glass wool plugs are inserted into both ends. The inlet leg of the column is not packed in the part which will be in the flash heater section of the chromatograph. Coiled columns are packed by plugging one end with glass wool and then applying slight vacuum to this end to suck the packing in increments into the tube. The tube is vibrated throughout to get a firm, uniform packing. When fully packed, the other end is plugged with glass wool.

The ultimate test of whether a column is well packed is its performance in terms of efficiency and peak symmetry. The number of grams of packing used per foot compared with efficient columns used earlier will indicate if the column is either too loosely or too tightly packed and therefore will allow prediction of column performance.

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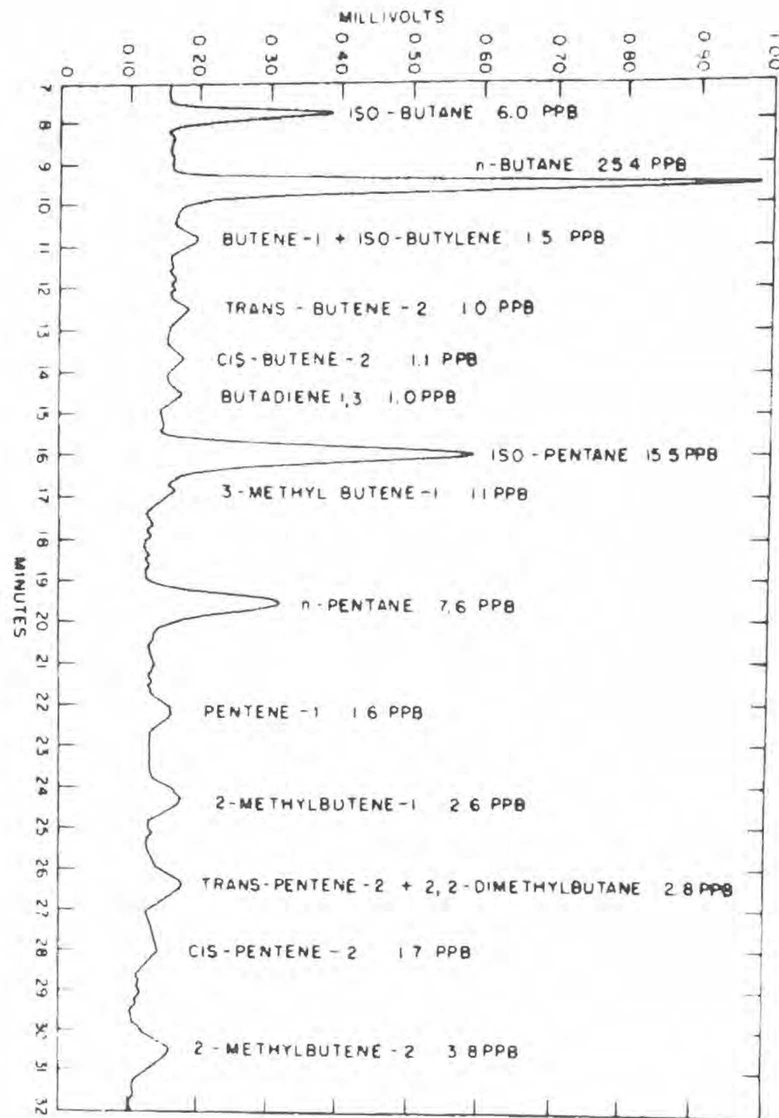

Figure 10. Gas chromatogram of aliphatic hydrocarbons in Cincinnati air. Column was stainless steel $\frac{3}{16}$ inch O.D., 0.035 wall thickness, containing 6 feet β-methyl ethyl adipate and 21 feet dibutyl maleate as the stationary phase. Temperature, 37 °C. Flow rate of helium was 26 cc/min. Sample, 200 cc air. Figure 1, page 245, Giddings/Keller, *Advances in Chromatography*, Vol. 5 "Atmospheric Analysis by Gas Chromatography" by A. P. Altshuller. Permission received to reproduce from Marcel Dekker, Inc., New York.

Both glass and metal columns should be cleaned before packing by rinsing with appropriate solutions such as acids and organic solvents. To dry before filling, attach one end to a vacuum line for 5 min and then flame lightly.

3. *Conditioning the column.* After packing, the column is conditioned to remove any excess liquid or volatile materials from the stationary phase. To do this, connect the inlet of the column to the chromatograph but do not attach the outlet to the detector. Pass carrier gas through the column and heat the column to within 10 °C of the maximum recommended operating temperature for the phase. Keep at this temperature for at least 12 hours. A separate column conditioner may also be used.

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VOLUME TWO
SECTION I

PRINCIPLES AND TECHNIQUES

I.III Liquid Column Chromatography
A. Liquid-Solid
B. Liquid-Liquid
C. Gel Filtration and Permeation
D. Ion Exchange

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Section I.III

LIQUID COLUMN CHROMATOGRAPHY[1]

This section will be divided into four parts according to the mechanism of separation, which reflects the nature of the stationary phase: adsorption, partition, gel filtration and permeation and ion-exchange chromatography. The boundaries between these different types of chromatography are not fixed, since, for example, certain separations on ion-exchange resins can be due to partition, adsorption (on the resin matrix) and molecular sieving effects as well as to simple ion exchange. Much of what is discussed in the section on adsorption chromatography will be generally applicable to all types of liquid chromatography, e.g., many of the procedural details, the theory, the design of automatic machines including the continuous detectors, etc.

A. LIQUID–SOLID (ADSORPTION) COLUMN CHROMATOGRAPHY (LSCC)[2]

For liquid column adsorption chromatography, the stationary phase is a surface-active solid (e.g., alumina, silica gel or charcoal) packed into a column, and the mobile phase is a solvent composed of one or more organic liquids. The separation of a mixture results from the differential adsorption of the components onto the surface of the solid. Weakly adsorbed solutes travel more quickly while strongly adsorbed solutes are retarded. The molecular interactions involved in adsorption can be of several types depending upon the nature (polarity) of the surface, the adsorbed solutes, and the solvent: London dispersion forces, hydrogen bonds, electrostatic forces, and charge transfer forces.

1. *Traditional Techniques*

Many successful separations are still performed today with simple apparatus of the type used by Tswett when he developed LSCC over 60 years ago. For example, Figure 1 shows the successful resolution of the plant pigments which can be obtained on a column of powdered sugar packed into a glass tube. Development can be performed with gravity flow in a tube such as shown in Figure 11(a). Chromatography tubes are fitted with a fritted glass disc or a plug of glass wool to support the solid, and they may or may not have a stop-cock. Development can be speeded up by applying pressure at the top of the column (Figure 11b) or vacuum at the bottom (11c).

If the zones are adequately separated when the solvent front has reached the bottom of the column, as shown in Figure 1, development can be terminated and the zones dug out with a long metal spatula. Each zone is then packed into a separate tube and the solute removed from the adsorbent by elution with a very polar liquid such as ethanol. Alternatively, development can be continued and each zone collected as it emerges from the column. It is often necessary to rechromatograph the separated solutes in order to increase their purity.

If the components of the mixture are not naturally colored as are the chloroplast pigments, the location of the zones must be detected in some way. Most often small fractions of the eluant are collected, and each fraction is analyzed by physical or chemical detection methods for each component. Another method is to carefully extrude the column of adsorbent and to treat it with a reagent which forms colored derivatives with the bound solutes or examine it under UV light if the solutes fluoresce. Columns with a standard-taper joint at the bottom facilitate this extrusion.

2. *Modern Instruments*

Due to an increased understanding of the ways in which separation efficiency varies with experimental conditions and the commercial availability of automatic instruments for separations at high pressures, liquid chromatography has entered upon a new era. These instruments provide generation and control of liquid flows at high pressures, efficient column performance, continuous general purpose detectors, and rapid analyses down to the picogram range. Separations by LSCC can now be faster and more efficient than comparable thin layer separations although they are still not as efficient nor rapid as separations by GC. However, LSCC and GC should be considered as complementary techniques since the former is useful for many samples (e.g., high boiling or heat labile) not successfully handled by GC.

In its crudest form, high pressure liquid chromatography can be carried out by pressurizing a solvent reservoir above a column with gas (e.g., Figure 11a). The basic components of a modern, rapid, high efficiency liquid chromatography instrument are constant flow rate pumps (mechanical or pneumatic; 500–1000 psi minimum pressure), sample injection valves, efficient columns, and detectors. A block diagram of one possible arrangement of basic components is shown in Figure 12. Additional equipment which may be desirable includes a solvent filter and pulse damper after the pump, a column pressure monitor and high pressure alarm, a guard column to protect the analytical column against dirty samples, and perhaps a fraction

[1] See "Modern Practice of Liquid Chromatography", J. J. Kirkland, ed., Wiley, N.Y., 1971.
[2] See "Principles of Adsorption Chromatography", L. R. Snyder, Marcel Dekker, N.Y., 1968.

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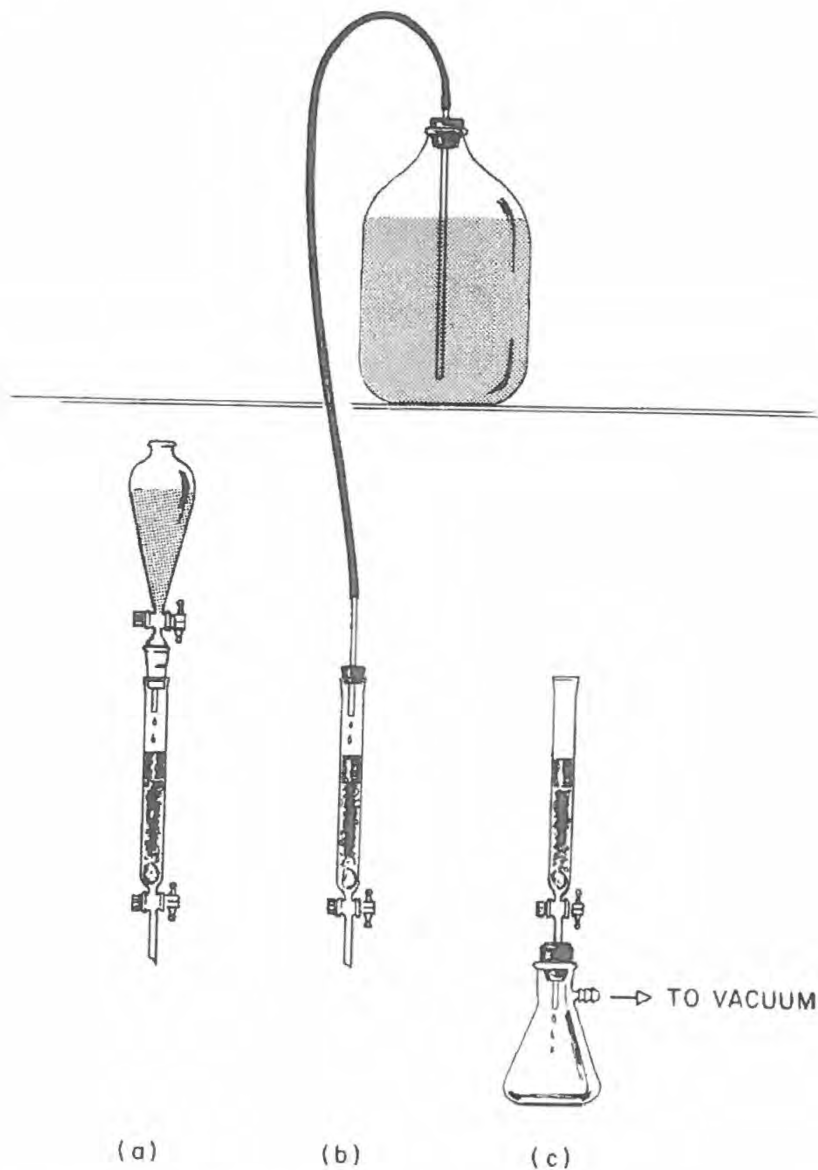

Figure 11. Chromatography tube fitted with a reservoir for holding the solvent (a); fitted with a tube attached to an elevated bottle of solvent for development under the pressure of the column of liquid (b); and inserted into a filtering flask which is attached to a source of vacuum (c).

collector. For high efficiency, the fittings and detector must have low dead volume. Means for precise control of the column and detector temperature may be desirable, especially for partition and ion-exchange chromatography. Precise control of carrier composition and flow rate is also desirable. A diagram of a commercial dual column split-flow instrument (The Waters Associates ALC 100) is shown in Figure 13. This instrument contains a variable-gradient solvent programmer and differential refractive index detector. A second pump and column provide dual-column split flow to the detector; when a UV detector is used, single column operation is satisfactory. In addition to Waters, other companies offering an assortment of LC instruments and accessories are DuPont, Nester Faust and Varian Aerograph.

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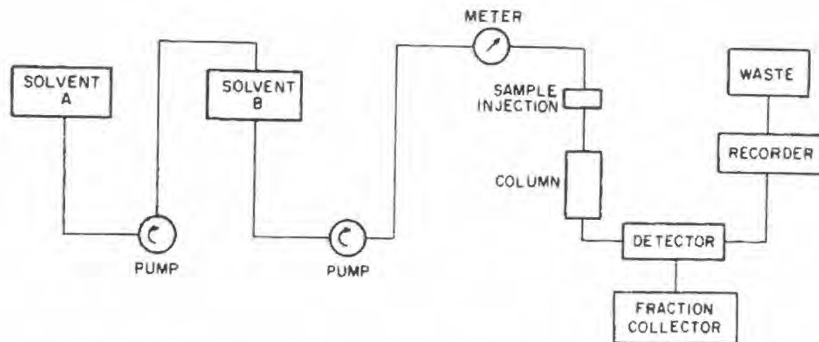

Figure 12. Block diagram showing the basic components of a modern liquid chromatographic instrument.

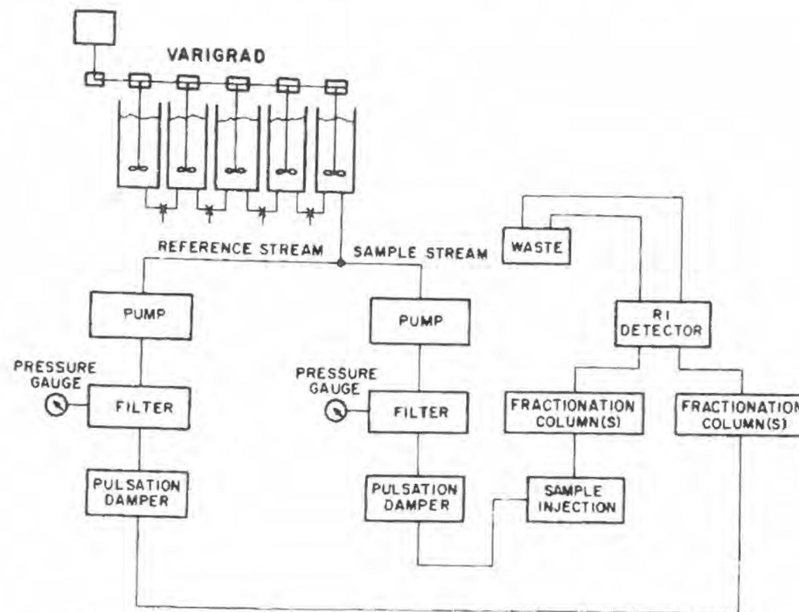

Figure 13. Block diagram of a dual column-split flow liquid chromatography system (The Waters ALC 100), after (*17*); introduced originally in reference (*18*). Permission received from International Scientific Communications, Inc, American Laboratory, and Elsevier Publishing Co.

3. *Theory*[1]

Much of what was written in the section on GC theory applies as well to LC, but additional considerations pertain to modern liquid chromatography. For example, the resolution of zones is directly proportional to zone center separation and inversely proportional to zone width. The former is dependent on the selectivity of the column, the latter on the efficiency of the column. The sources of zone spreading are again flow velocity inequalities, longitudinal molecular diffusion (generally unimportant in LC), and mass transfer considerations.

Gas chromatography is at present a faster and more powerful separation technique than even modern LC. The spreading factor is at least ten times greater in LC (i.e., speed of mass transfer is at least 10 times slower leading to broader peaks). However, LC has the added advantage of being able to utilize two phases for separation, and there are an enormous number of moving and stationary phases that can be used. LC

[1] From "Modern Liquid Chromatography", L. R. Snyder and J. J. Kirkland, American Chemical Society, 1971, Chapters 1 and 3.

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therefore provides a very large range in sample selectivity and can achieve elegant separations of complex samples in many situations. Modern LC has the advantages of greater convenience, greater separation efficiency and greater separation speed compared with classical LC methods (column, PC, and TLC).

In LC, the distribution ratio is defined as shown above for GC (equation [4]) as the ratio of concentrations for a given compound in the stationary (s) and mobile (m) phases

$$K = [X]_s/[X]_m.$$

The larger the K value the more likely the molecules are to be found on the stationary phase and the more slowly the compound will migrate through the column. Differences in K values lead to differential migration through the column.

Individual chromatographic bands are again characterized by t_r, the retention time, and t_w, the baseline band width in time units, or the equivalent volume units $V_R = Ft_r$ and $W = Ft_w$, where F is the flow rate of solvent and W is the band width in volume units.

Another important parameter in LC is the capacity factor (ratio) k' (or K')

$$k' = \frac{\text{total amount of } X \text{ in the stationary phase}}{\text{total amount of } X \text{ in the moving phase}} \qquad [23]$$

$$= V_s[X]_s/V_M[X]_m$$

$$= (V_s/V_M)K = \frac{V_R - V_M}{V_M} \qquad [24]$$

where V_s and V_M are the volumes of stationary and moving phases within the column.

Using the expression above, several other important LC equations can be easily derived. These are

$$t_R = t^0(1 + k') \qquad [24a]$$

where t^0 is the retention time of a nonretained band ($k' = 0$)

$$V_R = V_M(1 + k') \qquad [24b]$$

and

$$V_R = V_M + V_sK. \qquad [24c]$$

V_M, the volume of the moving phase within the column, is the retention volume of a nonretained band.

The above equations are related to peak position in LC, which is a function of the thermodynamics of the system. Equations related to peak broadening are the same as those in the section on GC:

$$N = 16(t_r/t_w)^2$$

or the equivalent

$$N = 16(V_R/W)^2 \qquad \text{(see equation [5])}$$

N, the number of plates, equals the total column length divided by HETP (see equation [7]). Resolution (R or R_s), as given by equation [6], is the distance between peak centers divided by the average peak width.

Adequate resolution between two peaks is a very important consideration in terms of sample analysis. As an example, for two bands representing a 1 : 1 ratio (equal size peaks), a resolution of 0.4 (equation [6]) does not provide observable band centers (both peaks merge into one large peak). If the resolution is raised to 0.6, two band centers are observable, but the apparent band centers do not coincide with the true band centers, leading to an error in the determination of t_r for qualitative identification purposes. Resolution would have to be further increased to 0.7 or 0.8 for the true and observed centers to coincide. An increase to R_s = 1.0 would be required if fractions of 98% purity were to be isolated for analysis by other means (cut point occurring at the valley between band centers). For quantitation based on peak areas accurate within 5% for the minor component, resolution must be adequate to reduce the valley between the two band centers to a value no higher than half the height of the minor band (e.g., R_s must be 1.0–1.25 for bands with a ratio of 4 : 1).

In view of the importance of resolution in sample analysis and preparative separations by LC, it is vital to understand how to control resolution. The basic resolution equation is

$$R_s = \tfrac{1}{4}(\alpha - 1)\sqrt{N}[k'/(1 + k')] \qquad [24d]$$

where α is the separation factor (ratio of k' values for two bands). This equation can be considered in three independent parts, i.e., resolution can be altered by varying α, N or k'.

Varying k'. It has been found that k' values between 1.5 and 4 provide optimum separation (adequate resolution, minimum time). Values of k' are altered by varying solvent strength in adsorption, partition and ion exchange, or the column packing in exclusion chromatography (the solvent normally has no effect on band migration in this method). For example, assume $R_s = 0.6$ for two bands and $k' = 1.0$. To increase R_s to 0.8, k' must be increased to 2.0, an increase that would be brought about, for example, by lowering

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solvent strength. It is not possible, at present to predict which solvent will give exactly the required change, so the change would be made by trial and error.

Varying N. If k' is in the optimum range, increases in plate number should be considered. The typical plot of plate height (H) *vs* solvent velocity (u) for LC indicates that H increases with u but at a decreasing rate. This is a different relationship than exists in GC.

N can be increased most simply by reducing the flow rate, but this leads to increased separation time. If instead the column length is increased (fixed column pressure), a proportional decrease in flow rate will result while separation time will increase with the square of the column length. Finally, N can be increased without increasing separation time by increasing pressure and column length simultaneously. This approach is not valid in GC for several reasons, e.g. because of the compressibility of the gas at high pressures, and is one of the keys that allows rapid, high resolution work to be done with modern LC.

For a comparison of these three approaches, assume initial conditions of 100 cm column length, (L), 1000 psi pressure, a separation time of 100 sec, a flow rate of 2 cm/sec and an H of 0.156 leading to 640 plates. A decrease in pressure to 180 psi would double the plate number to 1280 but increase separation time to 560 sec. An increase in L to 164 cm at 1000 psi would also provide 1280 plates, but with an increase in separation time to only 270 sec. This option is, however, generally less convenient. An increase in L to 320 cm and a simultaneous increase to 10^4 psi would provide 1280 plates with the original separation time (100 sec). The preferred option in modern LC for increasing resolution is to use the longest columns and highest flow rates possible, up to the limit of pressure of the available instrument.

Varying α. If k' is in the optimum range and the required change in N is impractical, resolution can be increased by changing selectivity. The approach is to hold solvent strength (k') roughly constant while changing the solvent composition by a trial and error approach. Neher has provided a guide for choosing solvents of different composition but with the same strength [R. Neher. *Steroid Chromatography*, Elsevier Publishing Co., 1964, p. 249].

Snyder has published equations relating the efficiency of presently obtainable columns to the variables p (column pressure), t (separation time) and d_p (particle size)(*1*). For normal columns with the optimum average value for the partition ratios of the components:

$$NQ^2 = 0.34P^{0.3}t^{0.7}d_p^{-0.3} \qquad [25]$$

where NQ^2 is the number of effective plates, a measure of column efficiency which is proportional to resolution. For regularly packed columns (if they could be obtained in practice) with the optimum average partition ratio value:

$$NQ^2 = 0.026P^{0.3}t^{0.7}d_p^{-0.8}. \qquad [26]$$

Equations [25] and [26] show that maximum efficiency (high values of NQ^2) is favored by large values of P and t and a small value for d_p. Since efficiency increases as the 0.3 power of pressure, a ten-fold increase in pressure is required to double efficiency. There is a similar small increase in efficiency as the particle size decreases (equation [25]). There are, of course, experimental difficulties involved with the use of very high pressures and very small particles. If regular columns could be packed with very small particles, equation [26] shows that a substantial increase in efficiency would result. Dr. Snyder predicts that in the near future, with the use of higher pressures (10,000 psi), smaller particles (1 μ) and adsorbents and packing techniques that provide regular packing structures, 20–50 effective plates per sec will be achieved in liquid chromatography.

Direct comparisons of plate efficiencies in GLC and LLC have been made in terms of reduced plate heights, h, and reduced fluid velocities, v:

$$h = H/d_p \qquad [27]$$

and

$$v = \mu d_p/D_g \qquad [28]$$

where H = the experimental plate height obtained from the chromatogram as explained in section IIB, d_p is the particle diameter, μ is the linear velocity of the mobile phase at the column outlet, and D_g is the diffusion coefficient of the solute in the mobile fluid at the column outlet. In one study of sorbed and unsorbed solutes on several supports the most extreme ratio of h values from GC and from LC on identical columns was 0.6 (*2*). Both in GC and LC, performance was found to deteriorate (h increased) when K' (equation [24]) was increased or when the particle size was decreased, the latter being due to the difficulties involved in uniformly packing small particles.

4. *Operational Considerations*

a. *Adsorbents* (Section II.III, Table 3). Adsorbents are activated by heating to remove water from the surface sites. The Brockmann Index is a scale of activity for rating adsorbents from grade I (most active) to V (least active) based on actual results obtained when standard azo dyes are chromatographed on the adsorbent. Grade I alumina, for example, is prepared by heating commercial basic alumina at 400° for 3 hours with occasional stirring. Less active grades are prepared by adding various amounts of water to the grade I

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alumina (3, 6, 10 and 15 ml for grades II–V, respectively). The activity of raw adsorbents can also be lowered by adding salts or by silanization.

Silica gel (SiO_2; also silica, silicic acid, quartz, porous glass) is the most widely used and versatile adsorbent. Grade I silica gel is prepared by heating at 160° with occasional stirring for 4 hr. The activity of this adsorbent is due to the presence of hydroxyl groups (attached to surface Si atoms and of several different types) which hydrogen bond with polar and unsaturated molecules. For modern LC, silica with a surface area of 350–400 m^2/g and a water content of 10% is recommended. The water content is controlled by activation and water addition. The water content affects retention times and so must be held constant. It also affects column efficiency (a dry column is one-third as efficient as one with 10% water), suppresses sample reactions and irreversible adsorption, and increases column linear capacity.

Basic alumina (Al_2O_3) strongly adsorbs all kinds of polar and unsaturated organic molecules. The mechanism of the adsorption is not yet definite, although surface hydroxyl groups do not appear to be involved. Acidic and neutral alumina can be prepared in the laboratory by treating the basic form with appropriate solutions. All three kinds are commercially available. Neutral alumina (pH ~ 7) has the widest range of applications and serves to separate hydrocarbons, steroids, alkaloids, esters, aldehydes, alcohols, weak organic acids and bases, etc. It is employed usually in systems with organic solvents. Basic alumina (pH ~ 10) adsorbs hydrocarbons, aromatics and other unsaturated compounds from organic solvents. It acts as a cation exchanger in aqueous media, adsorbing basic amino acids, amines, etc. Acid alumina (pH ~4) exchanges inorganic and organic anions such as amino acids, aromatic acids and carboxylic acids. The ion exchange behavior of alumina is opposite to that of synthetic organic exchangers (see Section I.III, D), in that resins with acidic groups function as cation exchangers and those with basic groups as anion exchangers.

Kieselguhr (Celite, diatomaceous earth) is a weak adsorbent used for the separation of very polar solutes. It is often used as a filter aid in mixture with more active adsorbents to achieve faster flow rates of solvent, or as a support for a liquid phase in partition chromatography.

Sucrose is a mild adsorbent useful, as described above, for separations of chloroplast pigments. It is best to rub commercial sugar through a coarse sieve prior to use, and to add additional cornstarch in humid weather.

Magnesia [MgO or $Mg(OH)_2$] is commercially available (Sea Sorb 43, Fisher Scientific Co.) in the form of a very fine powder which is mixed with an equal weight of a filter aid (e.g., Celite 545) before use. Magnesia exhibits preferential adsorption for unsaturated molecules and, unlike silica gel, sugar, cellulose, etc., can separate compounds differing only in the arrangement of double bonds.

Charcoal is of two types, nonpolar and polar (oxygenated). Nonpolar charcoal preferentially adsorbs large, nonpolar molecules, primarily by London forces. Oxidized charcoals are similar to alumina and silica gel in their preference for polar and unsaturated solutes.

Other adsorbents which have been used include magnesium and calcium silicate, calcium sulfate, calcium carbonate, zinc carbonate and hydroxyapatite [$Ca_2(PO_4)_3$]. Cellulose is also considered by some to be an adsorbent, but its properties will be discussed in section B3c below.

The ability of particular adsorbents to attract certain solutes is a function of the polarity of the adsorbent. Polar adsorbents such as alumina and silica gel have great affinity for polar compounds (e.g., amino acids, carbohydrates) and require highly polar wash liquids for development (see below). Polar solutes are usually better separated by partition chromatography than by adsorption chromatography. Hydrocarbons and other less polar solutes are better separated by adsorption methods.

b. *Column size*[1]. For a constant amount of adsorbent, column efficiency increases in proportion with the length to width ratio of the column. Ratios of 5 : 1 to 100 : 1 are currently used with traditional techniques. Glass tubes 1 cm I.D. × 25 cm or 2 cm × 30 cm, and 50 ml analytical burets have been conveniently used by the author for small-scale pigment separations, and tubes 8 × 40 cm and 5 × 60 cm for preparative separations. To pack a 30 cm column in the former tube requires about 3 lb of powdered sugar. For modern high pressure instruments, ratios of 100 : 1 to 1000 : 1 are sometimes used. Preferred column dimensions for such instruments are 50–100 cm in length and 2–3 mm I.D.; Straight columns are more efficient than coiled columns. Individual straight columns are connected in series using low dead-volume fittings (also used to connect the column to the detector) to provide longer columns with high efficiency. Columns are usually operated in a vertical position with an upward or downward solvent flow direction.

The ratio of sample size to amount of adsorbent should be roughly 1 : 20–100 for crude separations or preparative work and 1 : 200–2000 for analytical separations of complex mixtures.

Columns for use in LC instruments are usually composed of metal (e.g., stainless steel) or heavy wall glass. Kirkland has reported that the efficiency of "Trubore" glass and precision-bore stainless steel columns is higher than that of columns made with ordinary stainless steel tubing. Other workers have found this to be true only for one packing procedure, and have obtained equal efficiency for other tubes when the packing procedure was optimized for each tube.

c. *Preparation of the column*. Column packings used for both adsorption and partition columns are typically 100–200 mesh (~100 μ) for classical LC and 5–15 μ for high speed ion exchange and 20–50 μ for the other methods. As stated above, smaller particles improve efficiency because of fast mass transfer but greatly decrease flow rates causing higher pressures to be required. A small particle-size range also

[1] For a discussion of columns for modern analytical LC, see J. J. Kirkland, *Anal. Chem.* 43, 36A, 1971.

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improves column efficiency. Mixing a filter aid with a small-particle adsorbent often yields both excellent resolution and reasonable flow rates in classical LC.

Columns can be packed dry or wet. For dry packing, small amounts of adsorbent are introduced into the column and each portion is tamped firmly with a plunger having a flattened end. For coarser adsorbents, vibration of the column may be used in place of tamping. Some workers place the sample directly onto the dry column and then add the solvent, while others prewash the column with the first solvent (or a less polar solvent) before introducing the sample.

For wet packing, the adsorbent is slurried with a solvent (usually the first solvent to be used for the chromatography), and the slurry is poured into the tube. The tube is tapped gently to promote uniform settling. After each portion has settled, excess liquid is drained out (but the liquid level is always kept above the level of the solid) and more slurry is added until a column of the desired height is obtained. Usually the column is then washed with additional amounts of the initial development solvent, after which the liquid level is drained to the top of the column and the sample is introduced.

So that the top of the bed is not disturbed during sample or solvent addition, it can be covered with a circle of filter paper, a loose plug of glass wool, glass beads, sand, etc.

The "best" method for packing a particular column is an area of controversy. The particular packing method employed seems to be more important for wide columns than for narrow ones. For silica gel, some kind of dry fill-vibration-tamping procedure is usually employed; studies by Stewart, *et al.* (*3*) indicate that gentle tamping combined with a dry fill-vibration procedure becomes more advantageous in terms of efficiency as the particle size of the silica decreases. For very small particles, it has been suggested by Knox and Saleem that packing columns under vacuum (so that all particles fall at the same speed and air currents leading to the fractionation of particles of different sizes are eliminated) will lead to improved performance. Polystyrene resins used in ion-exchange and gel permeation chromatography are usually packed by gravity or high-pressure wet-slurry methods (see Section I.IIId below). Soft gels are packed by low-pressure slurry methods (see Section II.III, Table 4). Because columns may be used over and over in most cases, it is worth the initial time and effort to prepare the best one possible.

Alkali-treated open glass capillary columns have recently been employed for the liquid-solid chromatographic separation of DNS-amino acids (*4*). However, the widespread use of open columns does not appear likely for LC.

d. *Sample introduction.* For conventional separations, a small volume of the sample solution is applied as a narrow zone to the top of the column with a pipette. After the sample has just percolated into the bed, one or two small portions of the solvent in which the sample is dissolved are introduced to wash down the walls of the tube. Then a layer of developing solvent is added above the bed, a reservoir of this solvent is attached, if necessary, to the column and the chromatography is begun. As an alternative, the sample solution is slurried with Celite or some of the same adsorbent as in the column, and this solid is added to the top of the bed.

The solvent in which the sample is dissolved may be different than the developing solvent. For example, plant pigments are dissolved in very weakly polar liquids (e.g., petroleum ether) for the initial adsorption and the zone of mixture is then developed with more polar liquids or mixtures of liquids. In this procedure, compounds which fail to dissolve in the least polar liquids may be dissolved by a more polar liquid later on during development. If this does not occur, precipitation effects can cause poor separations or multiple zonation.

It is the best procedure to be sure that the solutes applied are in solution and that the developing solvents used will not allow precipitation on the column. If this requires such polar liquids that the fastest moving (least polar) solutes are then poorly separated, these should be collected and later rechromatographed in less polar solvents.

Injection techniques used with liquid chromatography instruments resemble those described above for GC. Injection of samples can be accomplished with a syringe through a rubber or Teflon septum port, or accessories for automatic injection, available with some machines, can be utilized. In general, use of a syringe is recommended for scouting work and a sample valve for routine work once a system is chosen. A back pressure of up to 2000 lb can be withstood by a syringe; for pressures in excess of this, the flow of liquid is stopped and injection carried out when the pressure falls to zero. Low solute diffusion in LC allows stoppage of flow during chromatography.

e. *Selection of solvents* (*wash liquids*). For elution (as contrasted to frontal or displacement) chromatography, the adsorbed sample is washed with a solvent which provides optimum values of k'. The developing solvent must therefore be selected in relation to the mixture and the adsorbent. In general, weakly-polar solutes require weakly-polar solvents and strongly-polar solutes require strongly-polar solvents. However, solvents that permit only moderate adsorption of certain solutes on a weak adsorbent (sugar, cellulose) will allow great adsorption on a strong adsorbent (magnesia, lime). Therefore, for a given mixture, less polar solvents would be used on sugar than on magnesia. A table ranking various solvents in terms of their elution strength is given below. This table is a compilation of several series published in the literature and should be taken only as a rough guide. In fact, each individual adsorbent has its own elutropic series. For mixtures of liquids, the polarity varies primarily with the concentration of the most polar substances. (See also Table I, p. 38).

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Solvents in Approximate Order of Their Polarity or Eluting Power

(least eluting)	Petroleum ether (hexane)		1,2-Dichloroethane
	n-Heptane		Chloroform
	Decane		*n*-Propanol
	CCl_4		Ethanol
	Cyclohexane		Methanol
	Diethyl ether		Water
	Carbon disulfide		Pyridine
	Benzene		Organic acids
	Esters	(most eluting)	Inorganic acids and bases
	Acetone		

Solvents of high purity should always be used. Commercial reagent-grade products are generally adequate although prior purification (by extraction, distillation or chromatography) may be desirable in some cases. As an example, chloroform contains about 0.75% ethanol as a preservative. If necessary, this can be removed by two extractions with equal volumes of water.

The solvent must not promote decomposition of the solutes; as examples, plant pigments decompose when developed with (or dissolved in) benzene if exposed to bright light; and chloroform-containing solvents may liberate HCl, which can alter chlorophylls and isomerize epoxy carotenoid pigments. The solvent should be compatible with the detection method being used, and should be volatile so that eluted solutes can be easily recovered. Finally, solvents of low viscosity promote fast flow rates and short analyses times when long columns and fine particles are being used.

For modern LC instruments, solvents are degassed by heating the solvent in a bottle while drawing vacuum. Failure to degas can adversely effect detectors and soft gel columns.

For preparative separations, the solubility of the solute in the solvent is an important consideration.

f. *Gradient elution (solvent programming)*. The use of solvent gradients is indispensable in liquid chromatography for increasing resolution and reducing analysis time. Solvent gradients in LC serve the same purposes as temperature gradients in GC: better resolution of poorly-separated, faster moving peaks and faster, sharper elution of well-separated, badly spread late peaks. Gradient elution also decreases tailing. Isocratic conditions are best for quantitative LC, just as are isothermal conditions for quantitative GC.

A major cause of tailed zones is the existence of a convex adsorption isotherm such as illustrated in Figure 14. (Other causes include overloading of the sample, excessive flow rates and chemisorption of part of the sample on active adsorbent sites.) Convex isotherms are quite common in adsorption and ion-exchange chromatography (especially at ordinarily-used solute concentration levels), while linear isotherms are usual in partition chromatography. Concave isotherms, which would lead to zones with fronting, are rare in chromatography.

In zones governed by convex isotherms, tailing occurs because the solute travels more rapidly when present in higher concentration than at lower concentration (i.e., relatively more solute is on the adsorbent than in solution at lower concentration). Due to effects such as diffusion, incomplete equilibrium, etc., it is normal for the concentration of a migrating zone to become uneven, the concentration being highest in the

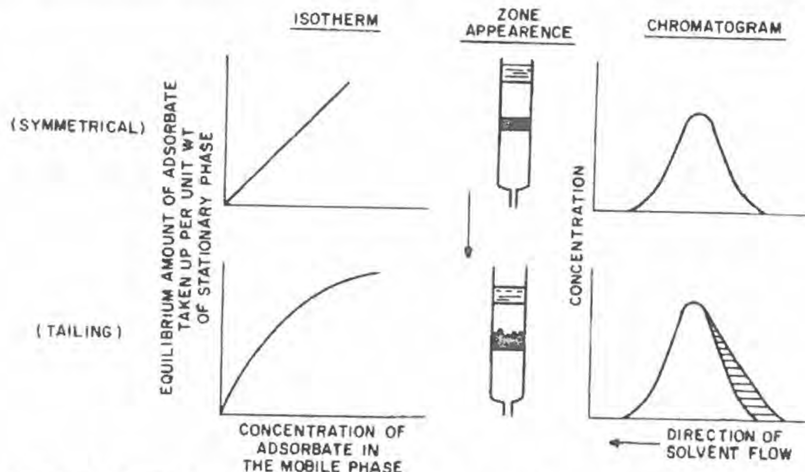

Figure 14. Effect of adsorption isotherms on peak shape [after (19)]. Figure 3, page 733, *J. Chem. Educ.*, 46 (11), 1969. Permission to reproduce from Division of Chemical Education, American Chemical Society.

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center and lower at the leading and trailing edges. When migration is governed by a convex isotherm, the concentrated zone center will move most rapidly, overtaking the front and keeping it sharp. The low-concentrated rear of the zone will move ever more slowly and be left behind as a tail (trail).

Gradient elution techniques involve a steady increase in the effectiveness (e.g., polarity) of the solvent during the run. This leads to the formation of a concentration gradient down the column so that the rear of the zone is maintained in a stronger eluting medium than the front. This causes the rear of the zone to catch-up with the front producing a sharper zone.

In practice, gradient elution can be carried out in a stepwise or continuous fashion. The former is often used for known mixtures where tailing is not significant or for less complex unknowns. It may involve elution of the sample in turn with solvents of the elutropic series, beginning with petroleum ether and increasing in strength, on a column of an active adsorbent. Development is continued with each solvent until nothing more comes off the column, and small volume fractions are collected all along the way. Or, development can be started with a weak solvent (A), one or several changes can be made to solvents containing A plus increasing amounts of a stronger solvent (B), and finally development with (B) is employed. If the mixture to be separated contains known compounds, preliminary studies with the individual, pure solutes can determine exactly what combinations of A and B and what volumes are required for a maximized separation of the mixture.

Continuous gradients are used in modern LC machines and are produced by beginning with a developing solvent of low strength and adding with mixing a stronger component to the solvent in a chamber which drains into the column. Continuous gradients are more convenient in that they are easily reproducible and, once set up, require no attention. A continuous gradient will give superior results for complex or unknown mixtures and badly tailed zones.

The nature of the gradient can be controlled in various ways, the simplest being to vary the absolute volumes and pumping rates of two pumps. If the volumes of the pumps are equal ($V_1 = V_2$) and the pumping rates are equal ($R_1 = R_2$), an almost linear gradient will result (Figure 15a). A useful rule of thumb is to

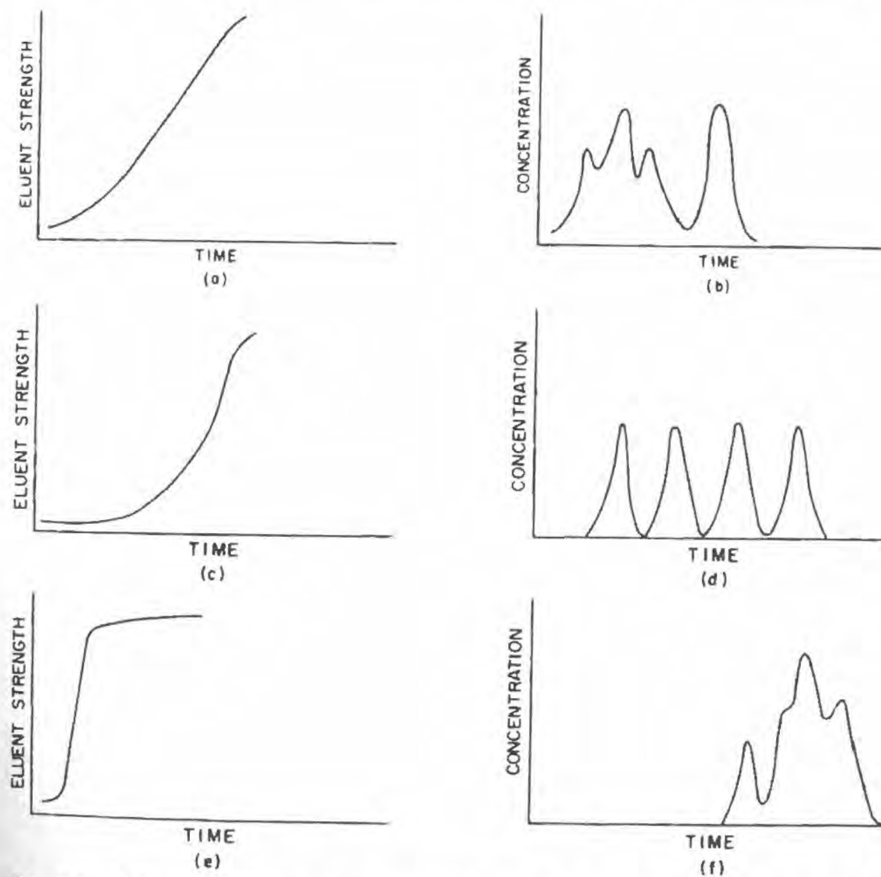

Figure 15. Elution gradients and resulting chromatograms [after (15)]. Permission received from American Laboratory and International Scientific Communications, Inc.

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begin with a solvent only strong (polar) enough to elute the least polar sample component and to end the gradient with a solvent just able to elute the most polar sample. If the chromatogram shown in (b) results from a linear gradient, this indicates that the solvent has become too polar too quickly. By making $R_2 > R_1$ (R_2 would be the rate of addition of liquid to the column, R_1 would be rate of transfer between pumps) a concave gradient (c) would be produced and result in a good separation (d). If the linear gradient causes the chromatogram shown in (f), a convex gradient (e), formed by making $R_1 > R_2$ and $V_1 < V_2$, would cause a more rapid increase in polarity but a lower eventual limit of elution power so that a separation similar to (d) would again result. Concave gradients are more generally useful. More complicated gradients sometimes involving more than two components have been produced in various other ways.

In addition to polarity gradients with organic solvents, pH and ionic strength gradients (separate or superimposed) can be generated in a similar manner. The latter are especially useful for ion-exchange separations (Figure 16).

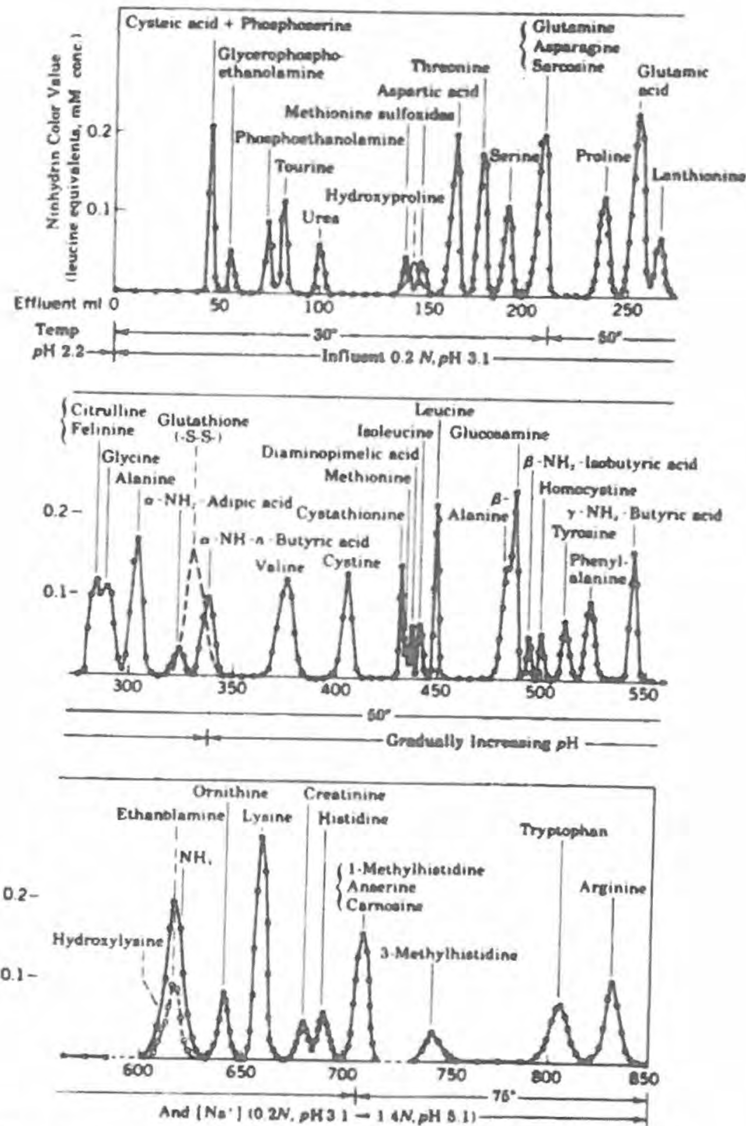

Figure 16. Separation of amino acids and related compounds on Dowex #50-X4 sulfonic-acid cation-exchange resin [after (20)]. Figure 1 from S. Moore and W. H. Stein, *J. Biol. Chem.* **211**, 893 (1954). Permission to reproduce received from authors and The American Society of Biological Chemists, Inc.

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At the end of the run, the column is regenerated by reverse programming. Passing several column volumes of the initial solvent through the bed will strip off the polar solvent and prepare the column for the next separation.

Solvent programming has been used most in adsorption and ion-exchange chromatography. It is generally not used in gel permeation chromatography (K' values are low so it is not needed) and can lead to difficulties in liquid-liquid partition column chromatography if the equilibrium between the stationary liquid and mobile liquid is not maintained as the mobile phase changes (see below).

g. *Mobile phase flow rate.* Although there is an optimum flow rate in terms of minimum plate height, most separations are carried out at flow rates in excess of this rate. Therefore, separations can usually be improved by lowering the flow rate if sufficient time is available. Typical operating conditions for contemporary high pressure column adsorption LC are as follows:

Flow rate	0.5–5 ml/min
Column	½–1 in. diameter, 15–30 in. length
Adsorbent	100–300 mesh
Pressure drop	30–100 psi
Separation times	20 min–12 hr or more

Column lengths can be much greater than shown here.

Solvent velocity changes can be made during the run, and indeed flow programming can be employed to obtain highest resolution and speed in some separations.

Flow rates through the column are controlled by varying the height of the solvent level above the column (gravity methods), by altering the pressure at the column inlet, or by applying vacuum at the column outlet. For qualitative and quantitative analysis by LC, it is important that the flow rate be known so that the time of the run and volume of effluent may be related. Constant flow rate pumps are used in LC machines for this purpose.

h. *Temperature.* Room temperature is most often used for column LC. Alterations in the adsorbent and/or solvent are generally easier and more effective than changes in temperature. Elevated temperature could be an advantage for a solute with a low solubility in the solvent, but the activity of the adsorbent would be decreased at the higher temperature. Temperature programming has been used in combination with solvent programming for separations of macromolecules by adsorption chromatography (the Baker–Williams Method). Temperature programming introduces many problems (e.g., many good solvents cannot be used because of low boiling points, solvent degassers are required at elevated temperature, viscosity changes can adversely effect the results of the separation, etc.), and in LC it is only a minor effect useful in special cases rather than an effective general substitute for solvent programming. Figure 16 shows an example of superimposed temperature, pH and ionic strength gradients used for the separation of amino acids by ion-exchange chromatography.

i. *Comparison of techniques for separating multicomponent samples.* Snyder (5) has compared several techniques on a theoretical and experimental basis for the liquid-solid adsorption chromatography of multicomponent samples which involve a wide range in retention volume values. Resolution per unit time was shown to decrease in order: solvent programming (best) > coupled columns (stationary phase programming) > temperature programming ≃ flow programming > normal elution (poorest). Only solvent programming was found useful for extremely wide range samples.

j. *Detection of resolved solutes.* In traditional procedures, fractions of eluant are collected from the column in constant intervals of time or volume. These fractions are then analyzed for the components of interest by physical (fluorescence, spectral absorption, pH, optical activity, refractive index, etc.), chemical (addition of a reagent to cause the formation of a colored or fluorescent product), nuclear (radioactive tracers, neutron activation) or biological (growth stimulation or inhibition) methods. The presence of the solvent in each fraction must always be considered. Tests on the fractions can be qualitative and aimed only at locating the positions of the zones or quantitative (e.g., a color-forming reagent is added to each fraction and the degree of spectral absorption related to that produced by standard amounts of solutes). Quantitative analysis is also possible on fractions known to contain only one solute by evaporating the solvent and weighing the residue.

Column separations can be monitored by performing paper, thin-layer or gas chromatography on a small portion of each fraction. The number of zones resolved (hopefully one, if the original separation is good) will indicate the composition of each fraction and the relative sizes of the zones will give an idea of the amount of each solute present in each fraction. With TLC, the same adsorbent and solvent as with the column can be used, or various solvents if the column is being developed by gradient elution.

Many types of continuous detectors for automatic LC machines are now commercially available. Short descriptions of some of these follow:

Refractive index detector. The differential refractive index between the solute and solvent is detected. Operation is based on measuring either the change in the bending angle of the light through a wedge-shaped sample of the flowing solvent or the intensity of reflected light, which varies inversely with refractive index (the latter are termed "Fresnel-type" refractometers). With proper solvent selection and instrument design, or solvent evaporation followed by continuous dissolving of the residue in a single solvent, the differential refractometer can be used with solvent programming.

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This is a so-called nonselective or "universal" detector, others of which are based on the measurement of infrared adsorption, dielectric constant and heat of adsorption. Conlon (*6*) states that the minimum sample size (mg) to be used for this detector is roughly the reciprocal of the difference in refractive index between the solvent and the sample. The sensitivity is approximately 10 μg/ml.

Ultraviolet absorption detector. These detectors can operate at several wavelengths, but the predominate 254 nm line emitted by a low pressure mercury discharge lamp is usually used. The detector is sensitive to compounds which absorb UV light (all compounds having π or nonbonding electrons, e.g., carbonyls, aromatic, olefins, nucleotides, nucleosides, and *N*-bases). If a high quality interference filter is used to isolate the 254 nm line, these detectors will have linear response within 1% over the range of 0–3.0 O.D. (optical density) units. This is a selective detector, as are continuous detectors based on fluorescence, acid titration and polarography. This specificity makes the detector relatively insensitive to flow and temperature fluctuations and solvent programming. A single column can be employed or a reference and analysis column. The sensitivity of detectors is usually increased by measuring the difference in a physical property between a reference and sample stream rather than measuring an absolute value of that property. The solvent must necessarily be transparent to UV light. According to Conlon (*6*), the minimum sample size is 10–100 mg, but Bakalyar has shown that the UV detector is capable of detecting 10^{-9} g (*7*), making them about two–three orders of magnitude more sensitive than the refractive index detector. Most UV detectors have flow cell optical path lengths between 1–10 mm.

The UV detector is not temperature sensitive, while the refractive index detector, like all bulk-property detectors, is temperature sensitive. The refractive index and ultraviolet detectors are the only two with any real importance for colorless samples at present. The refractive-index detector is used mainly in gel permeation chromatography, where the use of wider columns obviates its lower sensitivity.

Colorimetric detectors. As an example, carbohydrates and amino acids are detected by adding a color-forming reagent to the column effluent and then monitoring the stream with a visible absorption photometer. A time delay is involved in such a system to allow for mixing of the reagents and development of the color. This and the dilution effect of the added reagent may lead to broadening of the chromatographic zone. Commercial detectors of this type generally employ a visible (4000–8000 Å) source with an infrared filter, photocell detectors and interference filters available in 100 Å increments for the selection of the appropriate wavelength. Some systems have two channels for detection at either of two wavelengths (5700 and 4400 Å for amino acids).

Electrical conductivity detector. This detector is used mainly with aqueous systems. It responds to changes in conductivity between the solvent and sample, and under conditions of constant flow rate and temperature, amounts as low as 10 μg are detectable. This detector has found much use in gel filtration chromatography.

Adsorption detector. This is a differential detector containing two cells: the reference side is packed with a nonadsorbing material (e.g., glass beads), the active side with an adsorbent such as silica gel. Thermistors are imbedded in each cell and made part of a Wheatstone bridge. Temperature changes due to the adsorption and desorption of solutes in the active cell are detected, amplified and recorded as peaks which have the shape of the differential of a Gaussian curve. After early high hopes for this "universal" detector as a column monitor, it has been found to be nonquantitative and insensitive compared to a refractometer or ultraviolet detector under normal elution conditions, and little or no successful work has been reported with it.

Moving-wire flame ionization detector. This detector is at present only crudely quantitative. It is useful with gradient elution systems since the flame ionization detector responds to changes in mass. A moving chain or wire picks up drops of sample as they come out of the column and carries the sample, after removal of the solvent in an evaporating oven, to the flame of a GC flame ionization detector (see Section II, E, 2). In one variation of this detector the sample is burned directly on the wire; in another, the sample is removed from the wire in a pyrolysis chamber, and the gaseous pyrolysis products are swept into the flame. This detector is, of course, destructive.

Scott and Lawrence (*8*) have modified the detector so that the solute obtained after evaporating the mobile phase is not pyrolyzed but is burned in O_2 to CO_2 and H_2O. The CO_2 and excess O_2 are mixed with H_2 and passed over a nickel catalyst to convert the CO_2 to methane, which is detected by a flame ionization detector. An increase in ultimate sensitivity from 4 μg/ml to 1.1 μg/ml and a quantitative, linear response to solute mass are claimed for this modification. The sensitivity increase is especially marked for oxygenated compounds.

Radioactivity detectors. Continuous flow measurement of beta radiation over suspended scintillators provides advantages over the static counting of fractions (*9*). Continuous gamma ray spectrometer detectors have also been used.

With liquid detectors as with the gas detectors, the signal from the detector is amplified and fed to a recorder where peaks representing the eluted compounds are drawn. Retention times are used for qualitative analysis and peak areas for quantitative analysis as described above for GC.

Methods involving the collection of fractions are advantageous because the species present in each fraction can be characterized by several independent chemical and instrumental methods if desirable. The two modes of detection can be combined by continuously monitoring the solvent stream with a nondestructive detector and then collecting fractions at some point beyond this detector.

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k. *Loading and zone detection.* The amount of sample loaded onto the sorbent is intimately related to the detection of the separated zones. There is a minimum load for the determination of each solute after its migration and concomitant dilution. A less sensitive detection method can make a poor separation look better if only the zone centers, but not the more dilute edges of the zones, which might be overlapped, are detected.

In theory, the lighter the loading, the more effective is the separation. Unfortunately, at low loading there is more difficulty in detecting the zones, especially those of minor constituents. At higher loadings these minor zones would be more easily detected, but they might be overlapped by major zones.

In practice, a chromatographic system can be considered to be overloaded when the number of separated zones is less than that observed with lower loading (i.e., some zones have run together). It is underloaded when the number of detectable zones is less than that obtained with slightly higher loadings (i.e., some zones are lost). Another indication of overloading in chromatography is when the chromatogram obtained for a mixture is not simply the graphical sum of the chromatograms for the individual solutes alone. That is, each component should behave the same whether alone or as part of a mixture.

For equal lengths of a packed sorbent, comparable amounts of the sorbent are present in equal cross-sectional regions. This means that for the same degree of separation with the same distance of migration, the loading of a 1 cm I.D. column can be about thirty-one times that of a 1 cm spot in a 0.25 mm thin layer of the same adsorbent. A column 10 cm in diameter can separate 1000–10,000 times more mixture than would be separable from a spot in a thin layer.

In preparative work it is often advantageous to overload the column and sacrifice resolution, the separations being completed by readsorption of fractions of the mixture.

l. *Sample recovery.* Fractions of effluent containing the same compound are combined. The solvents are evaporated under vacuum (a rotary evaporator is convenient), and the residue is further purified, if necessary, by rechromatography and/or recrystallization.

With high speed machines, samples are conveniently collected manually. First a dye sample is injected into the instrument to measure the lag time between the detector and the collection point (typically a few seconds). Then one watches for the proper cut point to appear on the recorder during the separation of interest, adds the time lag, and begins manual collection.

m. *Prescreening systems by TLC.* Preliminary screening by TLC (see Section I.V) on layers of various adsorbents with a range of solvents can quickly provide information concerning the polarities of the solutes present in a mixture and the nature of the chromatographic system which might provide a column separation of the mixture. Results cannot be directly transferred between the two techniques, because the properties of TLC and column adsorbents are not identical. A solvent composed of two liquids, one polar and one nonpolar, which yields a separation of a mixture by TLC will often provide a good separation on a column of the same adsorbent, if the solvent is modified by somewhat lowering the concentration of the polar constituent. In some cases, TLC adsorbents have been used directly as column adsorbents with virtually identical results (*10*).

n. *Quantitative analysis.* Use is made of the internal standardization method when possible. The height or area ratio of the sample peak to the standard peak is plotted *vs* the concentration of sample (mg/ml). The plot should go through zero if the chromatograpby was carried out in a proper manner. Calculation based on peak areas is more accurate, assuming adequate resolution of the peaks.

5. *Applications*

Liquid column chromatography serves to separate and analyze complex mixtures of nonvolatile and thermally unstable substances. Adsorption chromatography is most useful for mixtures containing a wide range of solutes with different functional groups. Partition chromatography is applicable to separations of homologs, especially polar, hydrophilic compounds. Compounds of similar structure which differ in size or molecular weight are separated by gel permeation chromatography, and ionic substances are separated by ion-exchange chromatography (see the end of Section B for a further comparison of adsorption and partition chromatography). Figures 17 and 18 illustrate typical separations by column adsorption chromatography employing a modern LC instrument. Table I, page 38, shows information and guidelines useful in the selection of solvent systems for liquid chromatographic separations.

B. LIQUID–LIQUID (PARTITION) COLUMN CHROMATOGRAPHY (LLCC)

In this method, a column is packed with a solid support of high surface area which is coated with the stationary liquid phase. The mixture is applied as a narrow zone and is developed by passing a mobile liquid immiscible with the stationary liquid through the column. Separations result because solutes relatively more soluble in the stationary liquid migrate more slowly than those with a greater relative solubility in the mobile liquid.

In all cases the solutes are more soluble in the stationary than in the mobile phase. For the separation of polar mixture, therefore, a polar liquid (e.g., water) is the stationary phase and a less polar liquid (an organic solvent) is the developing solvent. This is called normal partition chromatography.

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TABLE I
PROPERTIES OF COMMON CHROMATOGRAPHIC SOLVENTS

Solvent	$E^{\circ}(Al_2O_3)^a$	Viscosity (cP, 20°)	RI	UVcutoff/nm
Fluoroalkanes	−0.25	—	1.25	—
n-Pentane	0.00	0.23	1.358	210
**Hexane*	*0.00*	—	1.375	210
*Isooctane	0.01	—	1.404	210
Petroleum ether, Skellysolve B, etc.	0.01	0.3	—	210
n-Decane	0.04	0.92	1.412	—
Cyclohexane	0.04	1.00	1.427	210
Cyclopentane	0.05	0.47	1.406	210
Diisobutylene	0.06	—	1.411	210
1-Pentene	0.08	—	1.371	—
Carbon disulfide	0.15	0.37	1.626	380
Carbon tetrachloride	0.18	0.97	1.466	265
Amyl chloride	0.26	0.43	1.413	225
*Butyl chloride	0.26	—	1.436	220
Xylene	0.26	0.62–0.81	~1.50	290
**i*-Propyl ether	0.28	0.37	1.368	220
i-Propyl chloride	0.29	0.33	1.378	225
Toluene	0.29	0.59	1.496	285
n-Propyl chloride	0.30	0.35	1.389	225
Chlorobenzene	0.30	0.80	1.525	—
Benzene	*0.32*	0.65	1.501	280
Ethyl bromide	0.37	—	1.424	—
Ethyl ether	*0.38*	0.23	1.353	220
Ethyl sulfide	0.38	0.45	1.442	290
*Chloroform	0.40	0.57	1.443	245
Methylene chloride	*0.42*	0.44	1.424	245
Methyl-i-butylketone	0.43	—	1.394	330
*Tetrahydrofurane	0.45	—	1.408	220
Ethylene dichloride	0.49	0.79	1.445	230
Methyl ethyl ketone	0.51	—	1.381	330
1-Nitropropane	0.53	—	1.400	380
Acetone	0.56	0.32	1.359	330
*Dioxane	0.56	1.54	1.422	220
Ethyl acetate	*0.58*	0.45	1.370	260
Methyl acetate	0.60	0.37	1.362	260
*Amyl alcohol	0.61	4.1	1.410	210
Dimethyl sulfoxide	0.62	2.24	—	—
Aniline	0.62	4.4	1.586	—
Diethyl amine	0.63	0.38	1.387	275
Nitromethane	0.64	0.67	1.394	380
**Acetonitrile*	*0.65*	0.37	1.344	210
Pyridine	0.71	0.94	1.510	305
Butyl cellusolve	0.74	—	—	220
**i*-propanol, *n*-propanol	0.82	2.3	1.38	210
Ethanol	0.88	1.20	1.361	210
*Methanol	0.95	0.60	1.329	210
Ethylene glycol	1.11	19.9	1.427	210
Acetic acid	Large	1.26	1.372	—
*Water	Larger	—	1.333	—
Salts and buffers	Very large	—	—	—

* Most commonly used with U.V. Detector.

[a] The Hildebrandt solvent scale is a list of common solvents used in liquid Chromatography in order of increasing energy of adsorption on alumina. The values are different, but the order is essentially the same on silica gel as on alumina.
The starting solvent selected for a given separation can be chosen by matching the relative polarity of the solvent to that of the sample. This is done as a first approximation by selecting the solvent to match the most polar functional group on the sample molecule. (e.g. alcohols for OH, amines for NH_2 etc.) From this first attempt the separation can be refined by the following procedure:
1. If the sample appears at the solvent front then the solvent is too polar to allow the adsorbent to retard the sample. Go to a solvent higher up (lower polarity) on the scale.
2. Conversely if the sample does not appear in a reasonable time go to a solvent or solvent blend lower down (higher polarity) on the scale.
Solvent blends are most useful when they are composed of pairs which differ in adsorption energy by no more than 100%. (Example propanol at energy 0.82 and chloroform at energy 0.40).
Solvent gradients should be generated on the same basis of no more than doubling the energy of the starting solvent. This range is more than sufficient for most if not all L C separations.
When changing solvents or when regenerating after a gradient, a minimum of 5 column volumes of the new starting solvent should flow through the system before another sample injection is made.

[b] [Information contained in data sheet supplied by Nester/Faust, 2401 Ogletown Rd., Newark, Del., 19711].

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1 5α – CHOLESTANE
2 CHOLESTEROL – PROGESTERONE
3 PREGNENEOLONE
4 TESTOSTERONE
5 UNKNOWN

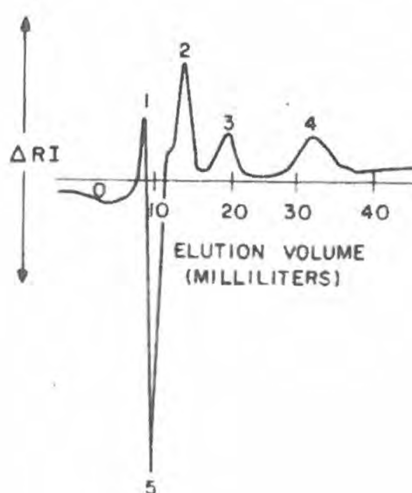

Figure 17. Separation of steroids by liquid-solid adsorption chromatography; instrument: Waters ALC-100 with differential refractometer detector; 1 ft × 1/8 in. O.D. Porasil 60 column; 5 mg sample; solvent: acetone-chloroform (1 : 9 v/v) at 0.9 ml/min; ambient temperature (*21*). Permission to reproduce received from Waters Associates, Inc.

In reversed-phase partition chromatography, nonpolar solutes are separated on a less polar stationary phase (e.g., mineral oil) with a more polar mobile phase (methanol).

The intermolecular forces operative in partition are of the same types as those involved in adsorption, namely hydrogen bonds, induced dipole forces, dispersion forces and specific interaction forces.

1. *Theory*

The theoretical considerations presented in the two sections above are applicable to LLCC. For a theoretical discussion pertaining directly to partition chromatography, the reader is referred to Chapter 6 of Heftmann's *Chromatography* (*11*). The choice of phases in LLCC is largely empirical. Useful guides are previous results from batch studies and paper chromatography. Solvent systems are chosen to provide k' values of 2–5 (see equation [23]).

2. *Columns*

Length to width ratios are similar to those for adsorption, but the minimum is about 20 : 1. The ratio of solute amount to sorbent amount varies greatly but is generally lower than for adsorption separations.

3. *Column Supports*

An ideal support will hold a large amount of stationary liquid but will not interact with the solutes. Various procedures are used to block active support sites, for example, silanization of silica gel and kieselguhr. Column efficiency is increased by reducing particle size and reducing the thickness of the layer of liquid on each particle. Particles with an average size of 30–40 μ are used for modern LLCC.

a. *Silica gel*. A good grade of commercial silica gel need only be dried at ca. 105° for about one hour before impregnation with the stationary liquid. Particles around 200 mesh are typically employed for partition chromatography. Impure silica gel should be washed in turn with 6 *N* HCl, distilled water and methanol prior to drying and impregnation. Ordinary silica gels can adsorb 50–75% of their weight of a polar liquid phase. Whatman SG 34 silica gel is specially prepared with a high pore volume for partition column chromatography. It remains free-flowing even when mixed with 90% of its own weight of water.

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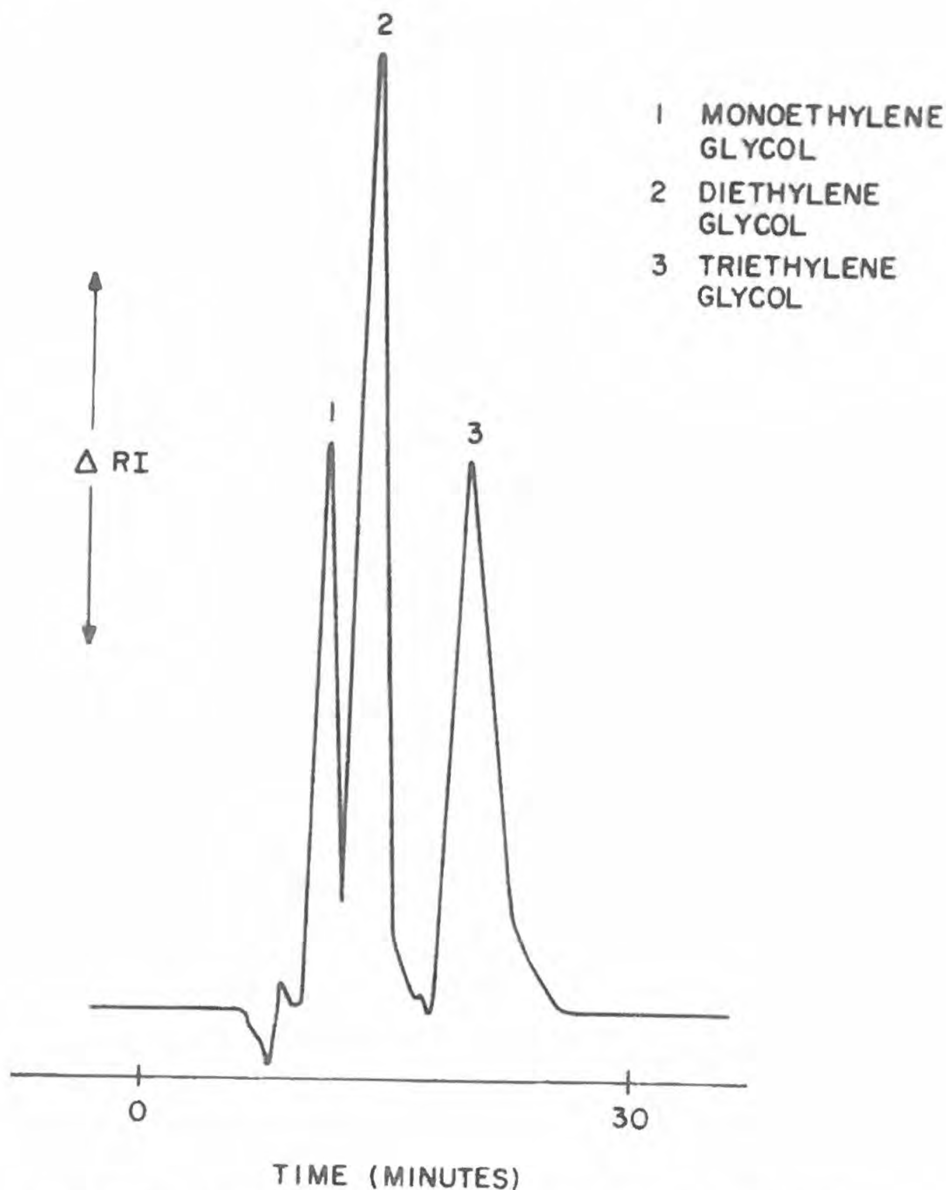

Figure 18. Separation of glycols by liquid-solid adsorption chromatography; instrument: Waters ALC-100 with differential refractometer detector; 1 ft × $\frac{1}{8}$ in. O.D. Porasil 60 column; 5 μl sample; solvent: 2.5% H_2O in methyl ethyl ketone at 0.4 ml/minute; ambient temperature (*21*). Permission to reproduce received from Waters Associates, Inc.

The pore structure of the support has an effect on mass transfer within the particle and therefore on bed efficiency in both adsorption and partition chromatography. Packings with deep pores can cause the formation of pools of stationary liquid leading to broadened peaks. Kirkland recommends the use of controlled surface porosity packings (spherical siliceous particles with a porous surface of controlled thickness and pore size and a solid, impervious core) on which a thin film of liquid is uniformly dispersed for packing more regular (efficient) columns. These coated beads have the advantage of reduced mass transfer distances within the particle leading to sharp peaks, a less steep dependence of h upon v (see eqs. [27] and [28]), and superior efficiency at high velocities. There is some loss in capacity and therefore analytical sensitivity with this type of packing.

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Karger *et al.* (*12*) have employed surface-etched glass beads (30 and 40 μ, Corning GLC-100) coated with 0.2% OPN and packed in Trubore glass columns (50 cm length × 3 mm I.D.) to achieve efficient columns with flow velocities up to 4 cm/sec (ca. 15 ml/min). High efficiencies are possible because of the easily accessible shallow pores of the packing.

b. *Kieselguhr* (*Celite*). This is a more inert material than silica gel and is a very widely used support for partition chromatography, especially the reversed-phase modification. High quality commercial kieselguhr is used as received without further treatment.

c. *Cellulose*. (See also Section I.IV A.) Whether cellulose chromatography is an adsorption or partition process has been argued for years. Because the exact nature of the cellulose surface is unknown, the term "water-cellulose complex" is often used, and chromatography on cellulose (column or paper) can be considered a process in which the rate of migration of a solute depends upon its overall attraction to this complex surface. This attraction can be due to adsorption on fiber sites or at liquid-liquid interfaces as well as to liquid-liquid partition between the mobile liquid and the stationary "gel". In either case, the same types of intermolecular forces are involved.

The polymeric chains of fibrous cellulose are composed of glucose units which are capable of forming many hydrogen bonds due to the presence of hydroxyl groups. Much of this hydrogen bonding occurs between cellulose chains so that fibrous cellulose is considered as a discontinuous linear crystal.

Both fibrous and crystalline Whatman cellulose powders are available from H. Reeve Angel & Co. The fibrous powders yield large-scale column separations which are similar to those that can be made on paper. There is a coarse grind available for more rapid flow rates and a standard grind (passes 200 mesh sieve) for a more closely packed column. Either is available in ashless quality if desired. The crystalline powder is a free flowing material which disperses easily in water. It is available in 100–200 mesh size and provides columns which are generally slower but more efficient than columns of fibrous cellulose. Schleicher and Schuell Co. supplies acetylated cellulose powder in addition to regular cellulose powder (see Section II.III, Table 3).

d. *Other supports*. Other "inert" supports which have been used for reversed-phase partition chromatography include Teflon-6, Kel-F and microporous polyethylene. Recent research has shown that Teflon is not truly an inert support for certain separations despite formation of symmetrical peaks.

Partition chromatography on beds of Sephadex (see Section II.III, Table 4) have been used to separate antibiotics, amino acids, etc. The Sephadex is swollen and packed in an aqueous liquid and the column is then equilibrated for long periods by the flow of an organic solvent.

4. *Solvents*

The solvent system consists of an equilibrated pair of immiscible liquids. One of these is placed on the support and the other is used to develop the chromatogram. A serious difficulty with partition chromatography is "bleeding" of the stationary liquid from the support. The liquids and the experimental conditions must be chosen so as to minimize this effect. Liquids should be chosen which have low mutual solubility (i.e., one should have a low "solubility parameter" and one a high parameter). The phases are saturated with each other by mixing in a separatory funnel before use, or a precolumn may be used to saturate the mobile phase with the stationary phase prior to elution. Polymeric stationary phases (e.g., Carbowax) have low solubility in many solvents, but they are highly viscous so that slow solute diffusion rates and inefficient columns may result.

The "brush" packings developed by Halasz and described above (Section IIC) are produced by attaching the liquid partitioning molecules onto the support by a chemical reaction. The partitioning phase is therefore attached chemically to the support at one end, with the rest of the molecule available to interact with the solutes. The permanently bonded liquid phase will not dissolve in the mobile phase, so bleeding is eliminated. Liquid chromatographic separations generating almost one effective plate per second have been reported with these brushes. Brush packings available from Waters Associates include OPN, Carbowax 400 or *n*-octane on Porasil C. A new Dupont support for LLCC (Permasphase) has chemically bonded silicone polymers which are nonextractable and thermally and hydrolytically stable.

Relative eluant strengths can be estimated for partition solvents using the "solubility parameter" of Hildebrand and Scott (*13*). The solutes should be much (ca. 1000 times) more soluble in the stationary phase than in the mobile phase. Although all the solutes will have a much lower solubility in the mobile phase than in the stationary phase, it is the relative solubilities in the two phases which determine if differential migration occurs. Stationary phases which have been used for normal partition chromatography include water; water + acid, base, or buffers; alcohols; glycols; formamide or dimethylformamide (with or without added water). Mobile phases include higher alcohols (e.g., butanols), hydrocarbons, chloroform, esters and ketones. For reversed phase systems, paraffin, mineral or silicone oil is often the stationary phase and water, lower alcohols or formamide the mobile phase.

5. *Preparation of the Column*

The support is coated with the stationary phase as described above for GC (rotatory evaporator or filtration techniques). A simple procedure useful for fragile supports (diatomaceous earth) involves stirring the support and dissolved phase in a shallow dish while blowing nitrogen or heating with a heat lamp until dry. As a general rule, the highest liquid phase loading which does not badly degrade column efficiency is

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used. This impregnated powder can then be packed dry into the column, after which the sample is applied and development with equilibrated mobile phase begun. Or, the wetted powder can be slurried with equilibrated mobile phase and packed by the slurry method described above. With kieselguhr, each portion of slurry must be tamped down because good columns do not usually result from the action of gravity alone. Cellulose columns can be packed by the slurry method with acetone. The mobile phase saturated with water is then passed through until the column is equilibrated.

For reversed-phase chromatography, dry support is treated with the nonpolar phase dissolved in a volatile solvent. The coated support is air-dried or oven-dried at 60–110°, and the column is then packed by tamping, or by the slurry method after the support is mixed with the equilibrated polar mobile phase.

Many variations of these methods are used by different individual workers to obtain "satisfactory" partition columns.

6. *Column Development*

The sample (25–100 μl for 2–3 mm I.D. columns, containing 1–100 μg solute), dissolved in equilibrated mobile phase, is added to the top of the column. If the sample is not soluble in the mobile phase, it can be dissolved in the stationary phase liquid. This is sorbed on a small amount of solid support which is then put on top of the bed. In general, however, it is best to dissolve the sample in the carrier liquid.

The sample is developed with a single solvent or a stepwise gradient can be used, each new solvent being pre-equilibrated with the stationary phase. Continuous gradient elution is more difficult to employ in LLCC because the equilibrium between the stationary and mobile liquids cannot be perfectly maintained as the mobile phase changes. Portions of the stationary liquid are continually removed from the support causing reduced column efficiency and increased difficulty with detection. Even so, some success has been reported with solvent programmed LLCC, notably in the separation of acidic and basic compounds whose degree of ionization can be controlled. For example, Freeman (*14*) has separated organic acids on silica gel impregnated with 0.5 *N* H_2SO_4 (to retard ionization of the acids) with a gradient of chloroform-*n*-butanol mixtures. Bonded phase supports appear to hold great promise for successful use in gradient elution LLCC.

In place of solvent programming, a systematic screening of the sample in widely differing solvent systems can be employed. Bombaugh suggests the following systems: isooctane-glycol, water-isooctane, isooctane-β,β'-oxydipropionitrile, water-chloroform, or the organic and aqueous layers of ternary mixtures such as water-alcohol-chloroform.

Presaturation of the carrier solvent with the stationary phase is obtained by overnight rapid stirring (use an Erlenmeyer flask and magnetic stirrer to form a vortex) of deaerated phases to produce turbidity. Use of a precolumn (e.g., ¼ × 50 cm) containing 20% stationary phase on 120–140 mesh diatomaceous earth, just before the analytical column and at the same temperature, helps ensure true equilibrium.

7. *Temperature*

The temperature during equilibration must be the same as that used for the separation so that the solubility characteristics of the system do not change during the run.

It is possible that in special cases selectivity could be controlled by temperature programming since the solubilities of the solutes in the two phases, and therefore their distribution coefficients, would be altered. Temperature programming is not generally practical or useful in LLCC, however.

Typical contemporary experimental conditions for partition chromatography as summarized by Gilding (*15*) are as follows:

Bed size	—	20–40 inches length, 1–3 mm diameter
Packing	—	surface textured spherical particles of 20–40 I.D. containing 1–3% liquid loadings
Flow rate	—	0.5–3 ml/min
Pressure drop	—	700–800 psi
Separation time	—	several minutes to 2 hr.

Eluant pressures up to 60,000 lb/in² were used in one study to obtain improved chromatographic resolutions (*16*).

8. *Applications*

Figures 19 and 20 indicate examples of partition separations possible with an LC instrument.

9. *Adsorption* vs *Partition Chromatography*

Presented below is a summary comparison of adsorption and partition chromatography:

a. Larger samples can be separated by adsorption. Low sample capacity can cause problems with detection in partition chromatography.

b. Partition yields symmetrical peaks, adsorption often yields tailed peaks; however, with control of water content on the adsorbent and reasonable sample sizes, linear isotherms and symmetrical peaks can be achieved in adsorption chromatography.

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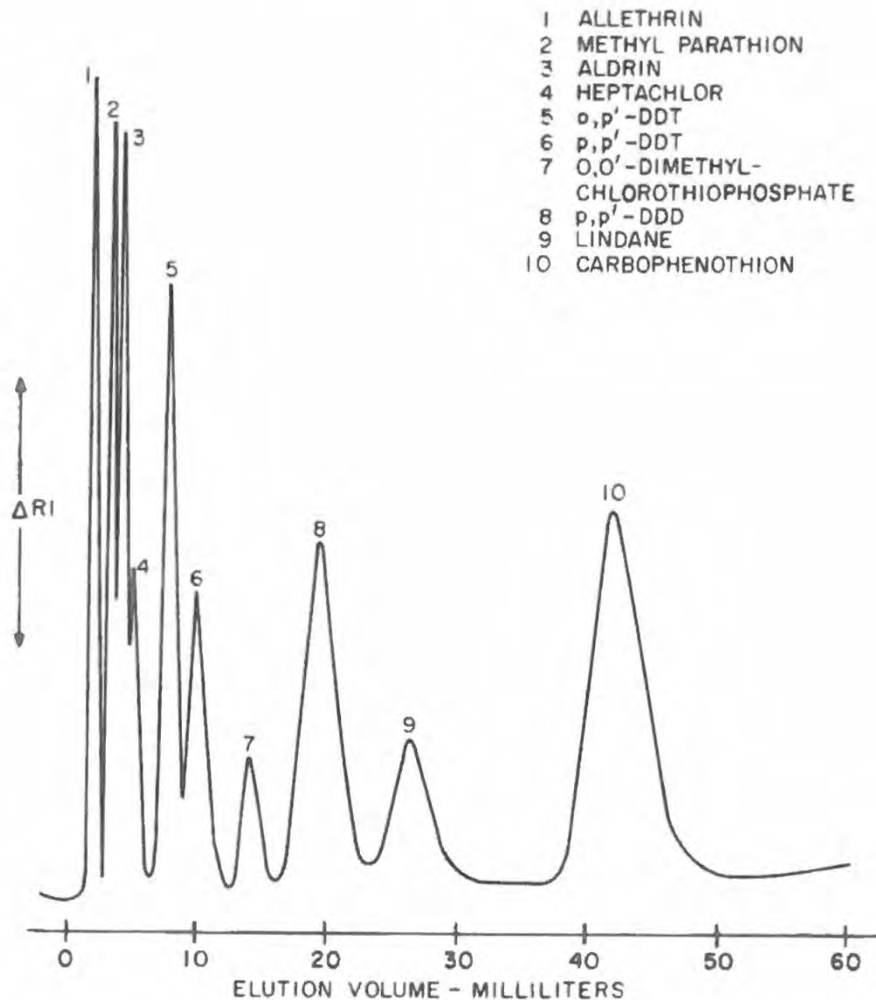

Figure 19. Liquid-liquid partition chromatographic separation of insecticides; instrument: Waters ALC 100 with differential refractometer detector; 4 ft × 0.09 in. I.D. column containing 10% β,β'-oxydipropionitrile on Porasil 60; 20 μl sample; isooctane solvent at 0.9 ml/min; ambient temperature. Permission to reproduce from Waters Associates, Inc.

c. It is easier to predict column results from batch studies in partition, although the literature of TLC is a very useful guide in designing adsorption separations.

d. Polar solutes are best separated by partition.

e. Partition is useful for separating polar, hydrophilic compounds, including homologs. Adsorption is best for relatively nonpolar solutes which differ widely in structure (isomers). Both methods are satisfactory for compounds up to molecular weight of about 10^3. By reversing phases, partition can be applied to nonpolar samples.

f. Adsorption more often causes chemical change of the solutes.

g. It is easier to prepare uniform partition systems than uniform adsorbents; however, it is often not easy to equilibrate partition systems, and in general adsorption is operationally more simple.

h. Partition chromatography is more efficient than adsorption. Partition chromatography permits the highest separation speeds of all four types of LC, is a gentle method, and allows preparation of very reproducible columns.

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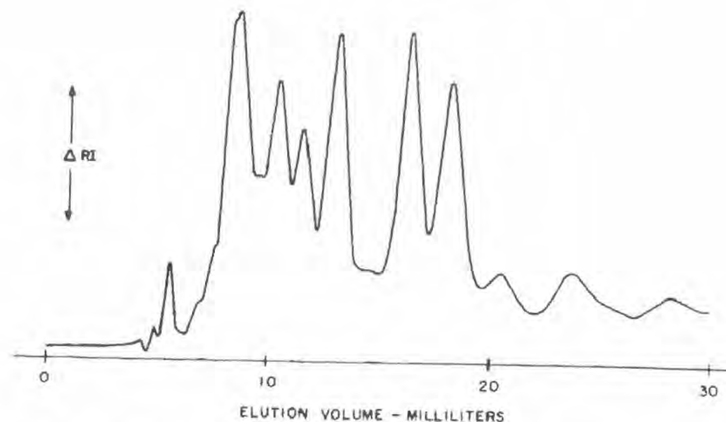

Figure 20. Liquid-liquid chromatography of chlordane on Waters ALC-100, 3 ft × 0.090 in. I.D. column as in Fig. 19; 25 μl sample; isooctane solvent at 0.25 ml/min [courtesy of Waters Associates, Inc.].

C. GEL FILTRATION AND GEL PERMEATION COLUMN CHROMATOGRAPHY

In gel filtration chromatography (GFC), the stationary phase is a solvent-swollen hydrophilic gel in the form of porous beads, and the mobile phase is an aqueous solvent. If the gel is hydrophobic and the solvent organic, the technique is called gel permeation chromatography (GPC). The generalized term "molecular exclusion (or inclusion) chromatography" can be used for both of these. The liquid outside the beads (the mobile phase) and that inside the beads is the same in each case, except that the liquid inside is immobilized.

When a sample containing small and large molecules is applied to the top of the bed and eluted through the column, the solutes will have a differential distribution between the liquid outside and inside the beads. The small molecules penetrate the gel particles and are retarded and eluted later than the large molecules, which will move only in the interstitial volume.

These techniques have had great application in industrial and polymer science (GPC) and biochemistry (GFC) for the determination of the molecular weight distribution of natural and synthetic polymers and for desalting and purification procedures (see "Applications" below).

1. *Theory and Mechanism*

Separations in GPC are based upon the molecular volume of the solute and the distribution of pore sizes available in the gel particles. The size of the molecule determines the percentage of the pore volume available to it; large molecules have no access to the beads and are therefore eluted quickly in the interstitial liquid. Smaller molecules can penetrate the gel to a greater or lesser degree and therefore travel down the column in a more tortuous (statistically longer) path and are eluted later. Polar molecules can be adsorbed by the substrate so that their movement would be slower than that predicted by size considerations alone.

The total volume (V_t) of a gel filtration or permeation bed can be represented by

$$V_t = V_M + V_s + V_g \quad [29]$$

where V_M is the volume surrounding the gel beads (the interstitial volume), V_s is the liquid space within the beads and V_g is the space occupied by the gel matrix. V_M and V_s correspond to the volumes of mobile and stationary liquid in LLCC. Molecules larger than the average pore size of the gel are excluded and remain in the V_M volume of the column. Molecules smaller than the pores are distributed between V_M and V_s and travel at a rate depending upon the fraction of V_s available to them.

The distribution coefficient K (often termed K_d) is then

$$K_d = \frac{V_R - V_M}{V_s} \quad [30]$$

where V_R is the elution volume of the solute. Large, completely excluded molecules have $V_R = V_M$, and $K_d = 0$. Very small molecules have about equal opportunity of being inside or outside the gel, so $V_R \simeq V_M + V_s$, in which case K_d is about 1. K_d values between 0 and 1 are to be expected then for molecules separated on the basis of size alone. K_d values >1 indicate the occurrence of adsorption between the gel matrix and the solute.

In practice, K_d values are difficult to determine because V_s and V_g cannot be easily measured. An alternate expression for solute migration, K_{av}, considers the whole gel ($V_g + V_s$) as the stationary phase rather

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than just the liquid inside the gel (V_s) as in [30]:

$$K_{av} = \frac{V_R - V_M}{V_t - V_M}. \qquad [31]$$

Values of K_{av} are easily obtained in the following manner: V_R for the solute is read from the chromatogram (Figure 6), V_t is calculated from the column geometry, and V_M is taken as the V_R of an excluded molecule, e.g., blue dextran 2000, molecular weight of ca. 2×10^6, for Sephadex gels. [Blue dextran is not eluted from Sephadex if an old solution is used. Further, blue dextran can accumulate on columns even when fresh solutions are eluted, so that protein samples may become bound to such columns causing anomalous results (*22*)].

The number of theoretical plates (N) in GPC, as in other methods, depends upon the conditions under which the column is operated and on the solute of interest. N is determined from the chromatogram using equation [5] as described in Section I.II. To obtain a rough estimate of N for a GPC column, the chromatogram of a single standard compound such as *o*-dichlorobenzene or tetrahydrofuran can be used.

In gel permeation chromatography, unlike sorption chromatography, peak width (W) is virtually constant as elution volume (V_R) increases. (In general, in GC and LC N is constant for different V_R values, i.e., band width increases in proportion to V_R.) For example, polystyrene ($K_d = 0.37$) eluted from 160 ft (40×4 ft columns) of Styragel at 873 ml had $W = 14.5$ ml while *o*-dichlorobenzene ($K_d = 1.0$) was eluted at 1579 ml with $W = 14.8$ ml (*23*). The number of theoretical plates increases with V_R, ranging from 100,000 for polystyrene to 180,000 for *o*-dichlorobenzene, but this represented only a range of 5000 to 36,000 effective plates (n) when correction was made for the dead (void) volume of the system. The fact that peak width values are independent of K_d indicates that all band spreading occurs in the mobile phase and not inside the gel phase in the Styragel system. The greatest difference between N and n values in GPC will occur for those compounds with the smallest K' values (see equation [24]), and the major difference between plate numbers in GC and GPC (in which N is customarily determined for the last peak with $K_d = 1$) is in the small K' values commonly available in GPC.

The peak capacity of a system is the maximum number of peaks resolvable between the volumes of elution of the first and last peaks. The peak capacity in GPC at constant efficiency (i.e., columns which yield equal plates N per unit length) is a function of column length and is equal to

$$\frac{V_R \text{ of last peak} - V_R \text{ of first peak}}{W}. \qquad [32]$$

In the absence of adsorption, the peaks in GPC are limited to a volume range between V_M ($K_d = 0$) and the total solvent volume $V_M + V_s$ ($K_d = 1$), which is roughly $2.5 \times V_M$. Fewer solute peaks can fit into this limited retention range so that with a column of similar dimensions and theoretical plates, the peak capacity is reduced about four times compared to GC and LC. For complex separations, GPC columns must clearly be operated under conditions leading to high plate numbers (e.g., increased column length; recycle), and since the elution range of GPC is limited as explained above, such required conditions can still allow reasonably fast separation times. The most efficient column packing and the optimum packing procedure must be employed.

Semilog calibration curves (molecular size *vs* elution volume) for exclusion packings are characterized by a straight line with a greater or lesser slope in the fractionation range (between V_M and $V_M + V_s$). Gels with a flat slope give better separation of molecules of similar size but cover a small molecular weight range. Gels with a steeper calibration curve cover a larger range but result in poorer separation of adjacent bands. For broad range samples, several different columns are combined to provide the average slope of the individual gels.

2. *Column Packings* (see Section II.III, Table 4).

Gel permeation and filtration column packings function as nonionic molecular sieves, the average pore size of the packing determining which molecules are excluded from entering the gel. A partial description of some packings which have been used follows:

a. *Polystyrene* (Poragel A, Styragel, Bio-Beads). Polymers prepared by cross-linking styrene with divinylbenzene are analogous to polystyrene ion exchange resins (Section III.D) without the ionic exchange groups. They are used for size separations of low molecular weight (ca. 600–50,000) hydrophobic materials with organic solvents. Also available is a Poragel P series containing different functional groups (aryl N, aryl O, hydroxyl and keto) for adsorption separations with aqueous and organic solvents, and Aquapak, a lightly sulfonated polystyrene for size separations with aqueous solvents.

b. *Porous silica* (Porasil). Spherical silica beads with controlled pore sizes and surface areas; can also be used as an adsorbent for LSCC or a support for LLCC. For GPC, deactivation of the silica beads is often necessary so that certain solutes [e.g., poly(vinyl alcohol)] are not adsorbed. Polystyrene is more efficient (by a factor of 4 or 5) than silica.

c. *Porous glass* (Bio-Glas and Corning CPG-10 LC packing). These are rigid glass beads with pore size ranges from ca. 200 to 2500 Å. They are quite inert and stable and are especially useful for separations at elevated temperature or where possible contamination of the sample is a problem.

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d. *Polyacrylamide* (Bio-Gel P). Designed for the separation of aqueous macromolecules below 400,000 molecular weight. They are prepared by polymerizing different amounts of acrylamide and methylene bisacrylamide.

e. *Agarose* (Bio-Gel A and Sepharose). For the separation of very high molecular weight aqueous macromolecules such as nucleic acids, proteins, polysaccharides, certain subnuclear particles and viruses.

f. *Polydextran* (Sephadex). Spherical beads of Sephadex are produced by cross-linking the polysaccharide dextran with epichlorohydrin to produce a highly porous, hydrophilic, sponge-like gel (Figure 21). The degree of cross-linking is controlled by regulating the amount of epichlorohydrin used during formation of the polymer, and this in turn determines the amount of water absorbed per gram (the swelling) and influences the size of the pores in the molecule. Hydrophilic Sephadex gels preferentially adsorb aromatic compounds and contain some carboxylic acid groups (10–20 μeq/dry g) which can interact with various molecules so as to distort gel filtration chromatograms.

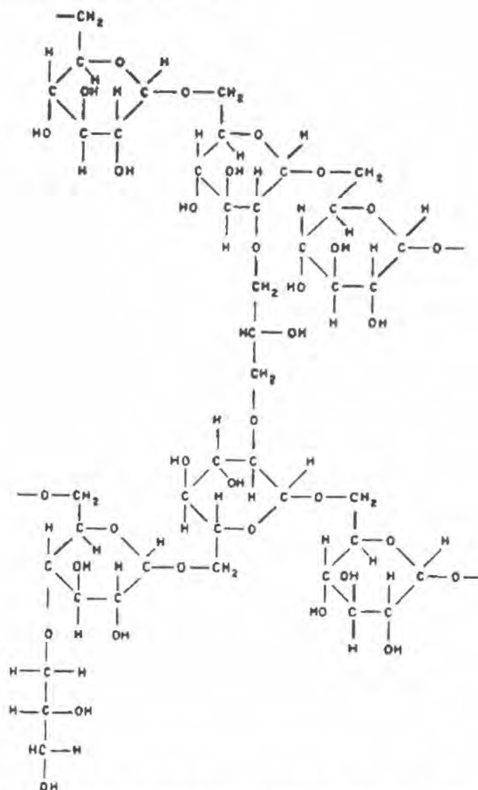

Figure 21. Structural formula of Sephadex after (34). Figure 11, page 35, "Sephadex-Gel Filtration in Theory and Practice". Permission to reproduce from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. and American Laboratory, International Scientific Communications, Inc.

The Sephadex G series is used for gel filtration in aqueous media. The LH-20 series is a lipophilic derivative of Sephadex (obtained by alkylation of most of the hydroxyl groups) useful for GFC in polar organic solvents (DMF, methanol, acetone, dioxane, etc.) and these solvents mixed with water for the separation of materials such as steroids, petroleum hydrocarbons, and other organic molecules. Superfine grades (10–40 μ) of the G series are available for TLC, as are Sephadex ion exchangers for the separation of various labile macromolecules (enzymes, hormones).

g. *Other packings*. Starch, vulcanized natural rubber and polyvinylacetate (Merckogel OR) have also been used for separations based on size.

3. *Equipment and Procedures*

Equipment can range from a simple open column to a modern, high temperature, pressurized LC instrument. Since gels for gel filtration are relatively soft, separations are generally carried out in rather simple glass columns with the flow of liquid produced by a difference in hydrostatic pressure. Gel permeation, which

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employs harder gels, is usually performed in a high pressure commercial instrument of the type shown in Figure 13. Major differences between GPC and general liquid chromatography are the use of loop injection and recycle techniques, and the absence of solvent programming (see Section h). Multiple columns are often used (Figures 28 and 29).

a. *Packing the bed* (see also Section II.III, Table 4). To pack Sephadex G-75-G-200, the tube (generally ranging from 0.9 × 15 cm to 10.0 × 100 cm) is mounted vertically and the outlet is fitted with a narrow piece of tubing (Figure 22a). A small amount of liquid is poured into the tube and the outlet is closed.

The gel is mixed with an appropriate solution to swell the beads. Sephadex gels are hydrated in the buffer solution which will serve as the eluant according to the following table:

Type of Sephadex	Minimum swelling time at room temperature	Minimum swelling time on boiling water bath
G-10, G-15, G-25, G-50	3 hours	1 hour
G-75	24 hours	3 hours
G-100, G-150, G-200	3 days	5 hours
LH-20	3 hours	—

(From "Sephadex-Gel Filtration in Theory and Practice," Table 3, p. 31, Pharmacia Fine Chemicals, Inc.)

A recent report (*24*) indicates that boiling Sephadex G-15 in 1 *N* HCl for 2 hr and washing with water before packing the column improves the chromatographic properties of the gel by increasing the number of theoretical plates, the internal volume and the effective pore size without any deterioration of the gel.

The buffer is chosen so as to be inert toward the sample and should not contain oxidizing agents or strong acids which could hydrolyze the gel matrix. The ionic strength of the buffer should be >0.02 to obviate the possible ion exchange effects due to the carboxylic content of the matrix. After hydration, fine particles are poured off from the slurry, and the buffer and slurry are degassed.

The slurry is poured down a glass rod into the tube. If possible, all the slurry required for the whole bed is added at once; an extension tube can be attached to the top of the chromatography tube if necessary. The slurry is stirred to remove air bubbles.

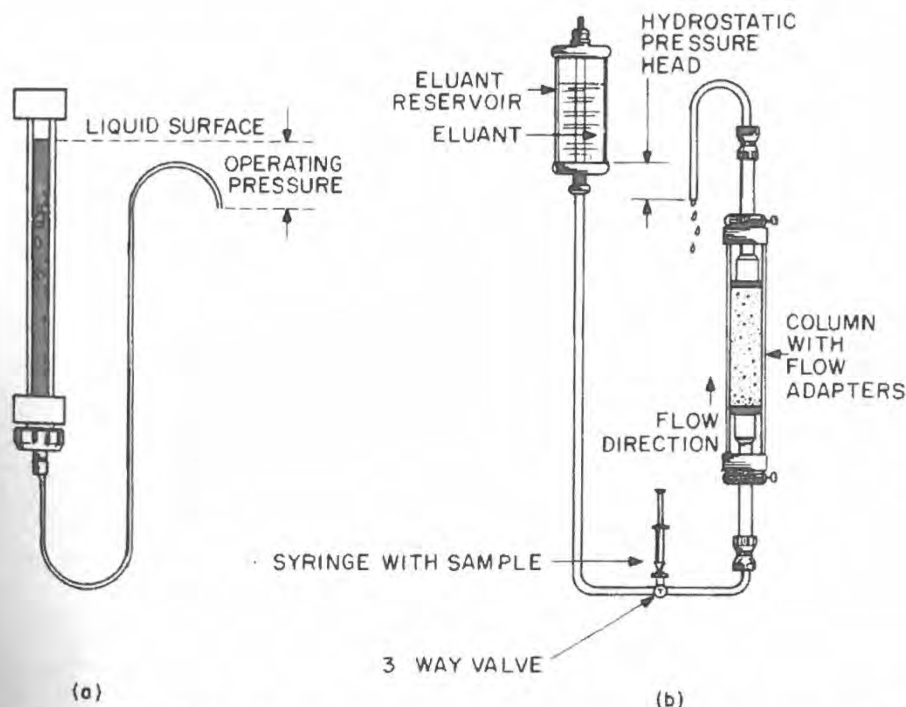

Figure 22. (a) Sephadex gel filtration column for use with downward flow of solvent; (b) a Sephadex closed-system setup for use with upward flow of solvent.

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The outlet tube is positioned as shown in Figure 22a and opened to let solvent flow out. If necessary, additional slurry is added as the solvent flows out, the gel settles and the column level rises. The tubing is finally lowered until the pressure to be used for elution is obtained (for Sephadex G-200, 100–150 mm pressure maximum is recommended). Sephadex G-10 to G-50 beds are easier to pack than beds of G-75 to G-100. The operating pressure can be higher so the exit tubing need not be so long. Columns of Sephadex LH-20 are packed as just described. Since the beads float in chloroform, a high flow rate is applied as soon as the slurry is poured into the tube, and a tube with a plunger-type flow adaptor is needed to keep the bed in position after the flow is stopped. Figure 22b shows a Sephadex column with two flow adaptors for use with gels that float or for upward flow or recycling chromatography.

A circular piece of filter paper or some other material is placed on top of the bed to stabilize it. Two or three column volumes of the eluant are passed through to equilibrate the bed, and then a sample of blue dextran 2000 is chromatographed to determine V_M and to check the quality of the packing by watching the colored zone move through the bed.

For GPC, polystyrene beads are first swollen in an organic solvent, packed into a tube with a slurry-pressure technique, and washed with the solvent in which they were swollen. Columns are made of glass or metal, are uniform and not coiled. The slurry-pressure technique of packing is difficult for a beginner and hard to apply reproducibly. It may be best to purchase these columns prepacked because efficiency is so vital in GPC. Waters Associates sells prepacked columns and also a column-packing unit which comes with a detailed description for this technique.

Excellent, reproducible columns containing controlled-pore glass material for exclusion chromatography may be packed by either a dry or wet (slurry) technique. With either method, it is most important that all pores be completely filled with the mobile phase. Correct procedures for packing and maintaining such columns have been provided by Pidacks (*25*).

b. *Sample application.* For analytical separations, a few mg of sample is applied as a dilute solution (ca. 0.5%) in the eluant. In gel filtration, application can be with a pipette after lowering the level of liquid in the column just to the top of the bed. Or, if the sample is more dense than the eluant, it may be applied with a pipette or syringe beneath a layer of eluant which is allowed to remain on top of the bed. Syringe injection can also be used (Figure 22b). Typical sample sizes are 0.4 ml for 15 mm columns and 1–2 ml for 25 mm columns. Applying the sample at a density above that of the solvent is a common technique in GFC so that there is a sharp boundary between the sample and the solvent. This is achieved by addition of a neutral salt or sucrose. Sample solutions with a high viscosity relative to the eluant can lead to skewed peaks with tailing or heading.

Sample injection with a syringe through a septum into the solvent stream and loop injection are used in GPC. Loop injection is performed as follows: a four-port valve is set to "fill" and the sample is introduced into the sample loop from a syringe; the valve is changed to "empty" and the sample is discharged as the loop becomes part of the eluant stream. The amount of sample loaded on the column is controlled by the concentration of the sample solution and the amount of time the valve is open. The sample should be injected as quickly as possible at a point just ahead of the column.

c. *Solvents.* Typical eluants for gel filtration include water (for uncharged substances), 0.025 *M* phosphate buffer (pH 7.0) and 0.1 *M* tris-HCl + 1 *M* NaCl (pH 8.0).

For gel permeation, tetrahydrofuran, *o*-dichlorobenzene, trichlorobenzene, perchloroethylene, chloroform, methylene chloride, carbon tetrachloride, toluene, benzene, cyclohexanone and others have been used. The eluting solvent and the gel should have similar polarity, and, of course, the solvent must completely dissolve the sample. The solvent should have low viscosity, a high boiling point, and should be compatible with the detector.

Solvents should be degassed prior to entering a heated column so that bubbles do not form within the bed. In GPC instruments, this can be done by passing the solvent through a vented heater placed just ahead of the solvent pump and held at a temperature just under the boiling point of the solvent.

d. *Elution.* A constant pressure closed-system set up for gel filtration is shown in Figure 22b. Upward flow is often used with Sephadex, and this sometimes allows higher flow rates than possible with downward flow of solvent. Downward elution in an open tube can also be used. Improved resolution has been obtained in one study with compressed beds of Sephadex G-50 (*26*).

e. *Temperature.* In some GPC separations, the solubility characteristics of the sample require the use of elevated temperatures. Increased temperature will improve efficiency if a viscous solvent is being used or if the sample solution is concentrated and therefore viscous. Temperature programming is not a generally fruitful experimental approach for increasing resolution in GPC.

f. *Flow rate.* Low flow rates and small gel particles lead to peaks with decreased widths in both gel permeation and gel filtration. Elution volumes are normally independent of flow rate.

g. *Recycle chromatography.* The effective length of the column can be increased without requiring large pressure increases by passing the column effluent back through the column both in gel permeation and gel filtration. Peaks well separated in the first cycle are taken off so that they do not remix with other peaks during later cycles. Recycle GPC can be carried out with a Waters ALC-100 LC (Figure 13) modified as shown in Figure 23.

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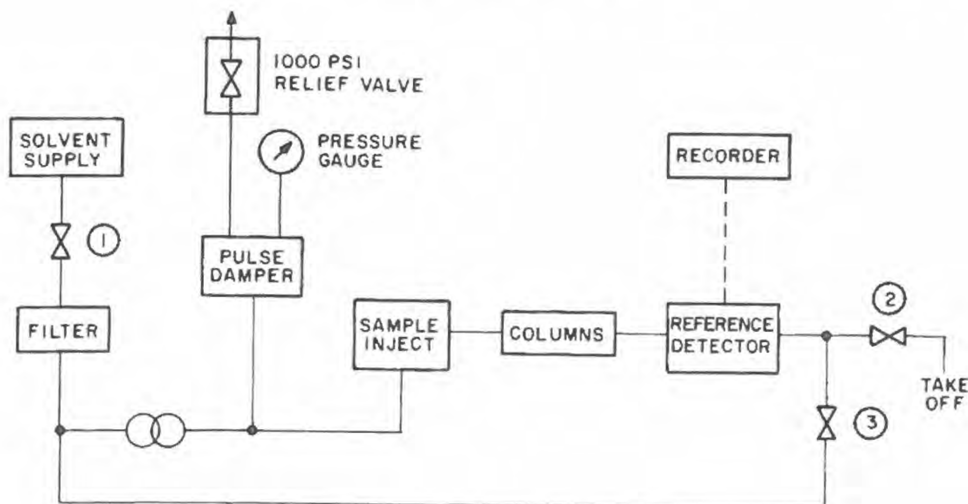

Figure 23. Schematic diagram of a recycle system [after (*23*)]. From *J. Chromatogr. Sci.* **7**, 43 (1969). Permission to reproduce from Journal of Chromatographic Science, Evanston, Illinois.

h. *Programmed exclusion chromatography.* Giddings and Dahlgren (*27*) have proposed a method for the continuous control of retention which substitutes for the techniques of solvent and temperature programming which are widely used in LC and GC, respectively, but are not effective in exclusion chromatography. Control is gained by adding to the incoming solvent a certain percentage level of a high-molecular-weight polymer whose molecules are too large to penetrate the pores of the gel. The added polymer will selectively alter the thermodynamic properties of the mobile phase because of interactions with the solute species. Solutes will be selectively excluded from the mobile phase based upon their molecular dimensions, leading to altered equilibrium constants and improved size-dependent separations.

i. *Detection and analysis.* Solutes are detected and estimated either continuously or by collecting fractions.

Continuous detectors are of the same type as for other kinds of liquid chromatography. These include refractive index (by far the most widely used for GPC), moving wire, infra-red, conductivity, radioactivity and ultraviolet detectors. These are used in conjunction with a recorder and integrator as described before.

Fraction collectors which measure volume rather than time are desirable because timing devices will not take account of changes in flow rate which will change the elution time but not the elution volume of a given solute.

A fully automated gel filtration system employing the Technicon Autoanalyzer for the continuous detection of eluted polysaccharides with the orcinol-H_2SO_4 reagent has been described (*28*).

j. *Regeneration and repacking.* Usually no regeneration of the bed is required between runs. Flow rates through beds of soft gels may be reduced with time as the particles compress. It may then be desirable to backwash or repack the column.

k. *Commercial equipment.* Suitable tubes for gel filtration can be purchased from commercial sources (e.g., Pharmacia Fine Chemicals, Inc., Figure 22) or can be constructed as described by Varley (*29*). These tubes should have a Terylene or Nylon support for the bed rather than sintered glass discs which can adsorb solutes (e.g., lipoproteins) and easily clog. Columns must also have a minimum dead volume at the exit to prevent remixing of the separated components. Columns for GFC are typically 1–5 cm diameter and 20–200 cm length.

Commercial chromatographs for GPC are manufactured by Waters Associates. Their ALC-100 is a general liquid chromatograph useful for molecular size separations at room temperature (Figure 13). The Waters Model 200 gel permeation chromatograph (Figure 24) is a high temperature, wide range GPC instrument. The operating procedure for the GPC-200 for determining molecular weight distribution curves, as taken from Waters' literature, is as follows:

A sample is prepared by weighing out a sufficient quantity of the polymer to make up a 0.25 or 0.50% mixture in a suitable solvent. The sample is dissolved in the solvent in an oven. A hypodermic syringe is then filled with 5 ml of the sample in the solvent and the valve loop flushed with this sample. The solvent reservoir

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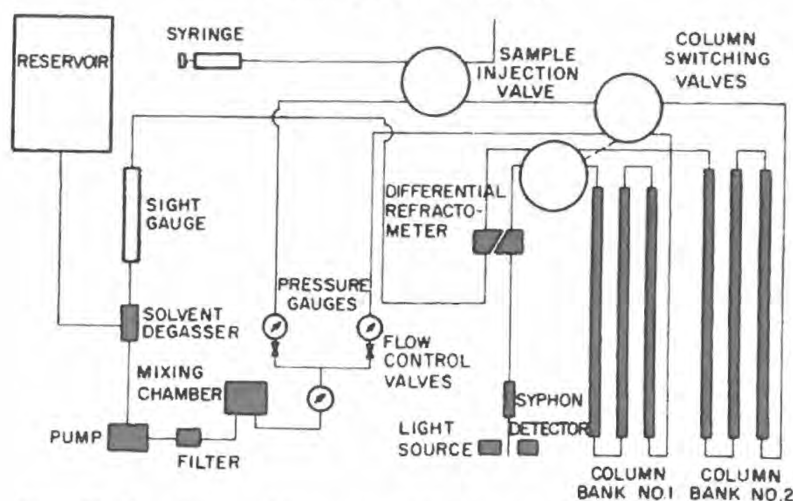

Figure 24. Flow diagram of Waters Model 200 Gel Permeation Chromatograph. Permission to reproduce from Waters Associates, Inc.

has been previously filled with the same solvent used to prepare the sample. The solvent is pumped from the reservoir through a heater to degas the solvent and then a filter to the columns. The solvent flow is controlled by means of control valves in each line. Flow rate through each bank of sample columns is set at 1 ml per min. The column switching valves are set to direct the sample flow through the desired set of columns.

When ready to place the sample on the column the valve is turned 90° to place the sample loop in the solvent line. The solvent then carries the sample from the valve loop on to the packed sample column. The valve is returned to its original position and solvent continues to elute the column. Depending upon the length of the column a molecular-weight-distribution curve will be obtained within one to three hours.

The effluent from the columns passes through the sample and reference cell to a differential refractometer. Since no solvent gradient is necessary for separation, the difference in the refractive indices between sample and reference cells of the refractometer is used to detect each fraction of the sample as it leaves the column. The solvent after passing through the reference side of the refractometer returns to the solvent reservoir. The sample passes to a 5 ml syphon and then to a fraction collector or suitable waste collector. When each 5 ml fraction is dumped by the syphon, the light beam through the syphon tube is interrupted and a pulse occurs on the recorder chart. In this fashion each 5 ml fraction is itemized on the recorder.

Gilding (*15*) summarizes typical experimental conditions for GPC as follows:

Three or four columns, each 4 ft × ⅜ in. diameter.
Sample injections of 3–5 mg.
Flow rate—1 ml/min.
Pressure drop—100–120 psi/16 ft of column bed.
Time of run—2.5–3 hr.
Mixtures of MW $> 10^3$ handled most efficiently.

The approximate molecular weight ranges normally handled by the various methods are <100 to 400 for GC, 7500 to >100,000 for GPC and 200 to 10,000 for LSCC and LLCC.

4. *Applications*

a. *Desalting*. Macromolecules are eluted in the interstitial volume and are separated from salts and other low-molecular-weight compounds which are eluted later. Examples are the removal of salts from proteins and phenol from nucleic acids. Gel filtration is faster and more efficient than dialysis for desalting, and denaturation of labile substances (nucleic acids, enzymes) is less likely. Centrifugal desalting with Sephadex is also useful for highly viscous polymer solutions.

b. *Separation of multicomponent mixtures*. Complex mixtures of substances with different molecular weights are separated by exclusion chromatography (Figures 25–28). Substances with similar molecular weights can be separated if there is preferential adsorption for one or more of the compounds (e.g., aromatic compounds).

c. *Molecular-weight determination*. Chromatography on Sephadex can be used to determine the molecular weights of unknown proteins. A plot of log molecular weight *vs* elution volume for a series of known proteins approximates a straight line. The unknowns are chromatographed under the same conditions and their molecular weights estimated from the standard curve based on their elution volumes.

GPC is used extensively to determine molecular-weight averages and distributions of polymers (Figure 29). The column is first calibrated with known molecular-weight standards. Polystyrene standards can be

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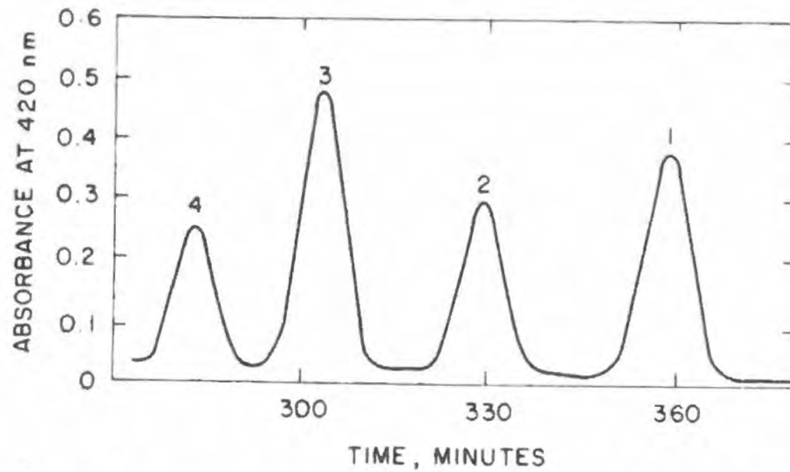

Figure 25. Gel filtration chromatography of glucose (1), lactose (2), raffinose (3) and stachyose (4) on Bio-Gel P-2, at 65 °C. Column 2.5 × 100 cm. Flow rate, 58 ml per hour. Eluant, water. 20 μl of a solution containing glucose, 320 μg; lactose, 240 μg; raffinose, 360 μg; stachyose, 200 μg, was applied to the column. After (*35*); see *J. Chromatogr.* **42**, 476 (1969) for later, improved separations of a similar nature.

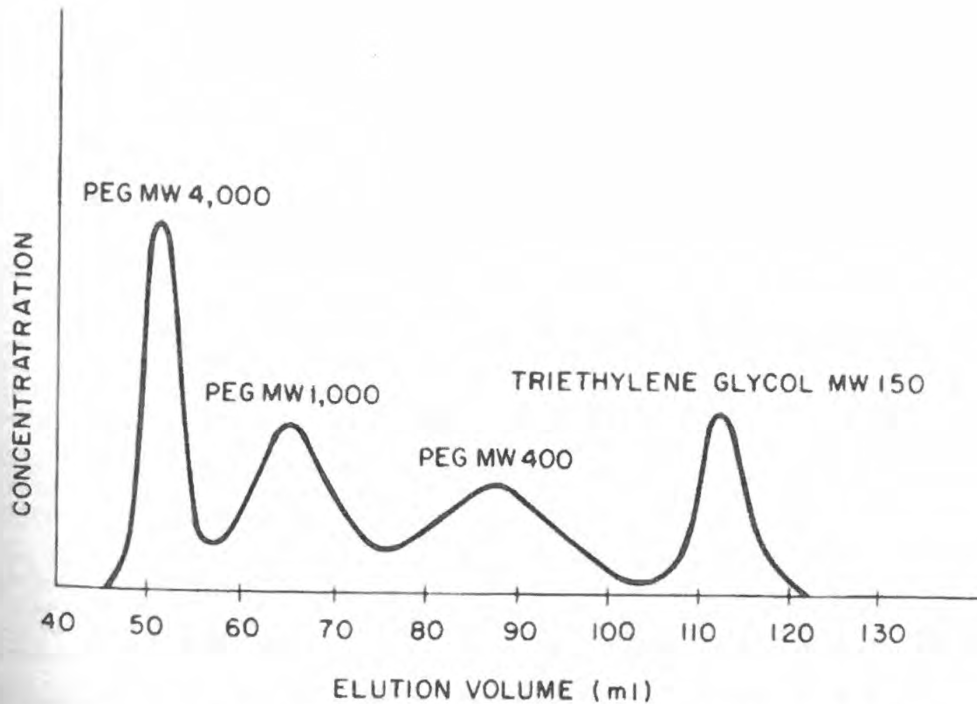

Figure 26. Elution diagram of a mixture of polyethylene glycols (PEG) on Sephadex LH-20. Solvent: ethanol. Bed dimensions: 1 in. × 30 cm. Sample: 2 ml containing 4 mg of each substance. Flow rate: 0.8 ml/min. [From (*36*).] From LH-20 booklet, page 4. Permission received to reproduce from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

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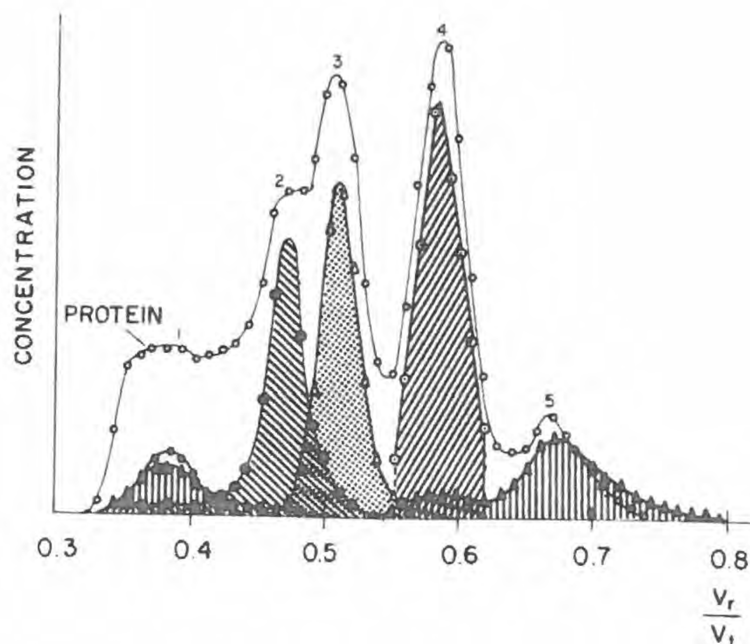

Figure 27. Fractionation by gel filtration of human serum proteins and hemoglobin on Sephadex G-200. Bed dimensions: 4.2 × 73.5 cm. Flow rate: 10–20 ml/h. Eluant: 0.1 *M* tris-HCl buffer, pH 8.0 and 1 *M* NaCl. V_r = elution volume, designated V_R in text. Peaks: 1. haptoglobin-hemoglobin complex, 2. γ-globulins, 3. ceruloplasmin, 4. albumin, 5. free hemoglobin; total protein concentration of the eluate is indicated by open circles [after (*34*)]. Reprinted with permission from American Laboratory, International Scientific Communications, Inc. and Pharmacia Fine Chemicals, Inc.

used in the determination of other polymers, or for more accurate work separate calibration curves must be made for each polymer. After calibration, the unknown sample is chromatographed, the resulting chromatogram is divided into equal sections by drawing vertical lines every 5 ml, and the average molecular weight of each section is obtained from the height of each line by referring to the calibration curve. The weight-average and number-average molecular weight of the polymer is then calculated. Applications include the characterization of polymers prepared under different reaction conditions, the comparison of two identical samples of a polymer after being subjected to different milling processes, and the differentiation of motor oils in order to relate performance and properties. A paper describing the molecular characterization of silicones by GPC has appeared (*30*).

d. *Separation of inorganic salts.* Inorganic salts have been separated on Bio-Gel P-2 and Sephadex G-10 by Pecsok and Saunders (*31*). Sorption isotherms, retention data and mechanisms of retention are discussed. A combination of adsorption on the gel, exclusion from internal water bound to the gel matrix, and Donnan exclusion of anions are all operative factors.

e. *Studies of molecular structure.* Cazes (*32*) has pointed out that adsorption between solutes and the substrate often retard migration during GPC and that GPC might be applied, therefore, to help characterize molecular configurations and stereochemical conformations of unknown structures. Molecules with permanent dipoles are often eluted earlier than expected based on their molecular volumes because of associative forces (solute-solute or solute-solvent). These attractive forces were studied for hydrocarbons, alcohols and other compounds by GPC. Molecular association has also been studied using GFC (*33*).

f. *Adsorption and ion-exchange chromatography.* Examples of the effects of adsorption or ion exchange in GFC include the retardation of aromatic compounds on Sephadex, fractionation of amino acids, fractionation of humic acid, separation of nucleic acid components, and partial resolution of stereoisomers on Sephadex (*33*). In most instances of exclusion chromatography, these effects are neither observed nor are desirable.

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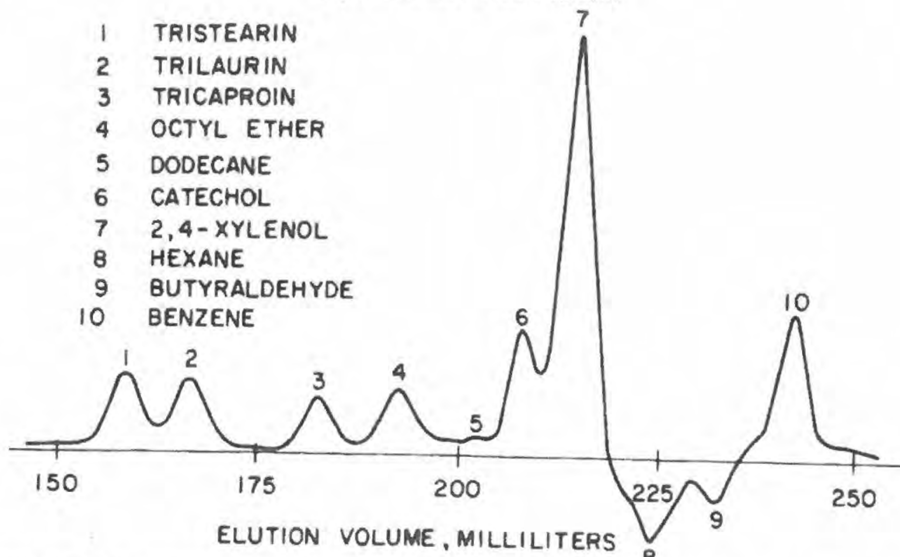

Figure 28. Molecular size separation of ten-component mixture, 78-891 molecular weight, on the Waters ALC-100 instrument with a differential refractometer detector; eight 3 ft Poragel A columns: 3-A1, 3-A3, 2-A25; 10 mg sample; tetrahydrofuran solvent at 1 ml/minute. Reproduced with permission from Waters Associates Inc. [After (*37*)].

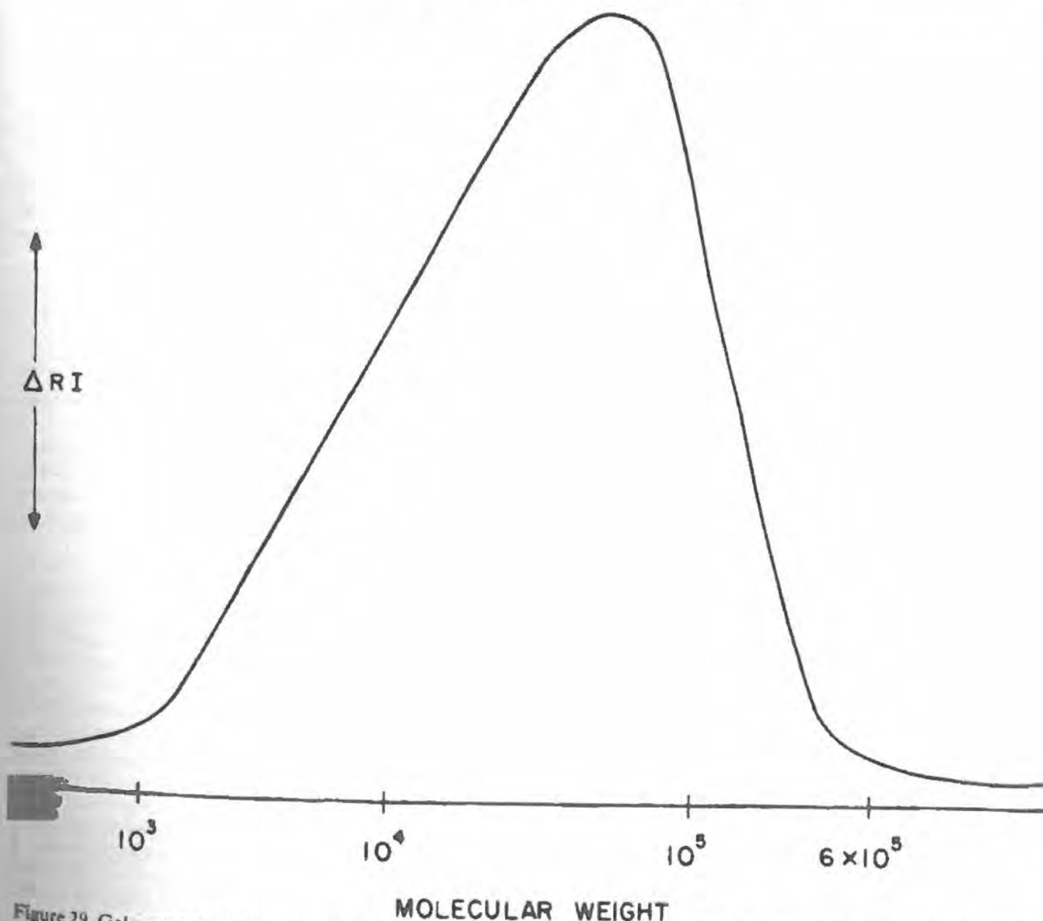

Figure 29. Gel permeation chromatography of polyethylene (overcap lid resin) on the Waters GPC-200 with differential refractometer detector; four 4 ft Styragel columns: 1×10^6, 1×10^6, 1×10^4, 1×10^4; 2.5 mg sample; solvent: 1,2,4-trichlorobenzene, 10 ml/min., 130°. [Unpublished work of W. A. Dark, courtesy of Waters Associates.]

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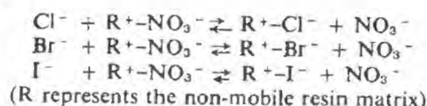
D. ION EXCHANGE COLUMN CHROMATOGRAPHY (IXCC)

In ion-exchange chromatography, the column is packed with an insoluble solid phase containing ionic groups which can reversibly exchange either cations or anions with a solution. A mixture of ions is sorbed on top of the column and is developed with an appropriate ionic wash liquid. The ions in the mixture, which are attracted to the stationary phase by electrostatic forces, are displaced by the developer and move down the column at different rates depending upon their relative affinities for the resin. Those most attracted to the resin will move the slowest and those least attracted the fastest.

An example of a simple ion-exchange procedure is the separation of the halides Cl^-, Br^- and I^-. The mixture, dissolved in a few ml of water, is poured onto a nitrate-form anion-exchange column and the following equilibria are established:



$$Cl^- + R^+\text{-}NO_3^- \rightleftharpoons R^+\text{-}Cl^- + NO_3^-$$
$$Br^- + R^+\text{-}NO_3^- \rightleftharpoons R^+\text{-}Br^- + NO_3^-$$
$$I^- + R^+\text{-}NO_3^- \rightleftharpoons R^+\text{-}I^- + NO_3^-$$

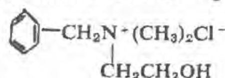
(R represents the non-mobile resin matrix)

The arrow lengths indicate that the resin binds iodide most strongly and chloride least strongly. The flowing nitrate eluant carries only free ions and not those combined with the resin. Each halide as it moves down the column will be sorbed by the reactions to the right but desorbed by the reverse reactions. Since the reaction for chloride lies mostly to the left, the chloride ions will be bound the least and will be eluted first; iodide ions will spend relatively the most time on the resin and will be eluted last. (See Figure 34.)

The differential attraction of the three ions to the resin is due to the *selectivity* (see below) of the resin. Very small differences in selectivity can be exploited by employing column procedures to yield many valuable analytical separations.

1. *Ion-Exchange Materials*

a. *Ion-Exchange resins* (see Section II.III, Table 5). By far the ion-exchange resins most used are those prepared by chemically adding functional groups to spherical beads of styrene which have been copolymerized with divinylbenzene. Reaction of the copolymer beads with a sulfonating agent (H_2SO_4) yields a resin with an average of about one $—SO_3H$ group per benzene ring, primarily in the para position. This product functions as a strongly acidic cation-exchange resin. Strong-base anion-exchange resins (Type I) are quaternary ammonium derivatives of cross-linked styrene. These are synthesized by chloromethylation and then amination of the styrene copolymer. The size of the beads is controlled by the rate of agitation during the preparation by suspension polymerization and the degree of cross-linking by the percentage of divinylbenzene mixed with the styrene. Figure 30(a,b) shows the chemical structures and preparation methods of these polystyrene-based resins. If amination is carried out with dimethylethanolamine instead of trimethylamine, a strong-base resin (Type II) with the functional group



will result. Type II resins are less basic than Type I resins and are recommended when a strong base resin in the hydroxide form is required (i.e., the Type II resins have greater affinity for hydroxide ion).

These ion-exchange resins behave like tiny beaded, porous sponges which shrink or swell when placed in various aqueous solutions. Exchange between ions associated with the resin and other ions occurs mostly inside the beads since only a small fraction of the ionic groups are on the bead surface. Some resins are produced in granular rather than bead form. These resins have greater surface areas and pack less regularly, the latter leading to columns with a higher interstitial volume and a lower pressure drop.

Other cross-linked polystyrene resins containing different functional groups, and resins with a different type of copolymer matrix and strong acid, weak acid or weak base groups have also been made (see Section II.III, Table 5). Two of these are shown in Figure 30(c,d). Also available are electron-exchange resins (vinylhydroquinone-divinylbenzene copolymers), chelating resins [cross-linked polystyrene containing iminodiacetate, $—CH_2N(CH_2—COOH)_2$, groups], and snake-cage, ion-retardation resins [$RCH_2N^+(CH_3)_3—OOCR'$, see Section 5c].

Strong acid and strong base resins are most useful because they are, for practical purposes, completely ionized in aqueous media at all pH values, and they undergo a wide variety of exchange reactions which are generally rapid. The chemistry of weak acid and base resins is analogous to that of acetic acid and ammonia, respectively, leading to significant differences in behavior between strong and weak exchangers. For example, strong exchangers will split neutral salts (e.g., $R—SO_3^-H^+ + NaCl \rightleftharpoons R—SO_3^-Na^+ + HCl$) while weak exchangers will not. Strong base resins react with weak acids of silica and carbon dioxide while weak resins will not react with weak acids or bases. The alkali metal salt forms of weak acid resins and the acid forms of weak base resins (e.g., $R—NH_2 + HCl \rightleftharpoons R—NH_3Cl$) are readily hydrolyzed when washed with water and liberate free bases and acids respectively. The conversion of a weak resin to a salt form is accompanied

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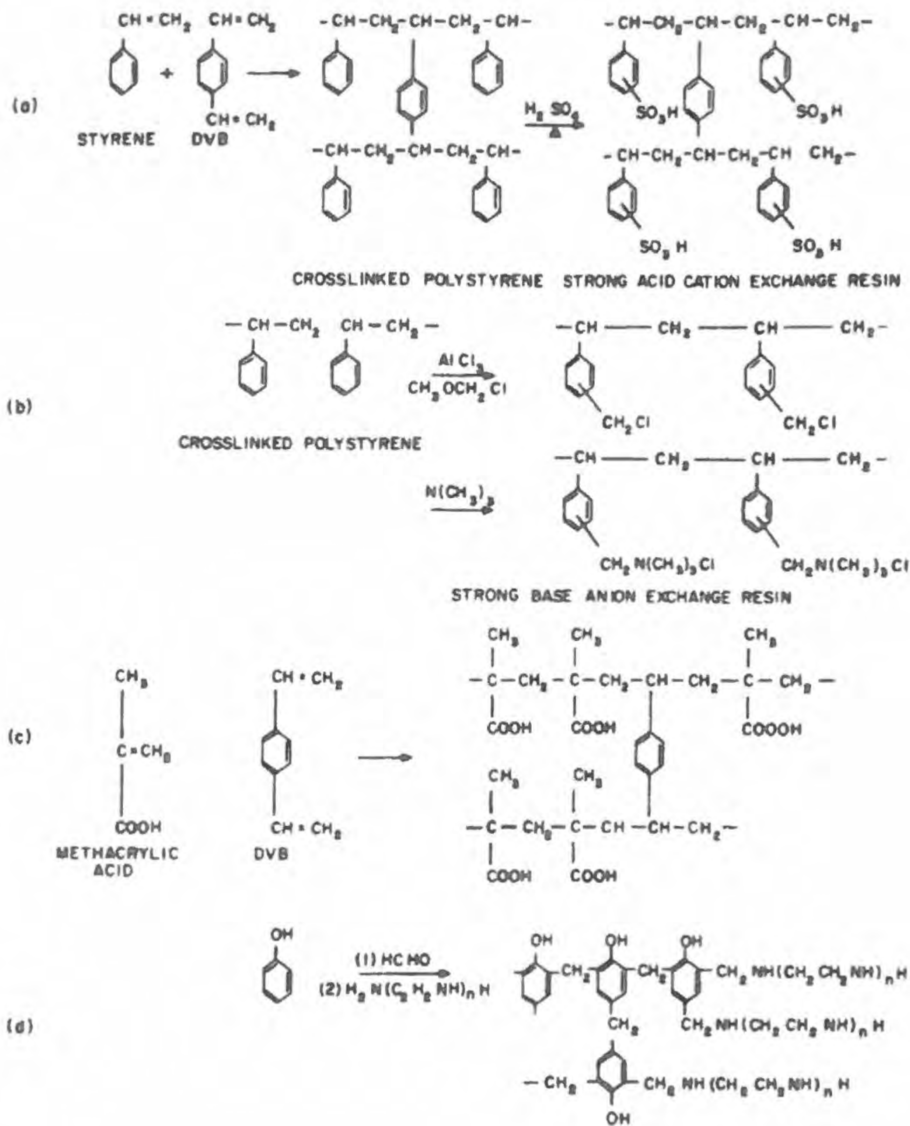

Figure 30. Examples of the preparation and structures of various exchange resins: (a) strong acid polystyrene cation exchange resin (e.g., Dowex® 50); (b) strong base polystyrene anion exchange resin (Dowex® 1); (c) weak acid crosslinked polymethacrylic acid resin (Amberlite® IRC-50); and (d) weak base phenol-formaldehyde condensation resin (Duolite A-7).

by considerable swelling not found with strong resins because the salt form is more ionic than the weakly ionized free-acid or free-base form and therefore the degree of hydration is greater.

Different polymerization techniques have led to the production of polystyrene copolymer resins with a rigid macroporous (or macroreticular) structure superimposed on the gel structure present in conventional gel resins. These macroreticular resins will therefore exchange with large molecules in nonaqueous media wherein gel resins will not swell and therefore will have negligible porosity. In aqueous solutions, gel resins with a relatively low degree of cross-linking will show a higher degree of porosity but low physical strength. Amberlyst resins (Rohm and Haas Co.) are macroreticular resins designed for use in nonaqueous or partly aqueous media, while Amberlite macroreticular resins are designed for improved stability in aqueous solutions (*38*).

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Weak acid polymethacrylic acid resins (e.g., Amberlite® IRC-50, Figure 30c) are synthesized by a completely different method than the resins just described, yet these exhibit a high capacity for some large molecules and are therefore classified as macroporous resins (*39*).

b. *Inorganic exchangers.* Among the earliest exchangers were aluminosilicates such as leucite ($K_2O \cdot Al_2O_3 \cdot 4SiO_2$) which can reversibly and stoichiometrically exchange potassium for sodium, calcium, ammonium or other ions. Other inorganic exchangers include zinc or dipotassium cobalt hexacyanoferrate(II); hydrous oxides of zirconium, tin, thorium, etc.; phosphates, molybdates, tungstates and vanadates of tervalent metals; salts of heteropoly acids (e.g., ammonium phosphomolybdates); and some sulfides. Most of these materials are cation exchangers, although zirconium oxide can exchange either cations or anions depending upon the pH of operation (in acid solution it carries a plus charge and can exchange anions).

Inorganic exchangers are precipitated from aqueous solution in the form of granular, microcrystalline aggregates. They are useful for separations of some metal cations (usually of group I and II) for which they exhibit special selectivity. They cannot be used at extremes of pH, have low capacities, and are in most cases too soluble to be as valuable as polymer resins for general ion-exchange work.

c. *Cellulose exchangers.* Since almost all of the molecules present in living systems are ionic in nature, biochemists have found ion exchangers to be especially useful for the separation of such compounds. Conventional and macroreticular gel resins have proved unsuitable as media for the separation of the many large ions which are of biochemical interest. Exchangers based on cellulose, however, do not have the disadvantages of a gel structure.

Table 6 (Section II.III) indicates some types of cellulose ion exchangers which have been prepared by attaching fixed ionic groups to the hydroxyl groups of cellulose by oxidation, esterification, ether formation, etc. Some of these materials are prepared by Whatman in three forms: fibrous, improved fibrous, and microgranular, the latter being preferred for analytical separations. Because the exchange groups are on the outsides of the fibers, they are accessible to very large ions. The nominal capacities of these materials for small molecules are relatively low, but the realized capacity for large biopolymers is in many cases very high. Further, the release of biopolymers by these exchange materials is very fast.

Applications include the extraction and purification of enzymes, high resolution separations of nucleosides and nucleotides, the analysis and preparation of γ-globulins from serum, etc.

d. *Sephadex ion exchangers.* Table 4 (Section II.III) lists Sephadex gel filtration materials in which ionic groups have been attached to the glucose units of the cross-linked polydextran matrix. They are available as beads which have good hydrodynamic properties and possess a certain pore size characteristic of gel filtration media. Sephadex exchangers are more porous than cellulose exchangers so that biopolymers can penetrate the gel as opposed to surface binding on cellulose. This means that somewhat higher ionic strengths are required for elutions compared to cellulose. This is an advantage for proteins which are more labile at low ionic strengths but will cause problems if the salt eluants lead to shrinkage of the pores so that molecules are trapped inside the gel. The nominal capacity of these exchangers is generally higher than ion-exchange celluloses, and they exhibit minimal nonionic interaction. Applications have been in the field of biochemistry, for example the chromatography of tissue nucleotides.

e. *Liquid ion exchangers.* Water insoluble liquid materials which resemble ion-exchange resins in properties have been used for separations by liquid-liquid extraction or reversed phase column, paper and thin-layer chromatography. The liquid exchanger is sorbed onto a porous support (a paper sheet, a silica thin layer, or a column of cellulose or an adsorbent), and an aqueous solution of an inorganic compound (usually a mineral acid) is used as the mobile phase. Examples are long-chain amines (tri-*n*-octylamine), quaternary ammonium compounds (Aliquat 336) and alkyl acid phosphates [di-(2-ethylhexyl)orthophosphoric acid].

2. *Resin Properties*

a. *Particle size and shape.* The particle size of the resin affects the flow rate (slower for finer particles at a given pressure), the rate of equilibrium (faster for finer particles), and therefore the shape of the chromatogram (sharper and more narrow for finer particles). The range and distribution of the particle sizes also affects the interstitial (void) volume and the wet capacity of the resin. Resins are commercially available in various mesh-size ranges depending upon the particular application. Products of 50–100, 100–200 and 200–400 mesh are generally used for analytical separations and coarser products (10–40 mesh) for industrial applications. For very difficult separations, small, uniform particles are required.

Most commercial resins are now manufactured in the form of spheres by suspension polymerization. Phenol-formaldehyde condensation resins are produced as granules by a process that yields resins with a high degree of porosity. Columns of granular resins pack less regularly and therefore have a greater void volume and pressure drop than columns of spherical resins with the same particle size distribution.

b. *Cross-linking.* Resins are three dimensional polymer networks tied together by the cross-linking agent. The weight percentage of divinylbenzene (ca. 1–16%) used in the preparation of polystyrene-based resins determines the cross-linking and many of the properties of the resin. A high degree of cross-linking lowers diffusion rates inside the beads and increases the time necessary for equilibrium to be reached. Lightly cross-linked resins imbibe much water and are therefore gelatinous, have a lower capacity per volume

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(i.e., the "wet capacity" is lower and the "dry capacity" is higher), allow larger ions to enter the resin, and are less selective. Resins with 4–8% DVB are generally used for analytical work. The density of ionic groups in a resin with 8% cross-linking is such that the insides of the beads have the properties of a solution 6–8 molar in the counter ion.

c. *Swelling*. When a dry resin bead is placed in water or a dilute aqueous solution of an electrolyte, the resin will swell primarily due to hydration of the fixed ionic groups. The degree of swelling is an equilibrium quantity which is a function of the kind and concentration of the external solution; the degree of cross-linking, the capacity, and the ionic form of the resin; and the temperature and humidity. For a given resin, maximum swelling occurs when the resin is placed in pure water, and shrinking will occur if the resin is transferred to a concentrated salt solution. Resins with low cross-linking show the greatest volume change when the surrounding solution is changed. Volume changes accompanying an exchange of ions reflect their degree of hydration and the selectivity of the resin for the ion. For a sulfonic acid cation-exchange resin, the volume order for alkali metal forms of the resin is $Li^+ > Na^+ > K^+$ and for alkaline-earth forms $Mg^{+2} > Ca^{+2} > Ba^{+2}$. These orders of swelling are the same as the orders of ionic hydration and inverse to the order of selectivity [i.e., among the alkali metals, Li^+ is the most hydrated (largest) ion and has the least attraction for the resin]. Carboxylic resins swell greatly on conversion from the acid form to the sodium form. The selectivity orders of carboxylic resins are not simply the reverse of the hydration orders.

d. *Donnan equilibrium*. If a sulfonic acid resin is transferred from pure water to a dilute salt solution of a nonexchange ion (e.g., a sodium form resin is put into a solution of NaCl), the resin would shrink because of osmotic forces and there would be only a *slight* penetration of ions into the resin bead according to the Donnan equilibrium equation

$$[(a_{Na^+})(a_{Cl^-})]_{resin} = [(a_{Na^+})(a_{Cl^-})]_{solution} \quad [33]$$

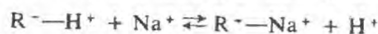
where a = activity. Because the activity of sodium ions inside the resin is very high relative to that outside, there is no driving force for the sodium ions to enter the resin phase; since the sodium-ion concentration in the resin is so high, the chloride-ion concentration must be low [recall that for electroneutrality, $(Na^+)_r = (—SO_3^-)_r + (Cl^-)_r$]. As the concentration of the external solution increases the Donnan penetration increases, the rate of increase being greater for resins with lower cross-linking.

The Donnan equilibrium concept explains the exclusion of dilute electrolytes by resins in a non-exchange form which is the basis for the separation of electrolytes from nonelectrolytes by the process of ion exclusion (see Section 5c).

e. *Capacity*. The capacity of a resin is a measure of the amount of exchangeable ion (milliequivalents) per dry gram or per unit volume of swelled resin. On a dry basis, the capacity of hydrogen form sulfonic acid cation-exchange resin is about 5 meq/g and the capacity of the chloride form quaternary ammonium anion-exchange resin is 3–3.5 meq/g.

f. *Equilibria and selectivity*. A fundamental property of ion-exchange reactions is that exchange occurs in equivalent quantities. Thus, if a solution of sodium ions is passed into a resin bed in the hydrogen form, the number of equivalents of sodium taken up by the resin exactly equals the number of equivalents of hydrogen released to the solution. The amount of sodium (or any other salt) added to the bed can be measured indirectly by washing the released hydrogen ions from the column with distilled water and titrating the effluent with standard base. The determination of salt content of a solution in this way is one important non-chromatographic application of ion exchange.

Ion-exchange reactions are reversible so that the law of mass action can be applied. For the reaction



$$R^-—H^+ + Na^+ \rightleftarrows R^-—Na^+ + H^+$$

(where R = the resin matrix plus the nondiffusible ionic functional group),

$$K_H^{Na} = \frac{[RNa]\gamma_{RNa}[H^+]\gamma_{H^+}}{[RH]\gamma_{RH}[Na^+]\gamma_{Na^+}}. \quad [34]$$

This equilibrium constant is a true, thermodynamic constant which is virtually impossible to evaluate because the activity coefficients (γ) inside the resin phase are unknown. In addition

$$K_H^{Na} = \frac{[RNa][H^+]}{[RH][Na^+]} \cdot \frac{\gamma_{RNa}\gamma_{H^+}}{\gamma_{RH}\gamma_{Na^+}} \quad [35]$$

$$= E_H^{Na} \cdot \frac{\gamma_{RNa}\gamma_{H^+}}{\gamma_{RH}\gamma_{Na^+}} \quad [36]$$

where E (sometimes termed K, K_c, K_d or Q) is the selectivity coefficient or selectivity quotient.

E values vary with the composition of the resin phase (so as to favor the sorption of the ion which is in the minority in the resin), and with the concentration of the solution if the exchanging ions are of unequal charge (concentrated solutions favor the exchange of univalent ions for polyvalent ions). Equation [36] shows that the E value varies primarily with the ratio of activity coefficients for the ions in the resin phase (assuming the ratio in the dilute solution phase is about 1). Resins with lower cross-linking are more

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"dilute" (more water is absorbed), the ratio of activity coefficients approaches one, and therefore such resins are less specific than resins with a higher degree of cross-linking.

Even though *E* values are variable, some generalities and selectivity orders can be stated:

1. For sulfonic acid polystyrene cation-exchange resins, cations with a higher charge are most strongly bound (e.g., $Th^{+4} > Ce^{+3} > Ca^{+2} > Na^{+1}$). If the charges are the same, the ion with the smallest radius is most tightly bound:

(*+1 ions*) Li (least bound) < H < Na < NH_4^+ < K < Rb < Cs < Cu < Ag < Tl.

(*+2 ions*) Be < Mn = Fe < Mg = Zn < Co < Cu < Cd < Ni < Ca < Sr < Hg < Pb < Ba < Ra.

(*rare earths and actinides*) selectivity coefficients decrease as the atomic number increases.

2. With weak acid resins, the order may be different. For example K^+ is less strongly held than Na^+ by carboxylic acid cation exchangers, and H^+ is very strongly bound by these resins.

3. The differences in selectivities are greater for anions than for cations. For type I polystyrene strong base anion-exchange resins, the order is OH^- (least sorbed) < F^- < acetate < formate < iodate < HCO_3^- < Cl^- < nitrate < bromate < Br^- < NO_3^- < chlorate < HSO_4^- < phenoxide < I^- < citrate < salicylate < benzenesulfonate. As with cation resins, there is a general increase in affinity with increasing valence and atomic number.

Ions containing aromatic rings (e.g., benzenesulfonate anion and benzylammonium cation) are strongly held by polystyrene resins because of adsorption on the resin matrix in addition to normal electrostatic forces. Many other cases of anomalous sorption have been reported wherein complex anions are strongly bound to cation exchangers in concentrated electrolytic mobile phases. Examples are the strong adsorption of anionic complexes of thallium (TlX_4^-) on Dowex 50-X8 in 5 *M* $NaClO_4$ and of palladiazo (a structural isomer of arsenazo III reagent: $[H_4L]^{-4}$ and $[H_5L]^{-3}$) on Dowex® 50W resin in 9–12 *M* $NaClO_4$, pH 6.5. Kraus and coworkers have found that some negatively charged inorganic complexes are quantitatively sorbed on cation-exchange resins in concentrated neutral saline media, while cationic species are unsorbed under these conditions. Nonionic molecules are sometimes sorbed by resin matrices (see section 5b).

When differences in their relative affinities are sufficiently great, ions may be separated by elution chromatography on a column of resin originally combined with the eluting ion. The eluting ion usually, but not always, has a smaller *E* value than any ion of the mixture. Thus, sodium and cesium are separated in this order on a column of hydrogen-form sulfonic acid resin by elution with 0.7 *M* HCl. Another example is the separation of halides on nitrate-form quaternary ammonium resin with 0.5 *M* $NaNO_3$, described in the introduction to this section D. If selectivity differences are not sufficiently great, eluants containing complexing or chelating agents and organic solvents can be used to enhance separation (alter distribution coefficients) by superimposing selective complexation and solvent extraction effects upon normal ion exchange. One example is the separation of the alkaline earth metals on a sulfonic acid resin by elution with citrate or lactate buffers (see Section 3c).

If a mixture is eluted with an ion having a stronger affinity for the resin than the ions in the sample, the process becomes displacement, rather than elution, chromatography. Development of a mixture of sodium and potassium ions on a hydrogen-form resin with cesium ion as the eluant yields overlapping zones in the order H^+, Na^+, K^+, Cs^+. This procedure is useful for preparative work rather than for analytical separations.

g. *Theories of selectivity*. It is beyond the scope of this treatise to describe the many theories proposed to explain and thermodynamically interpret the relative selectivities of ions for resins. Donnan equilibria between the beads and the solution have been considered, in addition to ion-pair formation, ion-sieve effects, swelling effects, activity coefficients, solubilization and salting-out effects, and the influence of non-exchange ions, neutral molecules, and water upon the exchanging ion.

h. *Kinetics*. The ion-exchange process involves: diffusion of the exchanging ions through the liquid layer around the resin bead, diffusion within the bead, the exchange reaction, diffusion of the displaced ion through the bead and finally through the liquid film. Diffusion into and out of the exchanger is not necessarily of equal speed. It is generally conceded that the actual ion-exchange reaction is usually fast and does not control the kinetics. For typical chromatographic systems with high (> ca. 0.1 *M*) external solution concentration, the slow step is particle diffusion; in very dilute solutions, film diffusion is the slow step for small ions. In either case, equilibrium is speeded up by the use of small beads, resins with low cross-linking, and a high temperature. Small, single-valent ions have the fastest exchange rates. Singly-charged ions also have higher diffusion coefficients than doubly-charged ions in the resin. Exchange rates are fastest in water systems and become slower with less polar solvents.

Weak resins are not completely ionized or fully swollen, and diffusion in them is slow. Conversion of these resins to a salt form is slow, but conversion from one salt form to another is as fast as with strong resins.

i. *Resin stability*. In salt forms, sulfonic acid resins are stable up to about 120°–200°, but in the hydrogen form, they lose sulfuric acid when heated with water above about 100°–150°. Quaternary amine resins are stable up to about 100° in most salt forms but only to about 30°–60° when in the hydroxide form. Tertiary amine resins are stable up to about 100° in the hydroxide form. Operation above these limits for a brief period may not cause appreciable decomposition. Exposure of resins to strong oxidizing agents (Cl_2, H_2O_2, HNO_3, chromic-nitric acid mixtures, dissolved oxygen plus a metal catalyst) causes degradation of the

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polymer matrix. Continued exposure of resins to solutions of high and low concentration can cause cracking and breakage due to osmotic shock. Most resins have sufficient physical stability to resist attrition losses in normal analytical column operation, although unusual conditions (e.g., deep, small-diameter beds; very high flow rates; frequent agitation or pumping) can cause breakage.

3. *Theory of Ion-Exchange*

The concept of the theoretical plate was introduced in the section on GC. The size of a theoretical plate is calculated from the experimental chromatogram, and, although true equilibrium is never achieved, the narrower the plate the closer the approach to equilibrium is assumed to be.

To increase the number of plates (N), the particle size and flow rate should be decreased and the length of the column increased. As stated above, however, resolution between zones increases only as the $\sqrt{N}$. Plate thicknesses are typically 100 times the average radius of the resin particles, and flow rates are 0.2–2.5 ml/cm^2/min.

The distribution ration or coefficient (usually designated D or C) is equivalent to the partition coefficient (K) defined above for GC:

$$D = \frac{\text{amount of ion in the resin of any plate}}{\text{amount of ion in the free solution of any plate}}. \qquad [37]$$

The volume distribution coefficient (D_v) is the amount of ion in 1 ml of resin bed divided by the amount in 1 ml of interstitial solution at equilibrium. The weight distribution coefficient (D_w) states the ratio in terms of grams of dry resin and mls of solution. Many authors use D for D_w as defined here. $D_v = dD_w$, where d is the density of the resin bed. The solute need not necessarily be an ion. One relationship between the elution volume of the peak and D is

$$D = \frac{V_R - V_M}{V_M} \qquad [38]$$

where V_R is the volume needed to elute the peak of the zone and V_M is the void or interstitial volume, the volume of solution between the resin particles (see Figure 6) equation [38] is identical to [24], so that the distribution coefficient as defined here is an expression of retention measured in column void volumes, and is identical to the capacity ratio K' as defined in equation [24]. An alternate equation for the volume distribution coefficient D_v is

$$D_v = V_{max} - i \qquad [39]$$

where V_{max} is the number of column volumes of effluent at the elution maximum and i is the fractional interstitial volume (usually about 0.40) *(40)*. D_v as defined in equation [39] and D as defined in [38] are related as follows:

$$D_v = \frac{DV_M}{\text{total column volume}} = Di.$$

D values can also be calculated *(41)* from elution data using the expression

$$D = \frac{V_R - V_M}{m} \qquad [40]$$

where m is the mass of the resin in grams.

Rieman and coworkers have modified and simplified the plate theory of Martin and Synge, and Mayer and Tompkins, to provide a practical guide for changing operational conditions in order to obtain improved separations by ion-exchange. This treatment assumes that equilibrium between the resin and solution exists in each plate during the elution (in fact, true equilibrium is never reached) and that the concentration of the sample ion in the interstitial volume and on the resin is negligible compared to the eluant ion concentration.

a. *Effect of eluant concentration.* It has been derived from equation [37] that for a simple ion-exchange system governed by the following equilibrium

$$zREL + B^{\pm z} \rightleftarrows R_zB + zEL^{\pm}$$

where R represents the resin matrix, EL a univalent eluant ion, and $B^{\pm z}$ a sample ion which does not complex with the eluant or combine with H^+,

$$D = \frac{WQE}{V_M[EL^{\pm}]^z} \qquad [41]$$

where W is the weight of resin in a plate, Q is the exchange capacity of the resin in meq/g, and E is the selectivity coefficient (Section 2f).

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Combination of equations [41] and [38] yields

$$V_R = \frac{WQE}{[EL^{\pm}]^z} + V_M. \qquad [42]$$

This equation allows one to calculate the effect of a change in eluant concentration on the position of elution peaks, since for a given ion on a given column

$$(V_R)_{c_1} = (V_R)_{c_2}\left(\frac{[EL^{\pm}]_2}{[EL^{\pm}]_1}\right)^z \qquad [43]$$

where c_1 and c_2 are two different eluant concentrations. The fact that the eluant concentrations are raised to the power numerically equal to the valence of the sample ion means that changing the concentration of the eluant is effective for improving the resolution of ions with different valences. If the ion of higher valence follows but overlaps the ion of lower valence, the eluant concentration should be decreased to increase the relative displacement of the higher-valent ion and improve the separation.

b. *Effect of eluant pH*. Equations [41] and [42] may be modified to yield an expression for the elution volume of a weak monoprotic acid or its anion

$$V_R = \left(\frac{WQE}{[EL^{\pm}]^z}\right)\left(\frac{K}{K + [H^+]}\right) + V_M \qquad [44]$$

where K is the ionization constant of the acid. For a diprotic acid

$$V_R = \left(\frac{WQ}{V_M}\right)\frac{(E_1K_1[H^+]/[EL^{\pm}]) + (E_2K_1K_2/[EL^{\pm}]^2)}{[H^+]^2 + K_2[H^+] + K_1K_2} \qquad [45]$$

where K_1 and K_2 are the dissociation constants for the two ionization steps and E_1 and E_2 the respective selectivity coefficients.

Separations of acids with different K values can be improved by adjusting the pH of the eluant. Decreasing the $[H^+]$ increases the ionization and the values of D and V_R for an acid, causing it to be eluted later. Acids with different K values are displaced selectively. The elution of strong acids is independent of pH. Certain organic acids (e.g., alkyl esters of phosphoric acids) are adsorbed to the resin by Van der Waals' forces even in the unionized state and their elution may not follow the above equations.

Buffered eluants are used to control the pH during the elution of acid mixtures. So that the buffer ion does not displace the counter ions from the resin, it should have a low affinity for the resin and its concentration should be kept as low as possible consistent with adequately controlling the pH.

c. *Effect of complexing agents in the eluant*. The separation of Ca^{++} and Sr^{++} by elution with 1.2 *M* ammonium chloride through a column of ammonium-form cation-exchange resin is not complete because the selectivities of these ions for the resin are quite similar. Elution with 1.2 *M* ammonium lactate, however, provides a good separation. Both metal ions are complexed by lactate, but the smaller calcium ions form the more stable +1 complex and so are less sorbed and eluted before the Sr^{++}.

Kraus and coworkers have developed methods for separating metals on chloride-form anion-exchange resins in chloride media. Chloride complexes and oxyanions formed by the metals are sorbed on the anion-exchange resins to varying degrees, depending on the chloride ion concentration (Figure 35). The volume distribution coefficient for metals in such a system is given by

$$D_v = \frac{DV_M}{V_b} = \frac{V_R - V_M}{V_b} \qquad [46]$$

where V_b is the bed volume. In the absence of chloride, the metals are not complexed and have no affinity (D_v or $D = 0$) for the anion exchanger.

d. *Effect of column length*. Rieman and coworkers have derived a Gaussian elution equation which in most cases allows the simple calculation of the ideal length of a column for a given "quantitative separation" (0.05% cross contamination):

$$\sqrt{H} = \frac{3.29}{D_2 - D_1}\left(\frac{D_2 + 0.5}{\sqrt{P_2}} + \frac{D_1 + 0.5}{\sqrt{P_1}}\right) \qquad [47]$$

where H is the column length, P is the number of plates per cm of column, and the subscripts refer to the components to be separated. The total plate number for each peak (N) in a given column is calculated from

$$N = \left[\frac{2D}{(D+1)}\right]\left[\frac{V_R}{V_a - V_R}\right]^2 \qquad [48]$$

where V_a is the average of the volumes at $C = C_{max}/e$ (Figure 31). A separate elution of each ion through the column yields the data required to calculate N, P and H.

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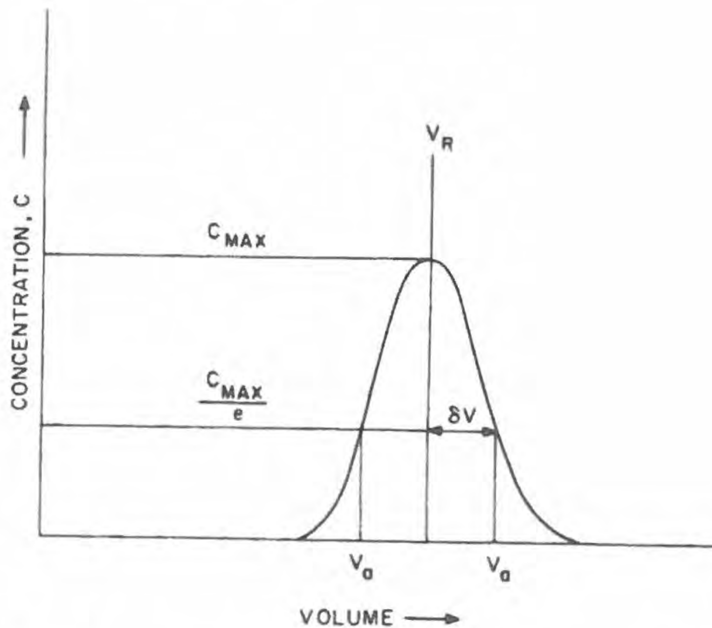

Figure 31. Ideal ion-exchange elution curve; e = the base of natural logarithms. The width of the band is defined here as the width for which $C = C_{max}/e$. Other ways of defining width are equally valid, e.g., the base of the equilateral triangle formed by the steepest tangents.

e. *Calculations of V_R with stepwise changes in the eluant.* The following equations may be used to calculate the elution volumes of peaks after stepwise eluant changes; if the distribution ratios of the solutes in a mixture are known from prior experiments for each concentration of eluant, an optimized separation can be easily designed. For a given solute in two eluants:

$$V_R = V_1 + V_M + D_2\left[V_M + \left(\frac{V_1}{D_1}\right)\right] \quad [49]$$

where V_1 is the volume of eluant 1 used and D_1 and D_2 are the distribution ratios of the solute in the two eluants. For $n + 1$ eluants (n changes):

$$V_R = \Sigma_1^n V_1 + V_M + D_{n+1}\left[V_M + \Sigma_1^n\left(\frac{V_n}{D_n}\right)\right]. \quad [50]$$

f. *The ideal elution curve.* Figure 31 shows the Gaussian shape of an ideal elution band. The width of the band (δ_v) is a function of the number of plates (N) according to the equation

$$\delta_v = V_R\sqrt{\left(\frac{2}{N}\right)}. \quad [51]$$

The maximum concentration of the band is also related to N:

$$C_{max} = \frac{m}{V_R}\sqrt{\left(\frac{N}{2\pi}\right)} \quad [52]$$

where m is the total mass of solute in the band.

4. *Experimental Procedures and Equipment*

a. *Columns.* Resin beds for analytical separations are usually about 15–20 cm long (or longer for more difficult separations) and 1 cm in diameter. A tube similar to that shown in Figure 22a can be used to hold the bed although backwashing during packing is facilitated if a bulb or other reservoir is at the top of the tube.

The conventional method of packing the column is to stir up the resin beads in an excess of the first eluant to be used, allow most of the resin to settle, and decant off the fines (the small particles). The stirring, settling and pouring-off are repeated once or twice. The remaining resin is slurried into the column and

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allowed to settle by gravity to form the packed bed. While the resin settles, the tube may be tapped gently with a small rubber mallet to expel air bubbles and improve the packing. Supernatant liquid is removed when necessary and additional slurry is added until the bed reaches the desired height. The column is then washed with several bed volumes of eluant (this is also done whenever a prepared column is not in use for 12 hours). The sample is added and washed into the resin after draining the supernate to the top level of the bed and adding a glass fiber or paper disk. The liquid level must never drop below the top of the bed or the column will have to be repacked or backwashed. A tube with outlet held above the top of the bed will prevent the bed from draining dry. Beds packed with small particles of resin exhibit capillary forces which prevent a water surface from sinking more than a few millimeters below the top of the bed.

Backwashing is accomplished by attaching a distilled water line to the bottom of the column and slowly introducing and increasing the upflow of water until the resin expands into the upper part of the tube. The water flow is maintained until all air pockets are removed and all particles are freely floating. The water flow is then stopped and the resin settles uniformly by gravity according to particle size: the larger beads will settle to the bottom and the smaller will be on the top. Some workers backwash every column after slurry packing in order to insure as regular a particle classification and eluant flow as possible. Backwashing must be performed if gas bubbles form in the bed. For difficult separations it may be necessary to screen the resin initially so that the entire column is of very uniform particle size.

With the conventional packing method described above, it is difficult to pack reproducible columns when the particle-size range of the resin is large, and it requires considerable time to pack columns of small resin particles ($< 20\ \mu$) which settle quite slowly. These problems are overcome by using two dynamic packing procedures recently described (*42*). In the first, a chamber or reservoir filled with a thick slurry (25–50 vol.% solids) of resin is connected to the chromatographic tube filled with clear liquid, and the slurry is displaced into the column with a liquid that is pumped into the top of the chamber with a linear velocity greater than the settling velocity of the largest particles. For packing small diameter columns of appreciable length or coiled columns, it is better to first pack a fixed bed into a reservoir of larger diameter and then extrude that bed into the smaller tube by displacing it with liquid.

b. *Choice and treatment of the resin.* Polystyrene strong acid and strong base resins, 4–8% divinylbenzene, 50–100 or 200–400 mesh, have been used for a vast majority of analytical applications. Other resins may be chosen for particular separations, e.g., carboxylic acid resins for separating organic bases, macroreticular resins for large molecules and/or nonaqueous solutions, and cellulose exchangers for large molecules of biochemical interest. Specially purified resins can be purchased from certain suppliers (e.g., Bio Rad, Inc.), or commercial-grade resins should be treated before use by repeated cycles of washing with 1–2 N HCl, 0.5–1.0 N NaOH and ethanol (with a water rinse following each) to remove metallic and organic impurities.

The ionic form of the resin should correspond to one ion of the eluant. For example, if the eluant is an aqueous solution of ammonium chloride, a cation exchanger would be used in the ammonium form or an anion exchanger in the chloride form. Resins are converted to a desired form by passing an appropriate salt, acid or base through the column until the effluent gives a negative test for the ion being replaced. For replacement of one univalent ion by another or a higher valent ion by one of lower valence on a strong acid or base resin, 1 M solutions are satisfactory. For replacement of a univalent ion by a polyvalent ion, 0.010 M solutions are better. Salt forms of weak acid or base resins are converted to the free acid or base form with 1 M HCl or 1 M NH_3. After the conversion is complete the bed is washed with distilled water to remove the excess conversion solution. If necessary, the resin is then extruded and repacked, or backwashed, before performing the separation.

If all the sample is eluted from the resin during the chromatographic run, the column can be used again for another sample without treatment. Any residue remaining on the column after the elution must be stripped off with an appropriate solution before the next addition of sample. If the ionic form of the resin is altered during the elution (e.g., the eluant contains more than one type of ion), it may be necessary to regenerate the column (convert it back to its original form) by passage of an appropriate solution.

c. *Column operation.* HETP increases with an increase in flow rate, since the ions are carried at a faster rate down the column and have less time to diffuse into the resin and reach all the exchange groups. Since peak widths (but not elution volumes) are proportional to HETP, good separations are favored by a slow flow of eluant. Flow rates commonly used in ion-exchange chromatography range from about 0.2–2.5 ml/cm^2/min. Gravity flow or operation under the pressure of a column of liquid have traditionally been used in this field. In modern instruments with columns containing fine resin particles, eluants are forced through the column with a pump.

Elevated temperatures (which lower HETP) and temperature programming (which changes distribution ratios) are sometimes used, especially in conjunction with solvent programming (e.g., aqueous buffers with pH and ionic strength gradients) to improve the resolution of complex mixtures (see Figure 16). These methods require the use of much more complicated equipment than is required for separations at room temperature.

High efficiency column packings consisting of an impervious core with thin coatings of ion-exchange resin have recently been introduced. Kirkland has found that columns of his controlled surface porosity ion-exchange packings (now commercially available from Dupont as "Zipax" supports) exhibited 3.5 (cation) and 8 (anion) theoretical plates per second at the highest velocities tested compared to 0.1 to 0.5

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theoretical plates per second for conventional gel resins. The high speed capabilities are due to improved mass transfer effects. Their hard, spherical nature permits operation at high column inlet pressures and carrier velocities (e.g., > 3 cm/sec) in narrow (2–3 mm I.D.) analytical columns loaded with small samples and connected to a continuous detector.

Figure 32 shows the flow diagram of the Technicon sequential multisample (TSM) amino acid analyzer. This is a dual column instrument capable of the totally automated, high speed separation and quantitative analysis of amino acids accurate to ca. ±3% at the 0.050 μmole level. The operation of this instrument

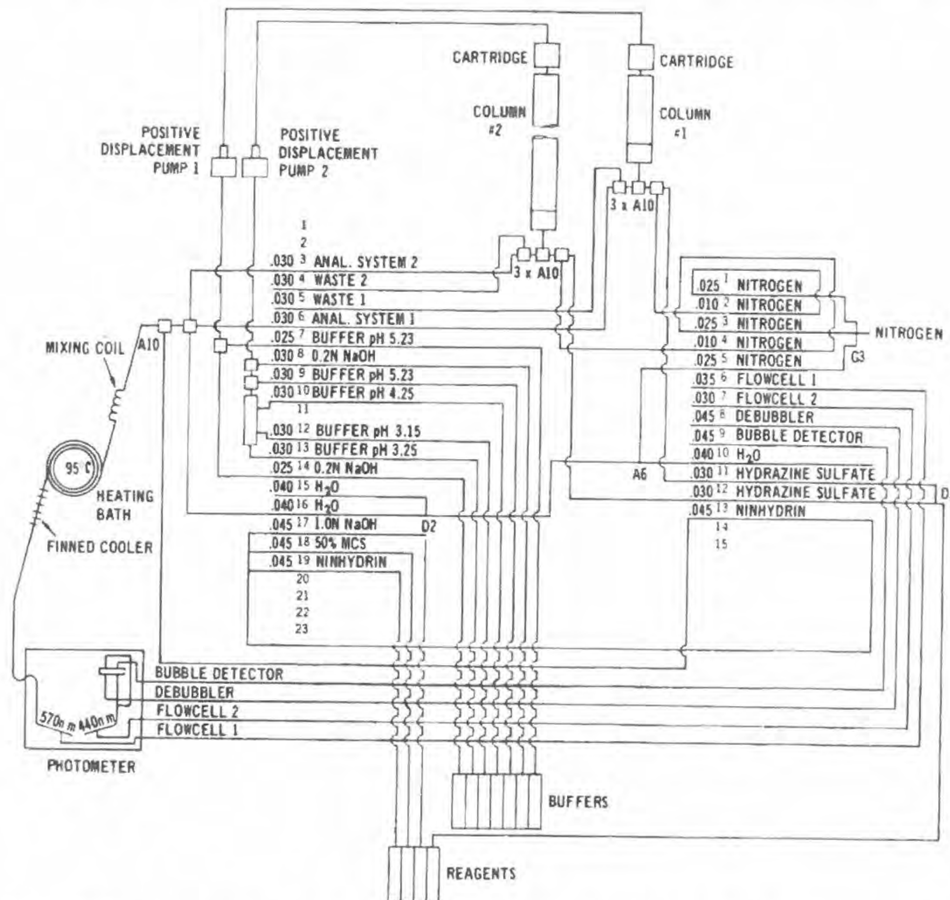

Figure 32. Complete flow diagram of the Technicon TSM Amino Acid Analyzer; the internal diameter of the pump tubes is given in inches. (Photograph courtesy of Harvey Adler, Technicon Corp., Tarrytown, N.Y.; reprinted from G. Ertingshausen and H. J. Adler, a New Accelerated Fully Automated System for Amino Acid Analysis by Ion-Exchange Chromatography, *J. Chromatogr.* **44**, 620–623 (1969) Figure 1.

has been described in two recent papers (*43*, *44*); Figure 33 shows a one-hour protein hydrolysate chromatogram obtained with it. The resin used in the columns (220 mm × 5 mm I.D. for the acidic and neutral amino acid column and 40 mm × 4 mm for the basic column) is a specially hardened, spherical, sulfonated polystyrene resin with 8% divinylbenzene which does not pack after loading. Samples are preloaded into resin-filled cartridges, up to 40 of which can be fitted into a sequential sampling device. When the instrument is in operation, the samples are presented in turn to the columns and analyzed with no further handling.

Amino acid analyzers are also available from other companies [e.g., Beckman (Spinco Div.) and Phoenix Precision Instr. Co.]. In addition, Technicon offers automated systems for the ion-exchange separation of sugars and peptides. The operation of an amino acid analyzer is in general as follows: The sample is introduced at the top of the column, and buffer is supplied to the column at a selected rate from reservoirs by a metering pump. Before the buffer enters the pump, trapped air is eliminated with a deaerator to insure constant-volume delivery. The columns are enclosed in thermostated circulating water jackets, and temperature changes can be made automatically during a run. As the buffer and sample are pumped through a

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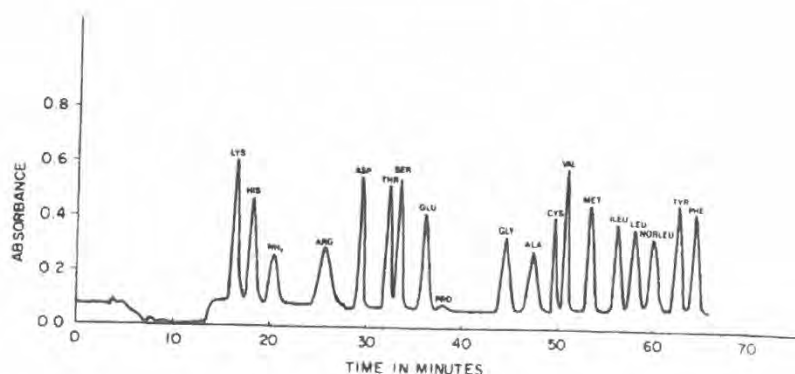

Figure 33. Chromatogram of an amino acid standard representing one complete automatic cycle of the Technicon TSM Analyzer. The sample contained 0.03 μmoles of each amino acid; the absorbance was read at 570 nm. (Reprinted from G. Ertingshausen and H. J. Adler, a New Accelerated Fully Automated System for Amino Acid Analysis by Ion-Exchange Chromatography, *J. Chromatogr.* **44**, 620–623, (1969) Figure 2.

column, the amino compounds in the mixture separate. The resolved compounds emerge from the bottom of the column and flow through capillary tubing to a column selector manifold. Here the column effluent is mixed with ninhydrin reagent supplied by a pump at a selected rate. The mixture then flows through a reaction coil contained in a reaction bath which is maintained at a constant temperature of 100 °C. The stream then passes through a photometer where the absorbance values at wavelengths 440 nm and 570 nm are measured for each reaction product. One or the other of these wavelengths is the absorption maximum of the ninhydrin reaction product for all amino acids. The absorbance values are monitored by sensitive photo-detectors which transmit the signals to a strip-chart recorder where the peaks are drawn. The instrument can be adjusted to automatically conclude an analysis, flush the lines, and put itself in stand-by condition for the next run.

Since all amino acids and many related compounds react with ninhydrin, a persistent problem in automatic ion-exchange chromatography of biological specimens is the identification of unknown peaks on the chromatogram. One procedure is to add standards of the suspected amino acids to the sample and then to repeat the analysis to see if the added standards appear in the same positions as the unknown peaks. Another is to use tracer amounts of marker amino acids labeled with carbon-14 to provide points of reference along the elution curve. The effluent stream is split before reaction with ninhydrin and one part diverted to a fraction collector. The labeled amino acids are detected in the fractions by liquid scintillation or gas flow systems.

In addition to the automated detection of compounds in the effluent by colorimetry, ion-exchange effluents can be continuously monitored by measuring refractive index, radioactivity, electrical conductivity, etc. Alternatively, fractions can be collected and subjected to analysis by chemical or physical means.

d. *Solvent* (*eluant*) *systems*. As mentioned above, simple salts, buffers or solutions of various inorganic or organic complexing agents can be used as eluants for various ion-exchange separations. Recently, eluants composed of aqueous-organic solvent mixtures have led to very selective separations of metal ions on both cation and anion exchangers. Typical solvents include an organic solvent such as methanol, ethanol, tetrahydrofuran or dioxane mixed with an aqueous solution of an inorganic complexing agent such as the acids HF, HCl or HNO_3; an organic agent such as tri-*n*-butylphosphine oxide or dithizone can also be added to the solvent.

Systems such as these are theoretically complex and the reasons for increased selectivities are not fully understood. Korkish has suggested that a bidimensional ion-exchange competition exists between a liquid exchanger in the outside phase and the solid resin phase, and he has termed methods using mixed solvents "combined ion-exchange-solvent extraction" (CIESE). Other factors which must be considered include changes in cation solvation (organic solvents in general make it easier to strip coordinated water from a metal cation to form a metal-chloride complex, so that many metals are strongly sorbed by anion-exchange resins that contain a much lower concentration of chloride than is required for their uptake from aqueous solution), altered electrostatic interaction between the fixed ionic groups and counter ions due to changes in the internal dielectric constant, and liquid-liquid partition effects due to the nonuniform distribution of solvent components between the resin and external phases.

The last factor explains why D values for metals increase in mixed solvents (except at very high organic constituent concentrations) for both cation and anion exchangers: the resin preferentially takes up the

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water from a mixed solvent so that the relative amount of water inside is greater than outside; since the resin phase is more aqueous, the inorganic complexing agent will prefer to go inside leading to increased complexing of the metal and higher D values. This type of partition effect, in addition to adsorption effects between the solutes and the resin, is also responsible for the separations of nonionic organic compounds by salting-out and solubilization chromatography illustrated in section 5b.

e. *Determination of distribution ratios.* Distribution ratios (D values) can be determined either by batch equilibration techniques or by elution of the compound through a column. In the former method, a weighed amount of resin is shaken with a known amount of a standard solution of the solute until equilibrium is reached (e.g., overnight). The phases are separated by filtration and either the solution phase, the resin phase or both are then analyzed, and, from the amounts of solute found in each, the distribution ratio is computed.

$$D_w = \frac{\text{amount of solute in resin phase}}{\text{amount of solute in solution phase}} \times \frac{\text{ml of solution}}{\text{g of dry resin}}. \qquad [53]$$

For determination of D values on a column, the elution volume of the chromatographic peak is determined and equation [38], [39], or [40] is employed. If columns containing exactly one gram of resin are used, the interstitial volume (V_M) is relatively small (< 1 ml) and the volume at the elution peak can be taken as the D value (i.e., $m = 1$ in equation [40]). Any error involved in neglecting V_M will be small and of no concern when comparing D values, i.e., when calculating separation factors. (A separation factor is the ratio of D values for the substances of interest; the largest D value is put into the numerator so that a large factor indicates good separability.)

An advantage of the column method is that the elution peaks of the solutes will be available for calculating the column height necessary for any desired separation using equations [47] and [48].

A method for determining very high distribution ratios is the preloaded column technique used by Kraus and coworkers: a tracer of the element is uniformly adsorbed on a weighed amount of resin and a column is prepared from the loaded resin and eluted; D values are computed from the tracer concentration in the effluent and its known concentration on the preloaded bed.

f. *Determination of the interstitial or void volume* (V_M). The external volume surrounding the resin is determined by filling the interstices of the column with a solution, washing out that solution completely with water and determining the amount that was washed off. Care must be taken to first remove and discard all of the solution above or below the resin in the tube. The solution used can be an electrolyte such as 0.01 M HCl (with a hydrogen or chloride-form resin), but a positive error will occur due to the Donnan penetration of the acid into the resin phase, especially with low cross-linked resins (< 4% DVB). A polyelectrolyte (e.g., sodium polyphosphate) which is excluded from the resin because of its size can be successfully used without correction. Cyclohexane can be used by filling the interstices with this liquid, which is then displaced from the column either by a water wash or by blowing with air and measured.

The theoretical interstitial volume, obtained from experiments with glass beads similar in size to resin particles, is 37% of the bed volume. Experimental values which have been obtained with polystyrene cation and anion exchangers (8% DVB) are 38% and 41%, respectively. Interstitial volumes decrease as the degree of cross-linking decreases and increase for beds of irregular particles.

g. *Determination of the selectivity coefficient* (E) *and total exchange capacity* (WQ). Selectivity coefficients are determined from batch experiments by analyzing systems in which the ion-exchange reaction of interest has come to equilibrium. Equation [42] can be used to evaluate E from an elution experiment if W, Q and V_M have been determined. WQ, the total exchange capacity, is determined by converting a column to the hydrogen (cation exchanger) or chloride (anion exchanger) form with approximately 0.1 M HCl. The excess acid is washed out with water. Approximately 0.1 M KNO_3 is then passed through to displace all the H^+ or Cl^-, and the column is again rinsed with water. An aliquot of the combined effluent and rinse, containing all the displaced ions, is titrated with standard NaOH (for H^+) or $AgNO_3$ (for Cl^-).

5. *Applications*

a. *Inorganic ions.* Figures 34–41 illustrate various separations by chromatography on ion-exchange resins under different conditions. Figure 34 shows the separation of three halides by elution with the simple salt $NaNO_3$. The change to a higher eluant concentration is made so that the iodide is eluted in a small enough volume for an accurate titration; if the elution is begun with 2.0 M $NaNO_3$, the chloride and bromide are not resolved.

Figure 35 illustrates the effect of complexing agents on the separation of metal ions on an anion exchanger. The metals are initially sorbed in concentrated HCl where all except Ni^{+2} are complexed as anions and distribution coefficients are maximal, Figure 35(a). Elution then proceeds with solutions of progressively lower concentration so that the distribution coefficient of one element at a time is lowered to about 1 or below and it is eluted. Figure 35a shows that the sorption (log D) of Mn is essentially zero below 10 M HCl, Co below 6 M, Cu below 3 M, Fe at 0.5 M and Zn in H_2O. Hence the eluant changes shown in 35b.

Figures 36 and 37 show the separation of metal ions achieved by cation exchange with concentrated electrolyte solutions and mixed aqueous-organic solutions, respectively, as eluants. In each case a stepwise change of eluants was made at appropriate points during the separation.

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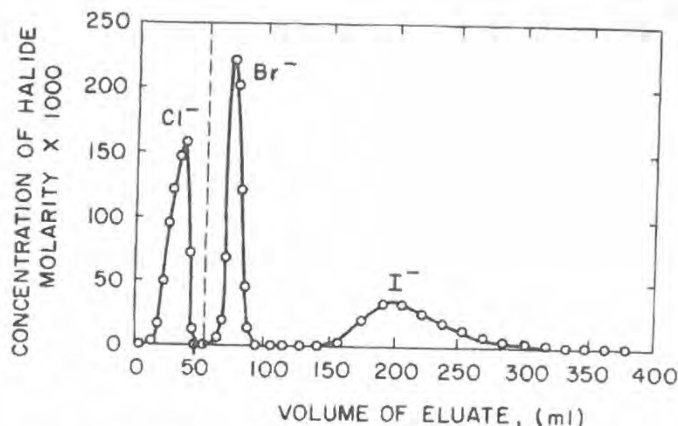
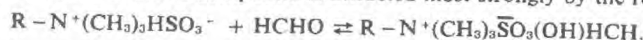


Figure 34. Separation of halides on Dowex 1-X10 nitrate form anion-exchange resin, 100–200 mesh, 6.7 cm × 3.4 cm^2 (2 cm diameter) column with 55 ml 0.50 M $NaNO_3$ and then 2.0 M $NaNO_3$ (starting at 50 ml) as the eluant at a flow rate of 1.0 cm per minute [after (*46*)]. Permission received to reproduce Figure 4, p. 89, vol. 15, No. 3 (1954), from Record of Chemical Progress, Wayne St. University Press, Detroit, Michigan.

b. *Organic compounds.* Organic substances can be separated by ion-exchange chromatography, an example of which is the separation of aldehyde compounds on bisulfite form anion-exchange resins with sodium bisulfite solutions of increasing concentrations used as eluants (Figure 38). The aldehyde which forms the most stable bisulfite addition compound is attracted most strongly by the resin, e.g.,



$$R - N^+(CH_3)_3HSO_3^- + HCHO \rightleftharpoons R - N^+(CH_3)_3\overline{S}O_3(OH)HCH.$$

Nonionic organic compounds may also be separated by partition chromatography on ion-exchange resins. In salting-out chromatography (Figure 39), water soluble nonelectrolytes are separated by elution with an aqueous salt solution through a resin in a nonexchange form. The compounds are selectively salted into the resin phase and are separated because of their differential distributions between the two phases. In solubilization chromatography (Figure 40), water insoluble compounds are separated by elution with a mixed solvent through a resin in a nonexchange form. Separations are based upon differential dissolving of the compounds from the resin to which they are strongly attracted by London dispersion forces.

Figure 41 shows a separation of amines by ligand-exchange chromatography: a column of cation-exchange resin is loaded with a metal-ammonia complex (e.g., nickel–ammonia) and the mixture of amines is added to the top. The amines coordinate to different degrees with nickel by displacing the ammonia and are selectively eluted with aqueous NH_3.

Already shown in earlier figures were the separation of amino acids with stepwise temperature, pH and ionic strength gradients (Figure 16) and with solvent programming in an automated analyzer (Figure 33). Amino acids are amphoteric compounds which contain both acidic and basic groups. Depending upon the pH of the solution, these substances will be positively or negatively charged or have no net charge and therefore will be more or less attracted to the cation-exchange resin. However, the separation of the amino acids shown in Figure 16, for example, is not due solely to differences in their ionic nature. The rate of travel on the column of resin is a function both of the charge possessed by the acid and the nature of its side group. The electrostatic attraction of the ionic part of the molecule and the physical attraction of the nonionic part are involved, and the rate of movement is determined by the pH and ionic strength of the eluant and its temperature.

The identification and detection of nanogram amounts of adenine and metabolites (including guanine) in body fluids has been carried out by high performance ion-exchange chromatography (*45*) on a 300 cm × 1 mm column of a pellicular cation-exchange resin, with 0.01 M $NH_4H_2PO_4$ as eluant, pH 2.40, flowing at 60 ml/hr (3000 psi) at 70 °C (15 minutes analysis time). This paper clearly illustrates how chromatographic conditions can be systematically varied to obtain optimized resolution of the compounds of interest.

c. *Ion exclusion and ion retardation.* Two methods for the separation of electrolytes from nonelectrolytes have been developed by the Dow Chemical Co. In ion exclusion, a mixture of, for example, sodium chloride and ethanol is added to a column of strong acid resin in the sodium form or strong base resin in the chloride form and eluted with water. The sodium chloride is excluded from the resin by the Donnan equilibrium principle described above and is eluted first while the ethanol can distribute between both phases and is eluted later. Since no ion exchange occurs, no column regeneration is required after the

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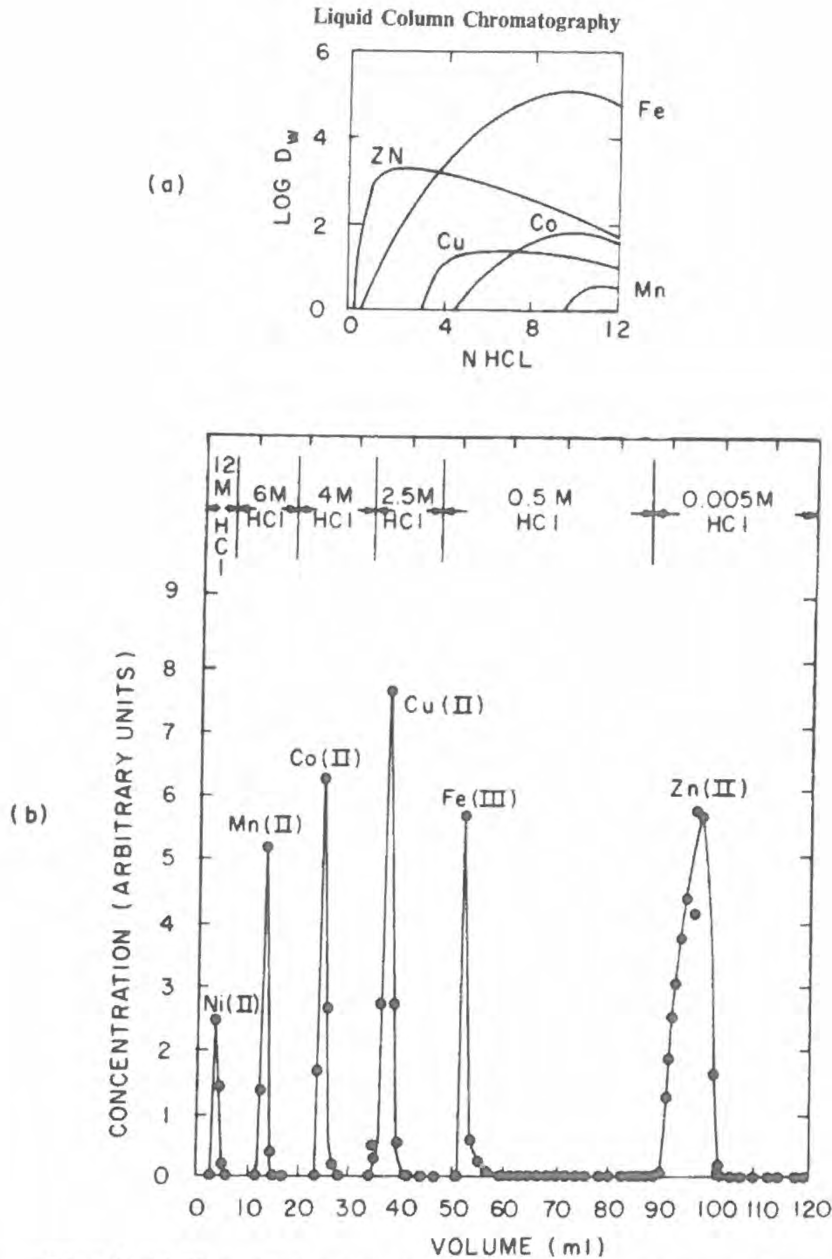

Figure 35. (a) Effect of HCl concentration on the distribution coefficients of various transition metal ions, and (b) anion-exchange separation of these metals on a Dowex® 1 column, 26 cm × 0.29 cm, flow rate 0.5 cm/min [after (47)]. Permission to reproduce received from American Society for Testing and Materials.

separation. In theory, if the electrolyte is completely excluded, it will be eluted by a volume of water equal to the interstitial volume. If the nonelectrolyte has a D value of 1 (is not adsorbed by the resin matrix), it will be eluted with a volume of water equal to the interstitial volume plus the solvent volume inside the resin particles (compare to gel chromatography). Other mixtures well separated by ion exclusion include (most ionic listed first): HCl from acetic, chloroacetic or dichloroacetic acid; trichloroacetic acid from acetic, chloroacetic or dichloroacetic acid; NaCl from ethylene glycol or formaldehyde.

As explained before, many nonionic organic compounds are sorbed by the resin matrix, so that these compounds will have D values > 1. If the D values among a group of compounds are sufficiently different, they will be separated by elution through a resin with water. An example is the separation of sucrose (D = 0.24), glycerine (0.49), triethylene glycol (0.74) and phenol (3.08) on sodium form Dowex® 50-X8.

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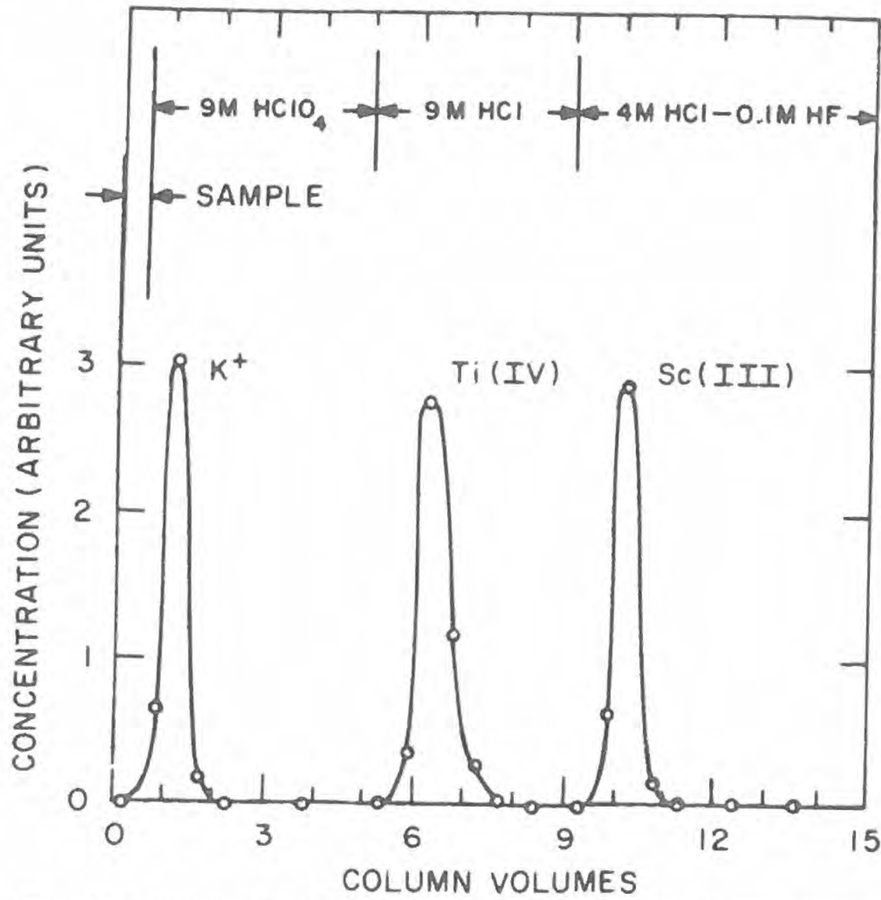

Figure 36. Separation of K^+, Sc(III) and Ti(IV) by cation exchange at high ionic strength; Dowex® 50-X4 pretreated with 9 *M* $HClO_4$, 3 cm × 0.2 cm^2 column. (Reproduced from F. Nelson, T. Murase, and K. A. Kraus, Ion Exchange Procedures I. Cation Exchange in concentrated HCl and $HCLO_4$ Solutions, *J. Chromatogr.* **13**, 503–535 (1964), Figure 11.) Permission to reproduce from Elsevier Publishing Co., Amsterdam, The Netherlands.

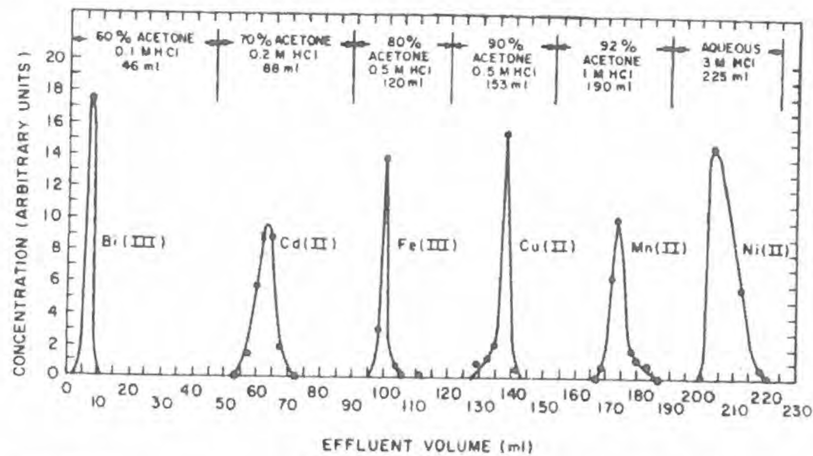

Figure 37. Separation of six metals by cation exchange in mixed solvents; Dowex® 50W-X8, hydrogen form, 100–200 mesh, 12.5 cm × 1.2 cm [after (*48*)].

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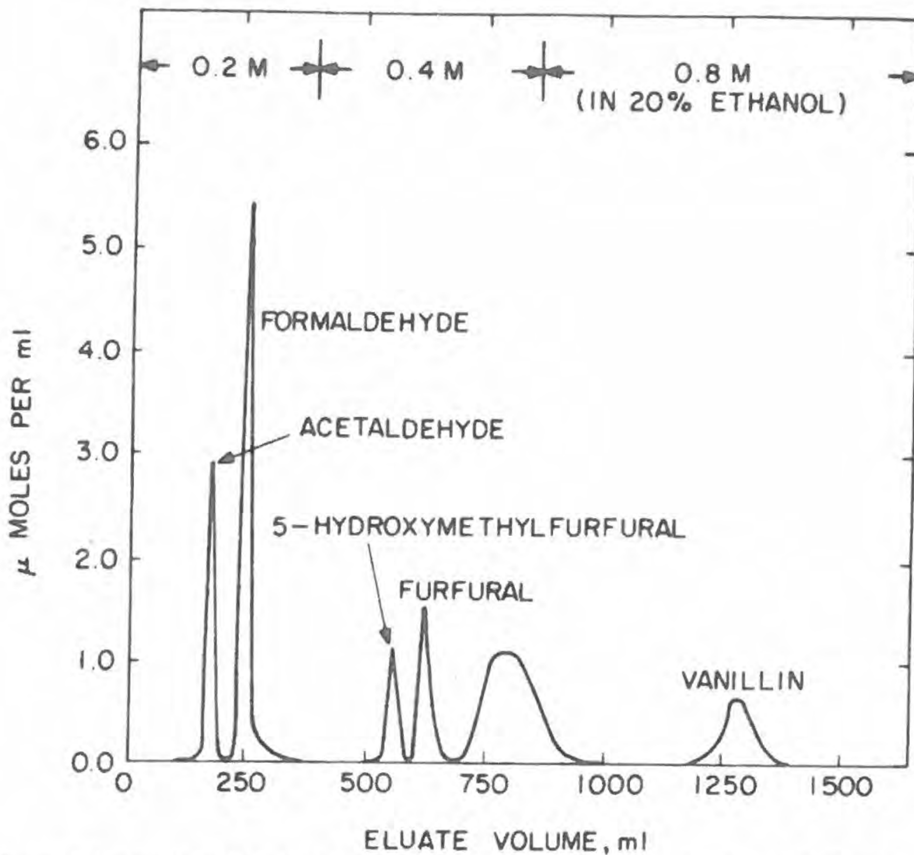

Figure 38. Separation of aldehydes by anion-exchange on bisulfite form Dowex® 1-X8, 150–300 mesh, 410 × 11 mm column, with $NaHSO_3$ eluants of different concentrations in 20% ethanol, flow rates 0.27, 0.27 and 0.52 ml/cm²/min [after (*49*)]; *Anal. Chim. Acta*, **33**, 285 (1965). Reproduced with permission from Elsevier Publishing Co., Amsterdam, The Netherlands.

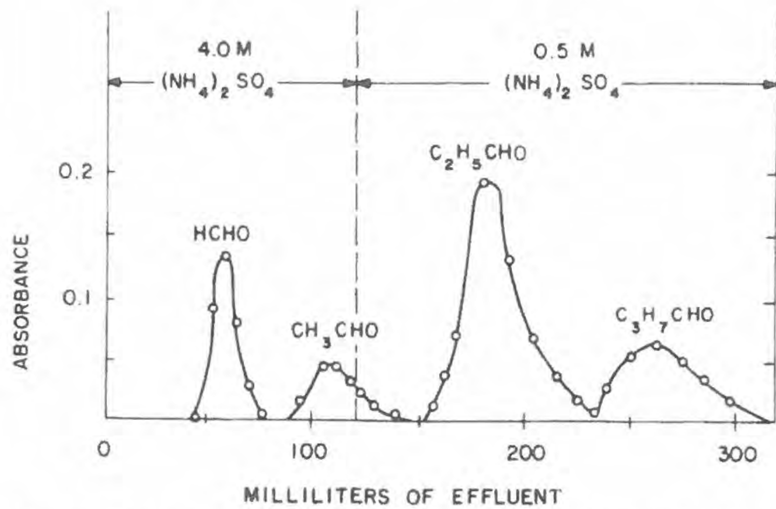

Figure 39. Salting-out chromatography of aldehydes on Dowex® 1-X8, 200–400 mesh, sulfate form, 30 cm × 3.88 cm², 0.5 cm/minute [after (*50*)]; *Anal. Chim. Acta*, **18**, 204 (1958). Reproduced with permission from Elsevier Publishing Co., Amsterdam, The Netherlands.

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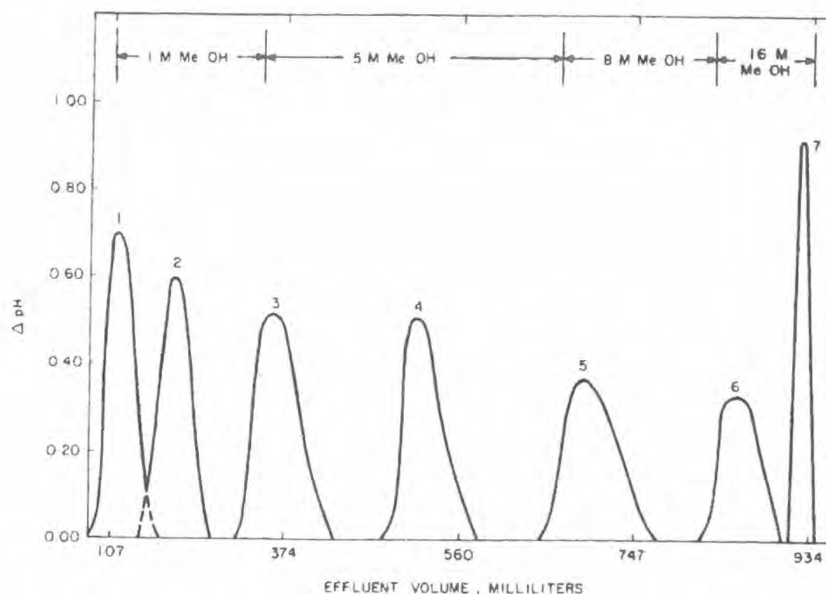

Figure 40. Solubilization chromatography of methyl ketones on Dowex® 50-X8, 200–400 mesh, hydrogen form, 54.5 cm × 2.28 cm^2, aqueous methanol eluants, 0.28 cm/min. 1. methyl iso-butyl ketone, 2. *n*-butyl, 3. *n*-amyl, 4. *n*-hexyl, 5. *n*-heptyl, 6. *n*-octyl, 7. *n*-nonyl [after (*51*)]; *Anal. Chim. Acta*, 33, 84 (1965). Reproduced with permission from Elsevier Publishing Co., Amsterdam, The Netherlands.

By using special ion-retardation resins (Dowex® 11A8), nonelectrolytes are eluted with water before electrolytes. This resin has both anionic and cationic sites which interact with the electrolyte and "retard" its migration while the nonelectrolyte is partitioned between the phases and is eluted earlier. Examples are the separation of sucrose from NaCl, and glycerine and polyglycerides from NaCl. Two electrolytes may interact with the resin to different degrees and therefore be separable by ion retardation. Examples are the separation of Fe^{+2} from Zn^{+2} and Na_2SO_4 from NaCl. Again, no regeneration of the resin is required. By methods analogous to ion retardation, salts or water-soluble nonionic substances may be separated from strong acids by water elution (acid retardation).

d. *Ion exchange.* Ion-exchange procedures (as contrasted to ion-exchange chromatography) make use of the ability of resins to exchange ions, but chromatographic separations are not involved. These applications are extremely important but since they are not chromatographic in nature they will only be listed: determination of total electrolyte concentration, water softening, separation of interfering ions, concentration of traces, and chemical conversions. Resins are also useful as catalysts for many chemical reactions.

6. *Selection of a Particular LC Method*

Some factors to be considered in choosing an LC method include:

a. Sample solubility—ion-exchange and partition work best with water-soluble samples, adsorption chromatography with fat-soluble samples. Exclusion chromatography has been applied to both.

b. Ionic nature—ion exchange is the first choice for ionic or ionizable compounds, and partition chromatography can also be used for these. Gel chromatography and adsorption chromatography are generally not used for ionic materials.

c. Molecular weight—very low-molecular-weight compounds are best handled by GC and very high-molecular-weight compounds by exclusion chromatography. Intermediate-molecular-weight compounds can be successfully separated by all four LC methods.

The following general scheme has been proposed by Kirkland [Chapter 5 in *Modern Liquid Chromatography*, L. R. Snyder and J. J. Kirkland, American Chemical Society, 1971] as a guide in selecting a mode of LC and a column type. Products mentioned are merely illustrative of types that would successfully be used.

a. Molecular-Weight Range of Sample greater than 2000.

1. Aqueous system—use gel filtration on Sephadex, Biogel, porous glass, etc.
2. Nonaqueous system—use gel permeation chromatography on Poragel, porous glass, Porasil, etc.

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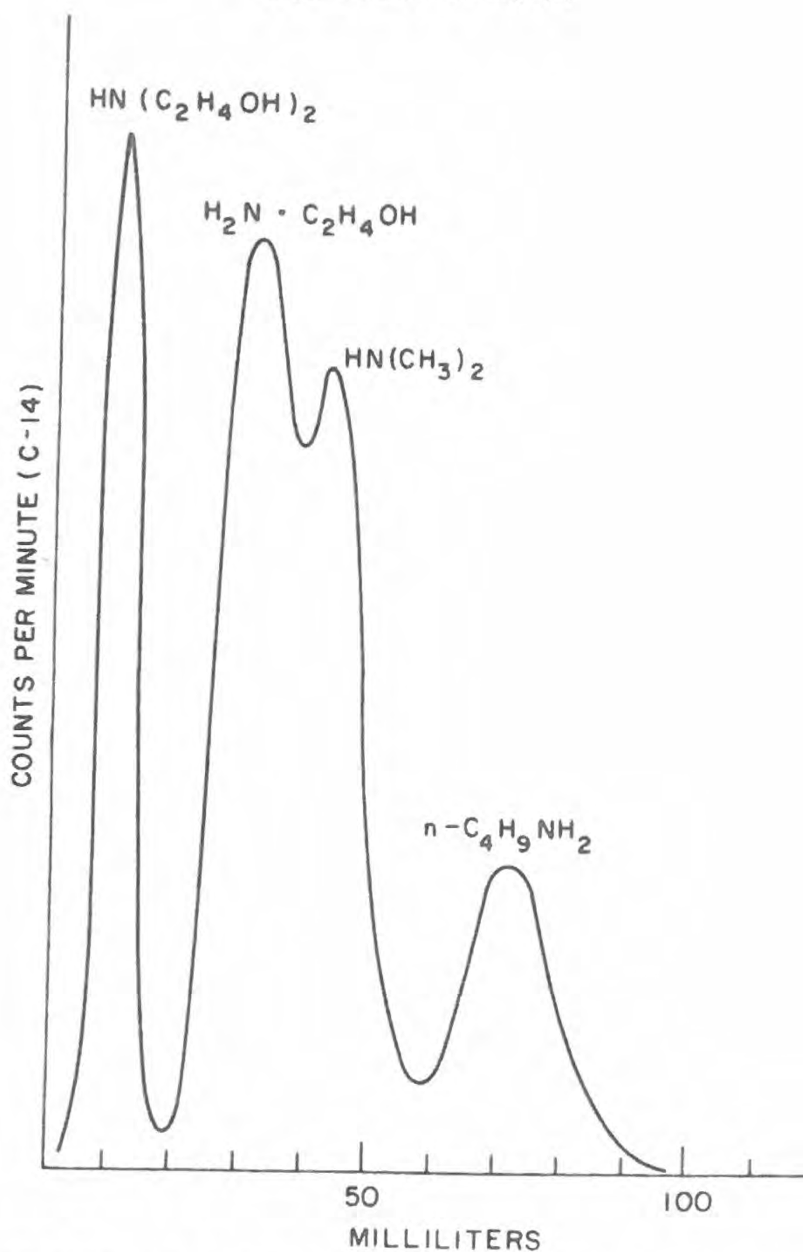

Figure 41. Separation of amines by ligand exchange chromatography on 2% cross-linked sulfonated polystyrene, 50–100 mesh, nickel form; 10.6 ml bulk column volume, 1.0 cm diam.; eluant, 0.94 *M* NH_3 to 50 ml, 1.8 *M* after that, 0.3 ml/min [after (*52*)]; *Anal. Chim. Acta*, **33**, 84 (1965). Reproduced with permission from Elsevier Publishing Co., Amsterdam, The Netherlands.

b. Molecular Weight less than 2000.

1. Aqueous system—use gel filtration on low porosity Sephadex or ion exchange on a gel or pellicular cation-exchange resin for basic compounds or on a gel or pellicular anion-exchange resin for acidic compounds.
2. Nonaqueous system—if size differences are significant, gel permeation chromatography on low porosity polystyrene beads or Poragel is used. For non-labile systems.or isomer separations, use adsorption chromatography on silica gel, alumina, Porasil or Corasil II. For labile systems

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and different compound types, use partition chromatography in the normal mode for polar compounds and in the reversed-phase mode for non-polar compounds.

LC methods can be readily combined for the separation of complex mixtures. A good general approach is separation first by exclusion chromatography followed by further separation of separated fractions by one of the other three high resolution methods.

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VOLUME TWO
SECTION I

PRINCIPLES AND TECHNIQUES

I.IV Paper Chromatography

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Section I.IV

PAPER CHROMATOGRAPHY (PC)

In paper chromatography, one spots a drop of sample near one end of a sheet or strip of filter paper. The paper edge nearest the spot is immersed in a suitable solvent contained in a tank. The spot must not be covered by the solvent. In the tank, the solvent moves along (up or down) the paper by capillarity carrying the components along at different rates. When the solvent front approaches the opposite end of the paper, it is removed from the tank and the zones are observed. In many cases, the complexity of the mixture makes for incomplete separation of its components in a single solvent. Two-dimensional chromatography is more effective because it utilizes the differential separating power of two solvents, run in two directions at right angles to each other, and because development occurs over twice the length of paper.

Differential migration occurs because the components of the mixture are selectively distributed between the stationary phase containing cellulose or attached to it and the solvent, which can be organic, aqueous or a mixture of these. For many applications, ordinary cellulose paper can be modified so that resolution is increased. The equipment for paper chromatography is inexpensive, the techniques are simple, and virtually every class of compounds can be separated.

A. FUNDAMENTALS OF PAPER CHROMATOGRAPHY

Cellulose paper consists of a partially oriented collection of cellulose fibers. These fibers are composed of approximately parallel carbohydrate chains strongly cross-linked together by hydrogen bonding in some regions to give a partly crystalline and partly amorphous structure. In the amorphous regions, water or other hydrophilic solvents are absorbed by the cellulose. This leads to the formation of pools of liquid connected by crystallite bridges. Water in these regions is of two types, one being chemically bonded to the cellulose fibers and the other being more loosely bound and available for partitioning the solutes.

1. *Theory and Mechanism*

As stated in Section I.IIIB3e, paper chromatography in systems with stationary water involves a combination of mechanisms, namely adsorption, partition and ion-exchange. Ordinary chromatography papers have ion-exchange properties due to the presence of a small number of carboxyl groups, and under certain conditions this effect can explain the retention of cations, the frontal analysis (demixing) of developers and the formation of multiple solute zones. Paper can be impregnated with various adsorbents, liquids, or ion exchangers to make the interactions with the solutes more selective.

Considerations discussed in earlier sections are valid in relation to the performance of paper chromatographic systems: a good system is one which has the ability to separate the centers of the solute spots and to keep the spots compact; three factors responsible for spot spreading are molecular diffusion (both in the mobile and stationary phases), eddy diffusion and resistance to mass transfer; and an average plate height over the distance of development can be calculated by an equation similar to the van Deemter equation for GC (*1*). Based on theoretical studies, a velocity of development near 1×10^{-3} cm/sec has been recommended by Stewart as being optimum for systems with stationary water. In practice, it is much more difficult to control the velocity of solvent flow in PC than in column chromatography. Some methods include location of the initial spot farther from the solvent reservoir, use of solvents of different viscosities, and wick-feeding of the solvent to the paper. Another problem is that there is usually considerable variation in the solvent velocity during the run, from the initial, rapid penetration into the paper to the final, slower arrival of the front some distance along the length of the paper.

Thoma (*2*) has made simulation studies of the chromatographic behavior of model, polar systems in order to devise general guidelines for systematically optimizing operating conditions. He stresses that chromatographic theory cannot be used quantitatively as a predictive tool in system design even though the effects of many individual factors (e.g., particle size and solvent velocity) on chromatographic behavior are well understood. The problem lies in the complexity of the overall chromatographic system which causes many parameters to change when only one experimental condition is altered. Qualitatively, the following are recommended: small sample loads should be developed on a finely-divided stationary phase with a solvent migrating as rapidly as permitted by the velocity at which distribution equilibrium is obtained; the solvent and sorbent should be chosen to provide specific interactions with the solutes; solvent proportions are chosen by trial-and-error to carry the spots $\frac{1}{4}$–$\frac{1}{2}$ the distance along the support for single development; development may be improved by altering the solvent, lengthening the support or employing multiple development in the same direction with the same or different solvents.

2. *R Values*

With one-way or radial development, the migration behavior of a substance is described relative to the solvent front (R_F values) or a standard substance X (R_X values) (Figure 42). The migration distance of the solute can be measured to the geometric center of the zone, the point of maximum concentration, or to both

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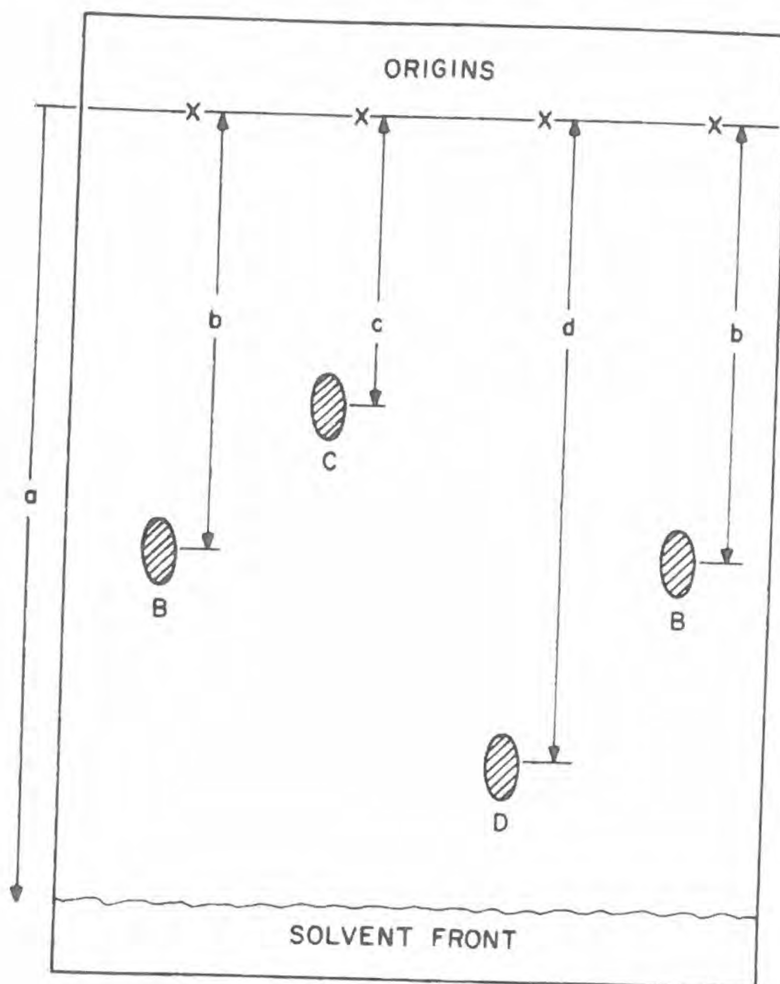

R_F OF COMPONENT C = $\frac{c}{a} \times 100$

R_B OF COMPONENT C = $\frac{c}{b} \times 100$

R_F OF COMPONENT D = $\frac{d}{a} \times 100$

R_B OF COMPONENT D = $\frac{d}{b} \times 100$

Figure 42. Method for calculating R_F values and R values relative to standard B (R_B values) of components C and D on a descending chromatogram.

the leading and tailing boundaries of the zone. The results of two-dimensional chromatography are best reported by listing the R values in each solvent and also presenting a drawing or photograph of the final chromatogram. Results can be reported without R values if sequences and separations are listed.

R_M values, where

$$R_M = \log\left[\left(\frac{1}{R_F}\right) - 1\right] \quad [54]$$

are used in studies of the relation between solute structure and chromatographic behavior [see, for example, Reference (3)].

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3. *Reproducibility of* R_F *Values*

R values vary significantly depending upon the experimental conditions and are not sufficient alone for the certain identification of an unknown substance. Factors which influence R_F values are the following: composition and aging of the solvent; nature, humidity and treatment of the paper; concentration of the solute; shape of the paper; direction, length and method of development; distance of the origin from the solvent reservoir; volume of solvent in the reservoir; air flow about the chamber; temperature; conditions of equilibration; change in composition of the solvent as it flows over the paper; irregular solvent flow; dimensions of the developing chamber; and impurities in the solvent, paper, or sample. R_X values are less affected by many of these variables and are more reproducible.

It is seldom necessary to sacrifice the inherent simplicity of paper chromatography in order to attempt to rigidly standardize operating conditions. R_F and R_X values should be considered as guides to possible separations whose numerical values cannot be exactly reproduced. Sequences and differences between these values are more significant than the actual numbers.

4. *Artifacts and Anomalous Zones*

Each solute should ideally be found in one compact, round or oval spot after development. In practice, zones may have double tails (Figure 43) or be diffused, streaked, flattened, swerved, or otherwise distorted,

Figure 43. Pigments in various amounts of leaf extract separated by one-dimensional migration on Whatman No. 1 paper from initial zones formed as a spot and as a streak; PE = petroleum ether, Bz = benzene, Ch = chloroform, Ac = acetone, IP = isopropanol, C = carotene, L = lutein, V = violaxanthin, a = chlorophyll *a*, b = chlorophyll *b*, N = neoxanthin, Y = yellow, O = orange, G = green [after (*10*)]. Figure 6, p. 481, *J. Chem. Educ.*, 46 (1969). Reproduced with permission from Division of Chemical Education, American Chemical Society.

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and a single substance may yield two or more distinct spots (multiple zonation). Even if the spots formed are not of ideal size and shape, the chromatographic system may be useful if it reproducibly provides a desired separation. Reasons for the formation of distorted zones are varied and not always understood. Interested readers may consult books on paper chromatography listed in the bibliography for detailed discussions of these topics.

B. THE PAPER

1. *Pure Cellulose Paper*

Various manufacturers provide commercial chromatography papers with a wide range of properties. Whatman Chroma Papers, especially manufactured for chromatography, are low in organic and inorganic impurities and uniform in physical characteristics. Whatman and other manufacturers supply various grades of filter papers "selected for chromatography", and the user should always specify that he desires such grades when placing an order.

Some workers prefer to personally pretreat commercial paper by an overnight descending development with a dilute solution of HCl, EDTA, diethyl ether, or the first development solvent they plan to use. This should be followed by a distilled-water wash and drying.

Many grades of paper are commercially available in a variety of sizes and shapes: rectangular, circular and other special shapes for certain procedures and apparatuses.

Table 7 (Section II.III) shows the types of pure cellulose and other papers available from selected commercial sources. As a general rule, Whatman No. 1 paper or an equivalent grade is suitable for general analytical work and the thicker Whatman No. 3 MM for preparative work.

2. *Chemically Modified Paper*

Partially acetylated paper, prepared in the laboratory or purchased commercially, is useful for separating hydrophobic substances. Papers with the carboxyl group content increased by chemical reactions have been used to separate various classes of polar compounds.

Copolymerized cellulose has been used as a support for reversed-phase chromatography. Wedge-compressed nitrocellulose membrane filters, either intact or impregnated with nonionic detergents or proteins, are a convenient medium for small-scale chromatographic separations or deproteinizations.

3. *Impregnated Papers*

Papers have been impregnated with hydrophilic liquids to facilitate separations of moderately hydrophilic substances (partition paper chromatography) and with olive oil, silicone oil, paraffin, rubber latex, etc., for the separation of hydrophobic solutes (reversed-phase partition paper chromatography). Papers impregnated with high-molecular-weight amine liquid anion exchangers, high-molecular-weight acid liquid cation exchangers, or neutral organophosphorus compounds serve for the separation of metal ions when developed with aqueous solutions of mineral acids. Chromatography on papers containing complexing or precipitating agents (e.g., 8-hydroxyquinoline, dithizone, CdS, quinalizarin, etc.) with various aqueous and aqueous-organic two- and three-component solvents also separates metal ions.

4. *Loaded Papers*

Loaded papers consist of a dispersion of powder, fiber or resin in a normal cellulosic paper network.

a. *Papers loaded with adsorbent powders.* These papers are used to separate less polar substances by adsorption chromatography using solvents similar to those used with thin layers of the same adsorbent. The presence of the cellulose along with the adsorbent may, however, lead to separations different than on a layer of adsorbent alone. Papers containing silica gel, alumina and kieselguhr are commercially available.

Polar compounds such as sugars and amino acids are separated on silica-gel papers using normal partition solvents developed for pure cellulose paper. By the proper choice of solvents, two-dimensional separations with an adsorption mechanism in one direction and partition in the other can be performed on silica paper.

b. *Papers loaded with ion-exchange celluloses.* Cellulose phosphate and carboxymethyl cellulose cation-exchange papers and aminoethyl, diethylaminoethyl and Ecteola cellulose anion-exchange papers are commercially available. Some properties of these papers are listed in Section II.III, Table 7. They have proven to be useful for a wide range of inorganic separations and applications in the biochemical field.

Knight (*4*) has pointed out that normal chromatographic techniques are often modified for use with ion-exchange cellulose papers. The papers are generally converted to the required ionic form by a descending wash with the developing solvent. Only with the "strong" cellulose phosphate exchanger can the treated paper be washed free of excess electrolyte after conversion, dried, and used in the normal "dry-start" manner without disturbing the established equilibrium. In other cases, the paper must be used with a "wet-start" technique in which the sample is applied directly to the wet, equilibrated paper as it hangs in the chamber. After application of the spots, descending development is carried out for a timed period since no solvent front will be visible. Ion-exchange papers are generally used with aqueous solvents and so are less sensitive to changes in conditions (e.g., temperature, humidity) during the development. All operations after development are the same as with ordinary paper.

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Amino acids exhibit both ionic and nonionic interactions with ion-exchange celluloses, and both influences are important in determining the sequence and resolution of the compounds. By the proper choice of solvents, two-dimensional amino-acid separations, in which ion exchange predominates in one direction (ionic solvent) and partition ± adsorption in the other (nonionic solvent), have been obtained on ion-exchange papers.

c. *Papers loaded with synthetic organic ion-exchange resins.* Commercial papers containing polystyrene strong and weak anion- and cation-exchange resins are available. Conversion of the resin to the same ionic form as the developing solution is obtained by a descending wash with, or soaking in, an appropriate electrolyte, followed by a distilled water rinse and drying. The normal "dry-start" development technique is used unless solvent demixing occurs during development, in which case the sample is applied, if possible, after the *bulk* solvent front has been allowed to migrate a few cm past the origin.

Development can be with various types of solvents: simple electrolytes, aqueous solutions of complexing agents, or mixed solvents with two or more components. The papers can be impregnated with various precipitating or complexing agents to improve resolutions.

Results obtained on ion-exchange papers are not necessarily equivalent to results obtained in batch equilibrium and column experiments employing the same resin and solvent because of basic differences in the techniques and the presence of the cellulose plus resin in the ion-exchange paper.

d. *Papers loaded with inorganic ion exchangers.* Papers may be impregnated with inorganic exchangers such as zirconium phosphate, hydrous oxides and ammonium molybdophosphate. Support-free cerium(IV) phosphate sheets have provided selective metal-ion separations when developed with aqueous solutions of $HClO_4$.

5. *Glass Fiber Papers*

These papers are useful in extreme conditions of temperature and acidity where cellulose papers are unsuitable. Corrosive detection reagents (e.g., chromic-sulfuric acid charring solutions) can be employed with these papers. Glass papers can be used untreated, or more often after impregnation with aqueous salt solutions, silica gel or alumina. Glass fiber papers already impregnated with silica gel and silicic acid are commercially available from The Gelman Corporation as ITLC media (see Table 8 in Section II.III).

C. SOLVENT SYSTEMS

The most important variable in paper chromatography is the solvent, the choice of which depends upon the nature of the substances to be separated. Solvent systems have been devised for separating members of virtually every class of compounds as can be seen from inspecting the data presented in Part III of this Handbook.

Solvents can be composed of two phases, the atmosphere of the chamber being saturated with the aqueous phase (or both phases), while the immiscible organic phase is used as the developer. Separations obtained in these systems are due primarily to the selective partitioning of the solutes between the two liquids. The phases can be reversed so that the more-polar layer is used as the solvent and the paper is impregnated with the less-polar layer (often an oil). In addition to two-phase solvents, "direct-phase" solvents consisting of an organic liquid saturated with a more-polar substance (e.g., phenol or *n*-butanol saturated with water), water-miscible solvents and even pure water have been used as the mobile phase. In general, polar solutes are separated in a polar solvent on cellulose which is unmodified or impregnated with a polar liquid; hydrophobic solutes are separated in reversed-phase systems (see Table 1).

1. *Aqueous Stationary Phase*

The paper is saturated with water by soaking and then blotting the excess, or water is taken up from the atmosphere by equilibrating hanging paper in a sealed chamber saturated with water vapor. Aqueous buffer or salt solutions are applied by dipping the paper, drying it, and then allowing it to equilibrate with water vapor in a chamber as just described.

Hydrophilic (e.g., sugars, amino acids) and some medium-polarity substances (e.g., ketonic, nitro) are separated on these papers by developing with solvents such as isopropanol-ammonia-water (9 : 1 : 2 v/v), *n*-butanol-acetic acid-water (4 : 1 : 5 v/v, organic layer) or phenol saturated with water. When two-phase solvents are employed, the chamber is saturated with both phases.

2. *Stationary Polar (Hydrophilic) Organic Solvent*

For volatile organic liquids (e.g., methanol), the paper is saturated from the atmosphere of the chamber and developed with an immiscible organic solvent. For less volatile liquids (e.g., dimethylformamide), the paper is drawn through the liquid dissolved in a volatile solvent (e.g., ethyl acetate), and the excess liquid is removed by blotting between two thick filter papers, squeezing the paper between rollers, or by simply holding the paper and letting the excess drip back into the vessel containing the impregnation solution. The paper is then hung in air until the solvent has evaporated (5–10 min). Many workers recommend saturating the mobile phase with the stationary liquid, but others suggest that this is not always necessary (if the two phases have very low mutual solubility) or even desirable (because the paper may extract additional impregnating liquid from the mobile phase, becoming overloaded and thereby causing lowered R_F values).

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TABLE 1

SOLVENT SYSTEM ACCORDING TO SUBSTANCE GROUPS AFTER DECKER

Substance group	Solvent systems
1. Strongly hydrophilic substances, such as amino acids, sugar, more readily soluble in water than in alcohol, R_F values in water-free butanol about 0	Organic solvent mixtures that are miscible with water to an unlimited or limited extent, with 10–40% water content. Possibly addition of acids, bases, or salts, especially buffers. Addition of water increases R_F value.
2. Moderately hydrophilic substances, more readily soluble in alcohol than in water, R_F values in water-free butanol frequently 0, in water about 1	Same as 1. Moreover, mixtures with a lower percentage of water, which contain homopolar solvents, such as chloroform, benzene, petroleum ether, ethyl acetate.
3. Aromatic and heterocyclic substances, such as phenols, dyes; R_F values in water, possibly after addition of acids, salts, or ammonia, 0.05–0.9	Same as 1, and 2. Moreover, mixtures of solvents with water 1 : 1 or 2 : 1 and various additions, such as acids, alkalies, salts (e.g., Na_2SO_4), salts reduce the R_F values.
4. Lipids, soluble in petroleum ether, insoluble in water; R_F values with water >0, with water-saturated butanol about 1	I. Same as 1. (water content will here reduce the R_F values) Same as 2. (petroleum ether and other hydrocarbons will frequently reduce the R_F values), however, frequently useless because of "tail" formation. II. Solvent mixtures, where the water is completely or in part replaced by formamide, glycol, etc. If necessary, the paper should be impregnated with the stationary phase. III. Reverse-phase system. Paper hydrophobized with silicones, oils, rubber, chlorinated rubber, or through acetylation. Solvent aqueous phase.
5. Acids and bases	I. Same as 1. and 2. and addition of stronger acids or bases for repressing dissociation. II. Same as 1. and addition of bases or acids for conversion into the hydrophilic salts.

(Reprinted from "Filtrak Papers for Chromatography", VEB Spezialpapierfabrik Niederschlag, Niederschlag, Erzgebirge)

The Zaffaroni systems employ paper impregnated with formamide or propylene glycol and developed with benzene, $CHCl_3$, cyclohexane or mixtures of these. These types of systems are chosen for the separation of moderately-hydrophilic, medium-polarity solutes such as many steroids.

3. *Stationary Non-Polar (Hydrophobic) Solvent*

Reversed-phase systems are used for the separation of hydrophobic solutes. The paper is drawn through a solution of Vaseline, kerosene, paraffin oil, or silicone oil dissolved in hexane, petroleum ether or benzene, hung in air until the solvent evaporates, spotted and developed with an immiscible, more-polar solvent such as aqueous isopropanol (70%), dimethylformamide-methanol-H_2O (10 : 10 : 1 v/v), or aqueous acetic acid. The mobile phase is usually saturated with the stationary phase prior to development.

Another technique for impregnating the paper is to dip the top into the impregnating solution which rises up the paper toward the origin. If the mobile phase to be used for development carries the solutes at its front on nonimpregnated paper, the impregnating solution is halted just short of the origin line. After evaporating the solvent, the sample is spotted below the impregnated area. The mobile phase will rise through the sample and carry it into the impregnated area whereupon an ideal thin, transverse origin is produced spontaneously.

Acetylation makes cellulose less hydrophilic so that systems employing acetylated paper are essentially reversed-phase systems. Such paper preferentially takes up organic components from equilibrating solutions in the chamber.

D. TECHNIQUES OF PAPER CHROMATOGRAPHY

1. *Sample Preparation*

Samples are dissolved in a small volume of a suitable solvent (often a volatile, organic solvent such as acetone, ethanol or chloroform; not necessarily the same as the development solvent) at a concentration level high enough so that a small spot can be applied to the paper.

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Extracts of animal or plant tissues and other biological samples are prepared by grinding or blending with a suitable solvent and removing insoluble residue by filtration or centrifugation. The extract must usually be purified and concentrated and often transferred to a different solvent before application to the paper. Removal of extraneous organic materials and inorganic salts, which can lead to anomalous results (e.g., streaking), is accomplished by solvent extraction, precipitation, electrolysis, or ion exchange.

Some chromatographic systems have been developed which allow biological samples to be directly applied to the paper without any preliminary clean-up. One example is the separation of amino acids by the development of untreated urine samples with three solvents in two directions on a 35 × 28 cm sheet of Whatman No. 1 paper (*5*).

Sometimes it is desirable to convert the substance of interest into a derivative before chromatography. This is true if the substance is more volatile or less easy to chromatograph, detect or to quantitate than the derivative.

Some methods for the preparation of samples suitable for chromatography are collected in Section II.II.

2. *Sample Application*

The amount of sample to be applied depends upon the sensitivity with which the solutes can be detected after migration and concomitant dilution. One to 20 μl of a solution containing 0.1–1% (w/v) of each solute is typical, and this amount of sample can be manually applied to pencil-marked origins with a micropipette, a platinum loop or by a dab of a wood applicator stick. The origins are situated about 6 cm from the end of the paper for descending chromatography and 2–3 cm for ascending. The spot is dried after application, either in air or with a portable hair drier, and should be about 5 mm in diameter (15 mm at most). Larger volumes of samples are applied by successive applications of a small volume to the same area of paper with drying in between to keep the spot diameter small, or, alternatively, as a streak along the origin line across the paper.

Application of the sample in the form of a streak rather than as a single spot can be beneficial in eliminating the double tailing of chlorophyll zones separated from leaf extract (Figure 43). When the loadings are comparable, the separation from the streak and in the central regions of the migrating spots are identical in this case. Figure 43 also illustrates that higher loading causes all the pigment zones to be pushed forward resulting in poorer resolution of the leaf extract. Many workers state that dependence of R_F values on sample concentration indicates that the system is governed by an adsorption rather than a partition mechanism.

The two most important general guidelines are that initial zones be kept reasonably small and as uniform as possible, and that solutions of standards and of unknowns must be as similar as possible and they must be applied in exactly the same manner.

3. *Development Procedures*

a. *Descending development.* The end of the spotted paper nearest the origin is placed into a glass or plastic trough supported at the top of the developing chamber on wires or on glass rods. The paper is anchored in the trough by means of a glass rod and passes over a second, elevated rod which prevents siphoning of the solvent (Figure 44). Solvent is added carefully to the trough through a hole in the top of the chamber and flows through the origins and down the paper.

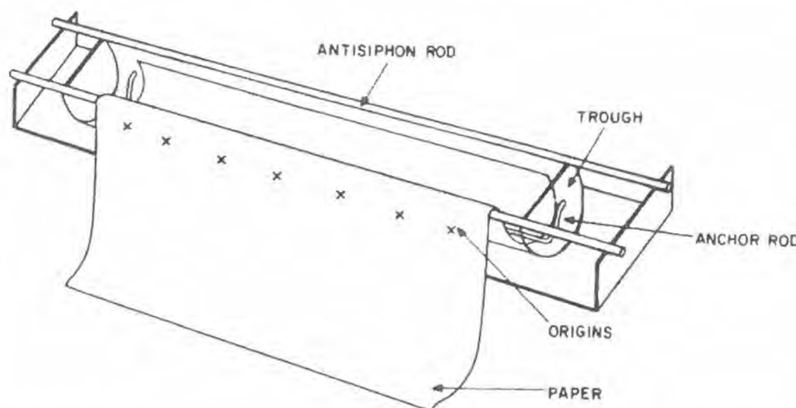

Figure 44. Arrangement of paper in a solvent trough for descending chromatography. A second piece of paper can be inserted under the anchor rod and over the second antisiphon rod for simultaneous development.

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The chamber must be tightly closed in order to maintain its saturation with the vapors of the solvent components. This saturation is obtained by placing a small dish of the solvent (both phases when two-phase systems are used) at the bottom of the chamber, or by covering the bottom of the chamber with solvent and lining the walls with filter paper dipped into this solvent. Care must be taken that the paper being developed does not dip into this extra solvent or touch the paper lining the tank.

The paper may be saturated for a period in the tank after spotting but before the solvent is added to the trough. However, most workers now apply the initial spots to the paper and begin development at once (without pre-equilibration of the paper) in a tank pre-saturated with the solvent vapors. With this procedure, the paper is equilibrating with the solvent throughout the run.

At the end of development, the paper is removed and the solvent front marked at once so R_F values can be calculated. Glass chambers are convenient so the front can be observed during the run. For slow moving substances, better resolution may be achieved by permitting the solvent to drip off the lower end of the paper during development of several days duration. Migration must then be related to a standard compound rather than to the solvent front.

Various commercial chambers are available for the descending development of sheets up to 18 × 22 in. Apparatuses have been devised for automatically adding solvent to the troughs and stopping the solvent flow at a preselected distance from the origin.

b. *Ascending development.* In this method, solvent is placed in the bottom of the chamber, and the paper is suspended above it from a wire or glass rods (Figure 45a). Alternatively, the spotted paper can be clipped into the form of a cylinder and stood in the solvent (Figure 45b). The top of the solvent must be well below the level of the origin line on the sheet in each case. Again, the chamber may be lined with extra filter paper to improve vapor saturation.

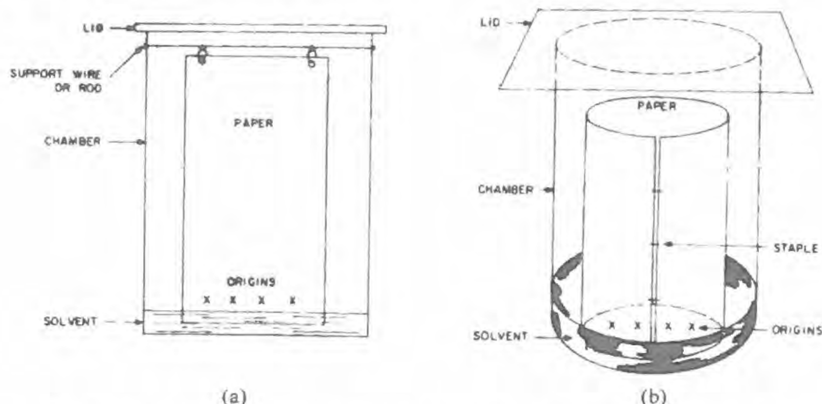

Figure 45. Apparatuses and arrangements for ascending paper chromatography.

Small paper strips may be stapled to the underside of a cork and developed in test tubes. Large paper sheets are developed in large commercial metal cabinets or glass aquaria fitted with glass rods running the length of the chamber at the top to which the paper is clipped.

The choice between ascending and descending development is usually a matter of personal preference since with equal vapor saturation results will be about the same. The rate of development is faster and more constant with the descending technique since the force of gravity restricts ascending development. In the ascending method, it is easier to maintain good vapor saturation, especially with very volatile solvents.

c. *Horizontal development.* A square or rectangle of paper is laid horizontally on glass rods or some other support, and the end nearest the origins is folded down and dipped into a solvent trough. Improved reproducibility of R_F values has been claimed for the horizontal method, and the chamber holding the support rods and trough can be made flat and quite compact so that it fits easily in an oven or refrigerator for development at elevated or reduced temperatures.

d. *Radial development.* In this method, the solutes are resolved into circular zones or arcs instead of the customary round or oval spots. Although circular zones are formed, circular paper is not required. The paper is usually developed in a horizontal position, but the method differs from that described directly above in that the solvent is fed to the paper through a wick or a restricted area of the paper. In one possible arrangement, horizontal, supported paper is in the shape of a square with a tab, which is bent down into the solvent, protruding from the center of one of the sides.

A simple apparatus for radial chromatography on a filter-paper disk consists of two equal-size Petri dishes slightly smaller than the paper. A narrow, parallel strip is cut from the center to the edge of the paper, and this is bent down at the center joint perpendicular to the paper to serve as a wick. The drop of mixture is placed on the paper disk at the joint of the wick, and the disk is placed between the two glass dishes, the

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lower containing the developing solvent. The rate of development is controlled by the width of the wick and the distance between the liquid surface and the paper. Dessicators can be used for larger tanks with the paper placed between the cover and the bottom part. Solvent is applied to the paper through a wick from a solvent reservoir below or fed to the paper by a pipette through a hole in the upper lid.

Multiple samples (e.g., standards and unknowns) can be applied as equidistant spots on a circle with a radius of 1–2 cm from the center of the paper disk; in this case, a series of segments results after radial development.

Resolution by radial chromatography is often superior to that by normal one-way ascending and descending chromatography because the arrangement leads to greater solvent flow at the trailing zone boundaries relative to the leading boundaries and therefore sharper zones.

e. *Overrun and unidimensional multiple development.* One method for gaining a greater effective distance of solvent flow without actually increasing the length of the paper is overrun development, where the solvent is allowed to continue flowing after the front has reached the end of the paper. This is accomplished by cutting drip points along the bottom of descending chromatograms or allowing the top of ascending chromatograms to protrude out of a slot in the chamber lid.

Another method is to develop the paper as usual by ascending or descending solvent flow, dry the paper, and repeat the development one or more times with the same, or a different solvent. The following equation is used to calculate the optimum number of developments with the same solvent in the same direction for obtaining the greatest separation between two substances

$$\eta_{opt} = \frac{\log[\log(1 - R_{F_2})/\log(1 - R_{F_1})]}{\log(1 - R_{F_1})/(1 - R_{F_2})} \quad [55]$$

where R_{F_1} and R_{F_2} are the R_F values of the two compounds after one development, $R_{F_1} > R_{F_2}$.

f. *Two-dimensional development.* The mixture is placed in one corner of a square sheet of paper and developed in succession with two different solvents in transverse directions. Development is by ascending or descending flow as described above; the chromatogram is thoroughly dried in a hood or chromatographic oven between the runs in order to remove all traces of the first solvent. By judiciously choosing solvents with different characteristics, this procedure yields the maximum resolution of complex mixtures. Figure 46 illustrates the separation of amino acids in a protein hydrolysate by development with an acidic and basic solvent.

Two-dimensional chromatography with reaction of the solutes on the paper after the first run is used to assess the effect of an enzyme or reagent on the solute or to aid in their identification. The same or a different solvent is then used in the second direction. To improve resolution, spots can be transferred automatically from one type of paper to another type or to a thin layer by attaching the papers or the paper and the thin layer together for two-dimensional chromatography. Already mentioned in Section B were two-dimensional separations with a different mechanism operative in each direction on adsorbent and ion-exchange papers.

g. *Gradient development.* Various methods have been described for changing the composition of the solvent continuously during the run [e.g., Reference *(6)*]. This is a specialized method, which like centrifugal-paper chromatography and some others, has not been widely used.

4. *Detection Methods*

After development and marking of the solvent front, the paper is removed from the chamber and dried in a hood with the aid of a fan or electric hair drier or in a chromatography oven. The solutes, if they are not naturally colored, must then be located.

a. *Chemical methods.* A suitable chemical reagent which forms a colored or fluorescent derivative with the compounds of interest is applied by dipping the paper into a solution of the reagent in a shallow tray or by spraying the solution onto the paper by means of an atomizer or aerosol bomb. The paper must sometimes be heated to complete the reaction. In some cases, two or three reagents are sprayed on the paper in turn to give the desired reaction.

The dipping technique is considered by some workers to be superior although it is probably not as widely used as spraying. The solvent chosen for the reagent solution must be one in which the solutes and their derivatives have very low solubility so that the spots remain fixed on the paper. Spray reagents are applied lightly and as uniformly as possible over the entire surface of the paper.

Section II.I lists a large number of reagents suitable for the detection of many types of compounds. Limits of detection for most compounds range from about 50 μg to <0.1 μg with reagents of this type, depending upon the type of compound, the type of paper, the compactness of the spots, etc.

Maximum information is gained from a single chromatogram if a series of reagents are applied sequentially either by dipping or by spraying. One example is the use of ninhydrin to detect amino acids followed by Ehrlich reagent in acid solution to detect indoles and finally anisidine reagent to detect imidazoles and hydroxyindoles.

b. *Physical methods.* Many organic substances absorb ultraviolet light between 240–260 nm and show up as dark areas when illuminated with such light. In some cases the sensitivity is improved if the paper is sprayed with fluorescein. This provides a fluorescent background against which the absorbing spots contrast sharply.

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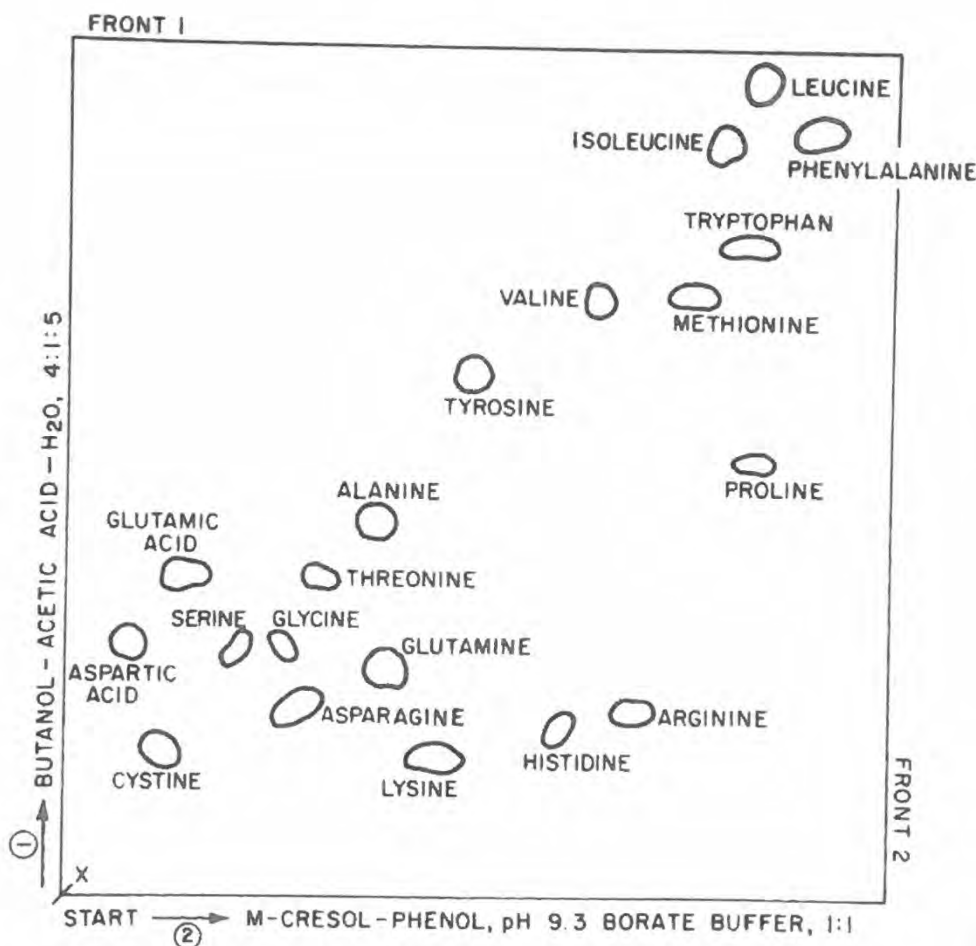

Figure 46. Two-dimensional paper chromatographic separation of amino acids [after (11)].

Other organic substances fluoresce when illuminated with light of about 360 nm (some only after heating or cooling the chromatogram) or phosphoresce when illuminated with 254 nm light (in some cases only in the wet state but not when present as dry spots on paper). Papers are conveniently examined in a darkened room under a long- or shortwave ultraviolet hand lamp or in a lighted room in a portable viewing cabinet.

c. *Radioactivity*. The positions of radioactive (labelled) substances can be determined by autoradiography or by scanning the paper with a hand-held counter or a mechanical scanner which graphically records peaks of radioactivity corresponding to the location of each zone. Both of these techniques are discussed in more detail in Section H.

d. *Biological methods*. Bioautographic methods of detection, based on the inhibition of growth of certain organisms, are specific for active antibiotics and do not detect biologically inactive artifacts, decomposition products or impurities. The procedure is to put the chromatogram on the surface of an agar plate which has been inoculated with a test organism (usually *Staphylococcus aureus* or *Bacillus subtilis*) which is sensitive to the antibiotic to be detected. After incubation for 15–20 hr at about 37°, clear zones become visible in the agar layer where the antibiotic has diffused in and inhibited the growth of the microorganism. The rest of the surface appears opaque. The time of incubation can be shortened and the growth-inhibition zones made more conspicuous by spraying the agar plate with a solution of 2,3,5-triphenyltetrazolium chloride and a solution of 2,6-dichlorophenol-indophenol after 4 hr incubation. After an additional 30 min incubation, inhibition zones are visible as blue spots on a colorless background.

Inhibitory zone diameters can be measured and related to concentration for the semi-quantitative estimation of antibiotics.

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5. *Documentation of Results*

Chromatographic results can be recorded by means of R_F or R_X values as described above or by making drawings, Xerox copies, UV photocopies or photographs of the actual chromatograms.

In many cases, the papers themselves can be retained for future reference. Storage in the dark or spraying with a certain solution can help retard fading of the colored spots with time. For example, Zweig has recommended a copper nitrate-nitric acid dip followed by a clear acrylic spray for the preservation of amino acid chromatograms detected with ninhydrin.

E. IDENTIFICATION OF SUBSTANCES

Chromatography can be used in the following ways to aid in the identification of a substance. As more of these methods are employed to gain information, the degree of probability of the identification is greatly increased.

1. Co-chromatography—tentative identification is obtained if the unknown is mixed with an authentic standard and the two cannot be separated in several diverse systems.
2. Use of selective color-forming reagents after development.
3. Recording of visible, ultraviolet and infrared spectra after elution of the spots from the paper. UV and phosphorescence spectra can be recorded for substances directly on the paper, although the spectra may differ from those obtained for the same substances in solution.
4. Chemical reactions performed directly on the paper before one-way chromatography or between developments in two-way chromatography. [See Reference (7) for a review of diagonal techniques.]
5. Systematic analysis—R_F values are obtained for the unknown in a series of solvents under very reproducible conditions. The "chromatographic spectrum" (a plot of the R_F values in different solvents) of the unknown is compared to the spectra of reference compounds.

F. PREPARATIVE PAPER CHROMATOGRAPHY

Milligram to gram quantities of pure substances can be prepared by scaled-up descending chromatography using thick filter paper. The separated compounds are eluted from the paper with suitable solvents, the solvent evaporated and the compounds crystallized.

Samples are applied as streaks or adjacent spots to the origin of the paper. Commercial applicators especially designed for preparative chromatography are available. Spots of sample and/or standards are applied near the edges of the paper. After development, marginal guide strips are cut from the paper and sprayed to locate the bands containing the compounds of interest in the main sheet. The guide strips are fitted back on the paper, and horizontal areas of paper corresponding to the markers are cut from the paper and eluted by attaching a paper wick from a solvent reservoir and a drip point (Figure 47). The resultant paper strip is folded into a supported trough and placed in a chromatographic chamber for descending development.

In addition to free paper sheets, paper disks tightly compressed into a column, paper pressed into a block, paper wound into a roll, or a rotating paper cylinder can be used for preparative chromotography.

G. QUANTITATIVE PAPER CHROMATOGRAPHY

Quantitative paper chromatography involves the application of uniform, reproducible initial zones containing a known amount of solution; separation by development with a solvent that yields regular zones; formation, if necessary, of a colored derivative of the substance of interest; and measurement of the solute either after its elution from the paper or directly on the paper.

1. *Formation of the Colored Derivative*

The detection reagent must be applied in a concentration high enough to react completely with the greatest concentration of solute that will occur. This may require the use of higher-concentration reagents than are needed for qualitative work. The background areas usually serve as blanks and so should be kept as uniform as possible; dipping techniques for the application of reagents usually give better results than spraying in this regard. Rapid reactions are chosen, if possible, for detection of the spots. Slow reactions are allowed to occur for a timed period which will give maximum difference between the color of the zone and the background. It may be necessary to protect the paper from the light and laboratory atmosphere during the reaction period. Zones that fade badly may be photographed at the height of their intensity and the quantitation made from the photographs. Plots relating the derivative concentration to the concentration of the original compound may or may not be linear; nonlinear reactions can often be used for accurate analyses as long as they are reproducible.

2. *Estimation of the Spots*

Once the colored derivative has been formed, the chromatogram is quantitated by direct or elution methods. In either case appropriate standards are always analyzed along with the unknowns. The concen-

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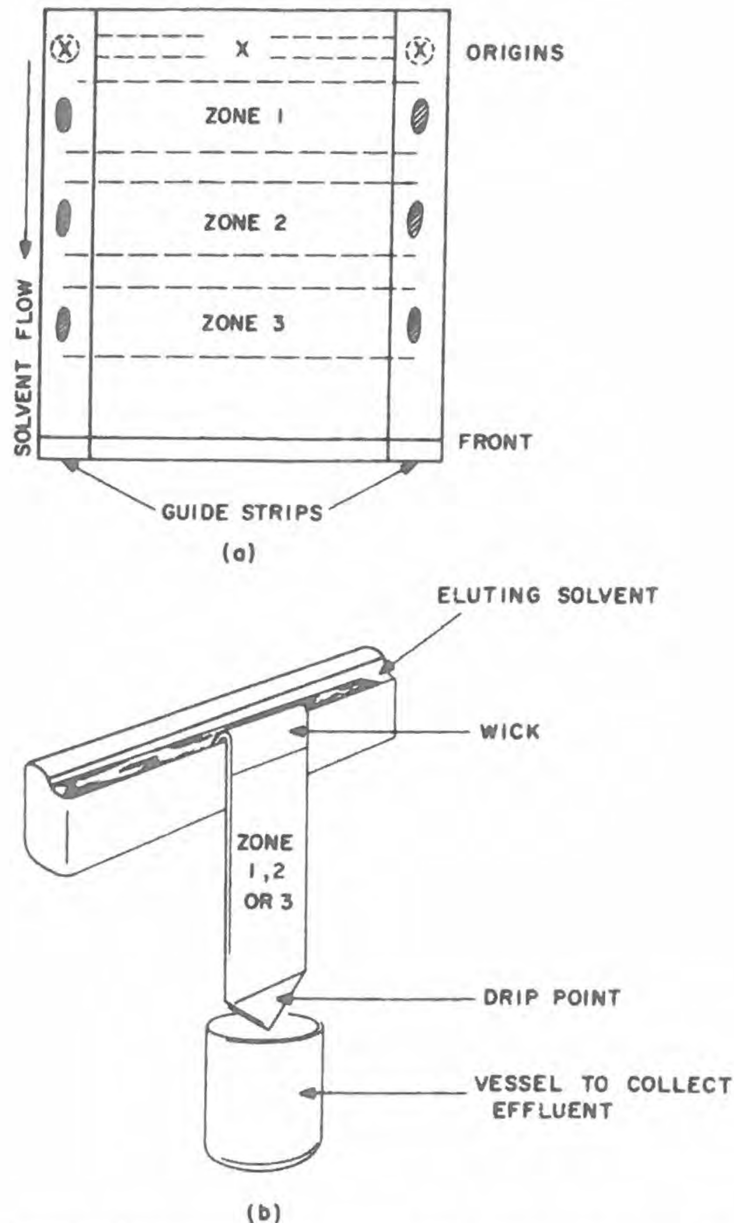

Figure 47. Arrangement of the paper sheet (a) and elution of the zones of substances from the paper (b) in preparative paper chromatography.

trations of standards are chosen so as to bracket the unknown, and the treatment of the unknown and the standards must be in all ways identical.

It is important for direct, comparative methods that the standard solutions be as similar as possible to the solution of the unknown so that the distribution of the compound of interest in all zones is the same. If the test solution contains impurities which could alter the zone distribution, the same impurities should be added to the knowns, or internal standardization should be employed.

a. *Visual estimation.* For many purposes, a rough estimation of the amount present in the unknown made by visual comparison with a series of standards is adequate. Some workers claim that optimum results are obtained when a range of standards of increasing concentration are spotted alternately with a range of dilutions of the unknown decreasing in concentration.

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b. *Spot areas.* Another visual method involves measurement of the areas of spots after chromatography and plotting the areas *vs* the logarithm of the amount of substance present in the spots. If a series of standard spots of the same initial size, covering a short concentration range, is used, these quantities are linearly related and the concentration of an unknown can be read from the graph. If the sample is applied as a streak across a paper strip, the length of the developed zone is proportional to the log of the zone concentration in many cases.

c. *Elution methods.* The separated solutes can be eluted from the paper and determined in the eluate by any appropriate analytical method (e.g., spectrophotometry, microtitration, microgravimetry, etc.). If the solute is naturally colored or fluorescent, the areas of paper containing the solutes can be easily located. If not, guide strips (Section F) are treated with color-forming reagents to determine which region of paper to elute. In some cases, the locating reagent is applied to the chromatogram itself, and the colored derivative is eluted and measured by colorimetry. This variation is faster since guide strips are not required.

Miniaturized methods similar to that shown in Figure 47b have been developed for the elution of individual spots from paper in a very small volume of solvent. Commercial micro-Soxhlet extractors are also useful for the removal of solutes from areas of paper cut from chromatograms.

Errors of 1–2% or below have been claimed for elution methods, as compared to 10–20% for the visual methods described earlier. Recent work indicates that to obtain this high level of accuracy, automatic, machine spotting of samples is required.

If the elution step is not quantitative, it must be at least reproducible. Cellulose fibers appearing in the effluent can be removed, if necessary, by filtering through a small bed of Celite. Unstable solutes must be eluted and determined as quickly as possible.

d. *Direct photometric measurement of spots on the paper.* The instrumental analogue to visual comparison is photodensitometric measurement of the intensity of standard and unknown spots directly on the paper. Densitometry involves the manual or automatic scanning of the chromatogram with filtered light (e.g., visible light of 500 nm or light in the UV or IR regions) in order to measure the amount transmitted through (or reflected from) the paper. The machine is zeroed on a part of the paper free of any solute (the blank), and the strip is then moved slowly past a photocell (Figure 48). The readout consists of a series of peaks whose positions correspond to the spot locations and whose areas correspond to spot intensities.

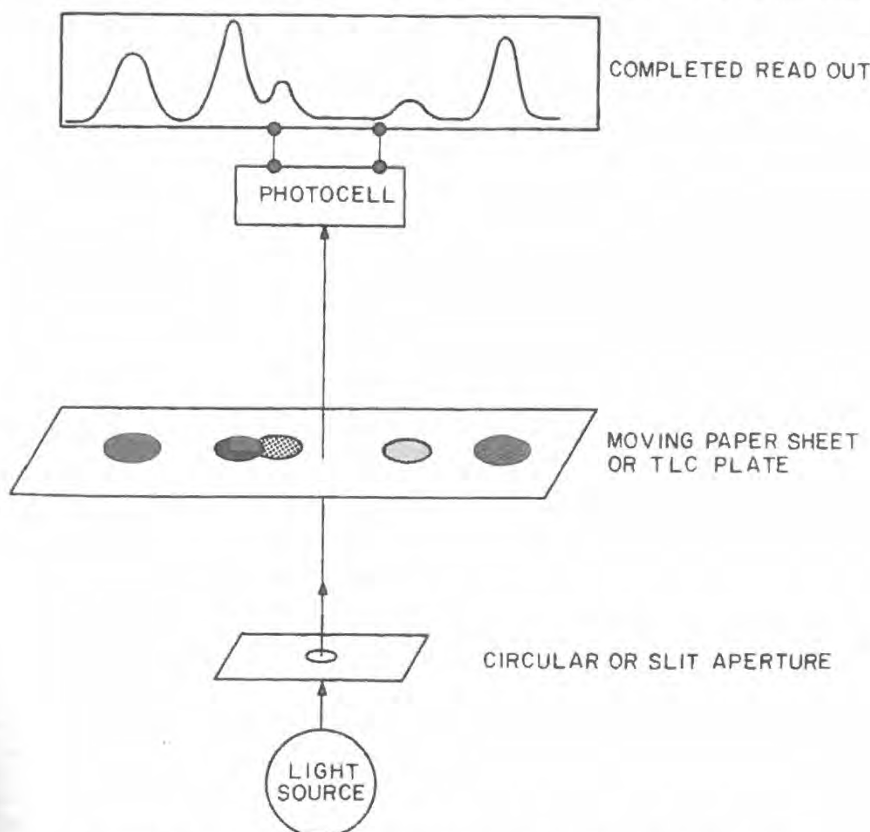

Figure 48. Direct photodensitometry of chromatograms (parts not to scale).

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Commercial densitometry systems, available from several manufacturers, incorporate automatic and continuous integration of the areas of the peaks as well as ways of compensating for deviations from the Lambert–Beer Law in the spectroscopic process.

The colored spots are generally scanned through the spot centers along the line of development. Scanning perpendicular to the line of development may be desirable if the spots are poorly separated or if impurities are located between the spots so that there are no blank areas between the spots. When scanning a number of spots in a row, care must be taken to ensure that the center of each spot is being scanned. Instead of the chromatogram itself, a photographic negative can be evaluated by densitometry.

Densitometry is more convenient than methods involving elution and requires less sample for analysis. Most workers report errors of about 5–7% for photometric *in situ* procedures, indicating that elution methods are in general more accurate.

H. RADIOISOTOPE TECHNIQUES

Radioactivity can be exploited for the detection, identification and quantitation of compounds separated by paper chromatography. Various aspects of some of these procedures will be briefly described.

1. *Autoradiography*

The beta-emitting isotopes ^{14}C, ^{35}S and ^{32}P are detected by placing the chromatogram in direct contact with x-ray film. The exposed film, developed by usual darkroom methods, shows the exact self image of the radioactive spots. Radioactive spots may be identified by co-chromatography in which the radioactive regions are eluted from the chromatogram and rechromatographed together with non-radioactive carrier of the suspected compound. Coincidence for a given spot of radioactivity and color formed by a specific reagent is sufficient for tentative identification. Densitometric measurement of the degree of blackening of the film allows semiquantitative evaluation of the chromatogram.

For tritium, x-ray film without the usual protective gelatin layer (which will absorb the low energy beta rays) is used. Alternatively, the paper itself is impregnated with photographic emulsion and developed photographically, or with a liquid scintillator to convert the beta energy into light energy which causes exposure of the film.

A much faster, but lower resolution, method than conventional autoradiography for the evaluation of ^{14}C, ^{35}S, and ^{3}H chromatograms is the spark chamber technique. See Reference (*8*) for a complete discussion.

2. *Radiometric Methods*

Both elution and direct evaluation methods are used for the quantitation of radioactive zones. In the elution method, the spots are located by autoradiography or scanning of the paper with a ratemeter, cut from the paper and eluted into a counting planchet for measurement by conventional counting techniques (e.g., G-M, proportional, gas-flow, or liquid-scintillation counters). Direct measurement involves automatically scanning the chromatogram by drawing it in front of a counter. Commercial instruments of various degrees of sophistication are available for scanning one- and two-dimensional chromatograms.

Liquid scintillation spectrometry is used for counting tritium as well as for ^{35}S, ^{32}P and ^{14}C. The paper can be examined by cutting it into small, equal sections and inserting each into a counting vial to which phosphor solution is added. Or, the whole spot can be cut out (sometimes after application of a color-forming locating reagent) and counted. Corrections are made to reflect the counting efficiency for each isotope and for quenching effects due to substances adsorbed on the paper or to the solute-reagent complex and the excess reagent if a detection test is used.

a. *Isotope indicators.* Solutes, after chromatography, may be treated on the paper with a radioactive reagent to form a radioactive derivative which can be located by the methods described above. Examples are the detection of metal ions by $H_2\,^{35}S$ and reducing sugars with $^{110}AgNO_3$.

b. *Neutron activation analysis.* The finished chromatogram is placed in a nuclear reactor, where it undergoes nuclear bombardment. Some of the atoms in the separated compounds become radioactive, and these compounds are determined by comparing the activities to those of similarly treated standards. Time is usually required for the background activity of the paper to drop to a low level. Activation analysis methods are highly sensitive and convenient in that the sample preparation, development, drying, etc., can be performed without the problems associated with handling radioactive compounds.

I. SOURCES OF ERROR

Studies by Fairbairn have indicated that the major sources of error in quantitative paper chromatography are caused by the delivery of the initial spots and differences between the slopes of the regression lines (relating measurement of the final spot with quantities applied to the initial spots) from sheet to sheet. These were overcome by using machine spotting (The Chromaplot Machine by Burkard Mfg. Co., Hertfordshire, England, was specifically recommended by Fairbairn) and the use of standard and test solutions for each sheet. No additional error was caused by the development except where adjacent spots had very dissimilar quantities of solute leading to lateral diffusion and some slight errors.

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For optimum results in densitometric methods, the volumes of the initial spots must be accurately measured, the initial spots should be of equal area, they should be dried in a current of cold air, tank conditions should be as constant as possible, and two quantities of the standard, one a multiple of the other, and two quantities of unknown, differing by the same factor, should be used for each assay. Color development is carried out by spraying on both sides of the paper or by dipping, depending upon which is most convenient for the particular reagent being used. Densitometry is always begun at exactly the same time after color development for each chromatogram. If these procedures are followed, errors of individual determinations are reduced to about ±6% (coefficient of variation about 2–3%; that is, 95% of the results will fall within 4–6% of the mean). If replicates are made, the limits of error are reduced by $1/\sqrt{n}$, where n is the number of replicates (*9*).

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VOLUME TWO
SECTION I

PRINCIPLES AND TECHNIQUES

I.V Thin-Layer Chromatography

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Section I.V

THIN-LAYER CHROMATOGRAPHY (TLC)

Although paper chromatography had made valuable contributions to all fields of biology and chemistry, the sole use of cellulose as the stationary phase imposed a great limitation on the technique, because paper is not universally satisfactory for all separations (e.g., some classes of lipids). Scientists began, therefore, to wonder how they could employ the adsorbents which were so useful in the Tswett column method in a convenient sheet form, like paper. The present-day system of TLC was first introduced in 1951 by Kirchner *et al.* (*1*), and additional work on modifications and applications for the method was published by these authors in a series of papers during the years 1952–57. The method became popular after Ergon Stahl helped develop commercial equipment and adsorbents for TLC.

The adsorbent is mixed with a binder, often plaster of Paris, made into a slurry with water, and coated on a glass plate using a spreading device. After drying, a rugged thin layer of adsorbent, usually 0.25 mm thick, is bound to the glass plate. This layer can be used for chromatography in a manner similar to a paper sheet. However, separations now depend on the properties of the adsorbent surface much as if the adsorbent were packed in a column. For this reason, thin-layer chromatography is sometimes called open-column chromatography.

The technical aspects (sample application, development, detection, quantification, etc.) of paper and thin-layer chromatography are very closely related. With the present commercial availability of flexible cellulose thin layers on plastic film as well as adsorbent-loaded chromatography papers, the borderline between the two methods is indeed indistinct.

In addition to the traditional adsorbents, systems employing layers of ion exchangers, exclusion gels, liquid-coated supports, and combinations of these are now used in conjunction with many different varieties of solvents for the separation of all types of compounds. TLC is a highly sensitive, versatile analytical method, resulting in generally sharp and rapid separations.

Much of what is written in the preceding sections concerning theory, methods and materials applies to TLC. Depending upon the type of layer employed, the mechanism of separation can be adsorption, partition, ion exchange, exclusion or a combination of these. Many of the techniques are the same as those used in paper chromatography and most of the coating materials have already been described.

A. COATING MATERIALS

Table 8 (Section II.III) shows the TLC coating materials available from various manufacturers. These are similar to the sorbents used for column chromatography except that they are generally of a smaller particle size. Some of these contain a binder such as plaster of Paris ($CaSO_4$) or starch to improve the adherence of the film to the plate. An inert fluorescent indicator (e.g., zinc silicate) which fluoresces when illuminated with 2540 or 3660 Å UV light so as to aid in the detection of separated spots is also sometimes included. The presence of a binder may change the properties of the sorptive layer. Some materials (e.g., cellulose) are completely free of additives and still adhere well.

Silica gel is by far the most used adsorbent for TLC. In choosing a sorbent system, one is guided by the characteristics of the compounds to be separated such as the acidity or basicity, ionic character, solubility, and possibility of chemical reaction with the layer or the solvent. In general, lipophilic compounds are separated on aluminum oxide, silica gel, acetylated cellulose and polyamide. Hydrophilic substances are separated on cellulose, ion-exchange cellulose, kieselguhr and polyamide. This generalization is merely a rough guide since, for example, fat-soluble chloroplast pigments are successfully separated on cellulose as well as on alumina, magnesia and silica gel.

Activated silica gel is an acidic adsorptive medium especially suited to the separation of acidic and neutral compounds. Classes of compounds readily separated include aldehydes and ketones, alkaloids, sugars, phenols, steroids, and amino acids. Nonactivated silica gel contains enough water to permit separations based on a partition mechanism to be carried out. Aluminum oxide (alumina) is a basic adsorptive medium (acidic and neutral alumina are also available) useful for the resolution of basic and neutral compounds including polycyclic hydrocarbons, alkaloids, amines, fat-soluble vitamins, and aldehydes and ketones. Alumina may catalyze the decomposition of many organic compounds.

Kieselguhr is a neutral sorbent widely used as a support for partition separations. Cellulose powders for TLC are available in both fibrous and microcrystalline forms and can be used for all separations which can be done on paper. Magnesium oxide, the properties of which are strongly dependent upon the method of preparation, is selective for compounds with aromatic and conjugated double bonds.

Cellulose and resinous ion exchangers are used in thin-layer ion-exchange chromatography. The latter are also useful for thin-layer separations of organic compounds by a partition mechanism (section IIID5b). The resin can be bound to the plate with starch or can be mixed with cellulose powder prior to spreading. Silica gel and cellulose layers are impregnated with liquid ion exchangers (e.g., HDEHP, triisooctylamine) to achieve similar results.

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Dextran gels are used for thin-layer gel-filtration chromatography with descending solvent flow only (no capillarity is operative). Development times are relatively long.

Polyamide powder is used for both column and thin-layer chromatography. Separations are usually based upon hydrogen bonding between the polymer chains and the solutes, although other factors are involved in some cases. Polyamide powders are prepared from Nylon 66 (polyhexamethyldiamine adipate), Nylon 11 (polyaminoundecanoic acid), Perlon (polycaprolactam) and acetylated Perlon. Classes of compounds separated include nitroanilines, benzophenones, phenols, flavonoids, amino acid derivatives, etc.

Alginic acid has been introduced as a support with ion exchange and complexing properties. Separations of various metal ions are possible by elution with aqueous acids (*2*).

Powdered glass (Corning) has been recently used as a successful adsorbent for TLC. The glass is ground to 200–250 mesh and mixed with plaster of Paris before spreading the layer. Polyacrylonitrile, polyacrylamide and poly(*N*-acetyl acrylamide) have been used for the separation of water soluble substances.

Layers can be modified in various ways to suit a particular separation: Two sorbents can be mixed prior to preparing the layer; adjacent layers of different sorbents can be placed on the same plate; buffers, precipitants (e.g., sulfide for the separation of metals), complexing agents (e.g., borate for sugars), chelating agents (for inorganic ions), $AgNO_3$ (for unsaturated compounds), hydrophobic materials, etc., can be incorporated; layers with activity gradients in various directions can be prepared [see References (*3*), (*4*)].

B. PREPARATION OF LAYERS

Various commercial apparatus are available for applying sorbent layers to glass plates (20 × 20 cm or 10 × 20 cm) or polyester film. As an example, Figure 49 shows the Kensco multi-thickness applicator which has interchangeable gates for producing layers of 250, 275, 500, 750, 1000, 1500 or 2000 μ thickness. An adjustable gate for layer thicknesses from 50 to 3000 μ in steps of 50 μ is also available. [It has been generally found that within a reasonably wide range of layer thicknesses (ca. 0.15 to 2.0 mm) R_F values are constant.] A gradient-thickness layering gate for producing a layer that gradually decreases in thickness allows heavy sample loading on the thicker part of the layer while the more rapidly moving trace components in the sample may be more easily visualized and recovered in the thinner portion. Adsorbent reservoir dividers allow the production of two to five parallel sections of different sorbent layers on the same plate; if sample development is carried out with the sections aligned horizontally, the sample will travel through each section in turn and fractionations not successful on one sorbent could occur on another. Or, the sections can be aligned vertically with samples being developed simultaneously on each for comparison under identical conditions.

The Camag plate coater (Figure 50) works in a different manner, such that the applicator is stationary and the plate is moved under the applicator. Layers of 300 and 500 μ thickness are produced, the 300 μ layer shrinking on drying to 250 μ.

Slurries are prepared by mixing the sorbent powder with an amount of water specified by the manufacturer (e.g., 30 g Mallinckrodt SilicAR TLC-7GF in 55 ml of water) in a mortar until the slurry is homogeneous and free of lumps or air bubbles. For the preparation of uniform layers of cellulose, homogenization of an aqueous suspension with an electric blender for 30–60 sec is often recommended. If a binder is present in the sorbent, coating must be carried out before the slurry hardens. Some workers prefer to use organic solvents in place of some or all of the water required to prepare the slurry, and basic silica gel layers for the separation of alkaloids can be produced by using dilute NaOH in place of water.

Layers of silica gel and alumina are first air-dried at room temperature for about 15 min without disturbing the support. They then may be dried vertically in a chromatography oven for 1–2 hr at 100–120 °C. Stahl recommends preliminary air-drying followed by 10 min drying by hot air in a vertical position and then 30 min at 110 °C. Truter states that air-drying overnight gives a layer activity about the same as drying at 100 °C. The temperature and time of drying are varied depending upon the activity required. Activity is determined by chromatographing a commercially available standard dye mixture. The plates are stored before use, if necessary, in a dessicator over silica gel. Convenient storage racks are commercially available.

Layers of cellulose, polyamide, and ion-exchange materials are thoroughly air-dried but are not usually oven heated. Sephadex gels are used at once in the swollen state.

Procedures for impregnating thin-layer plates with formamide, silicone, paraffin, etc., for partition separations are given as part of Table 8 (Section II.III).

If a commercial spreading device is not available, satisfactory layers can be produced in various other ways. Layers of masking tape may be placed on two opposite edges of a glass plate at a height equivalent to the desired layer thickness. The slurry is poured on the plate, a glass rod is drawn in a parallel direction over the taped edges to smooth out the layer, and the tape is later removed after the layer has set. Alternatively, tape can be put around the ends of the glass rod to raise it the required distance from the glass plate during the spreading operation. A slurry can be sprayed onto a horizontal glass plate with various laboratory spray devices, although the resulting layer will be nonuniform and of unknown thickness. Carefully cleaned microscope slides can be coated by dipping two slides back to back into a well-stirred nonaqueous slurry of sorbent. The slides are lifted out slowly, and excess adsorbent is drained. The slides dry

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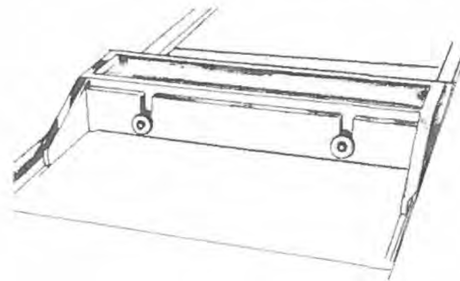
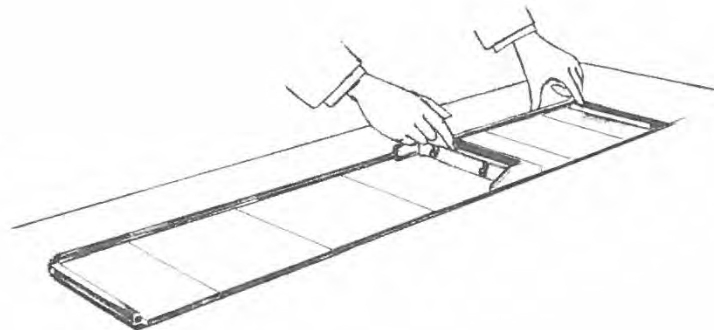


(a)



(b)

Figure 49. (a) Multi-thickness applicator with interchangeable gates. A choice of easily interchangeable gates is used with the multi-thickness applicator. Each gate has a different aperture which determines the layer thickness to be applied. When slipped into position on the applicator the gate moves freely in a vertical direction and produces a uniform layer across the plate by measuring from the surface of each successive plate. (b) Applying adsorbent layers with the multi-thickness applicator. Slurry is spread with a single smooth movement of the applicator along the guide bar. Matched glass plates are not required. Each plate receives the same adsorbent layer regardless of nominal variations in glass thickness or dimensional irregularities. The procedure for applying adsorbents to film is the same as coating glass. A single piece of film 48 inches long by 8 inches wide is coated with one continuous layer. After drying, sections of any dimension can be cut off with scissors, and the remaining film can be rolled without damage to the layer and stored. [Permission to reproduce from p. 4, Catalog K-400, Kensington Scientific Co., Oakland, Calif.]

rapidly. They are separated, the edges wiped clean with tissue, and, if necessary, the plates are steamed briefly to obtain a more strongly bound layer.

Precoated layers of many sorbents are available from various manufacturers (Section II.III, Table 8) on glass plates, plastic film and aluminum foils. The plastic films cannot be used with all organic solvents and not all brands will withstand corrosive, charring detection sprays. Coated glass tubes are also used.

Ultrathin liquid films and vacuum evaporated thin films (1 μ) of metal oxides provide some very rapid and sensitive separations [see References *(5)*–*(7)*].

C. CHROMATOGRAPHIC TECHNIQUES

1. *Sample Preparation and Application*

Samples are prepared for chromatography by procedures similar to those used for paper and liquid-column chromatography (Section II.II). Plates coated with a small strip of strong-acid ion exchanger in cellulose at the origin while the rest of the plate is covered with pure cellulose can be used to analyze the amino acids in urine without desalting the sample: The sample is spotted in the resin area and two develop-

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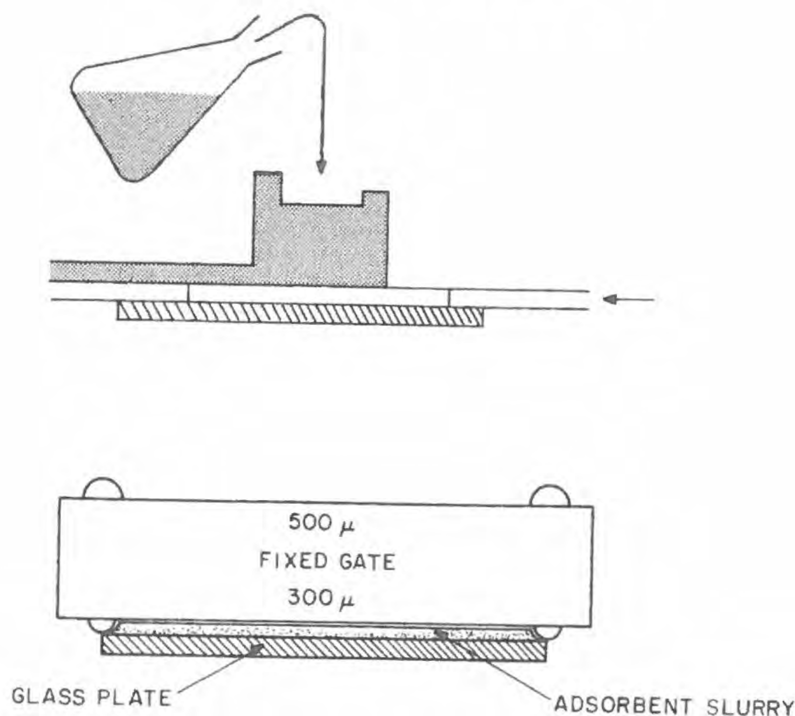

Figure 50. The Camag finger-tip plate coater. Reproduced with permission from Camag, Inc., New Berlin, Wisconsin.

ments in one dimension with water and a basic solvent, followed by an acid solvent in the second dimension, isolates the unwanted salts in the resin and separates the amino acids from each other on the cellulose.

Another method involving cleanup on the layer for drugs in blood is as follows:

First spot serum directly on a flexible, precoated, silica-gel thin-layer sheet. Then develop to a distance of 3 cm above the sample origin with a one-to-one mixture of ethyl alcohol-chloroform to denature and precipitate the protein and to extract the drug with the solvent front. Finally, remove the sheet. Dry it, cut off the first centimeter containing the protein residue, and remigrate the remainder of the sheet in a solvent system suitable for resolution of the drug and standard reference compounds. Application of reference materials in normal serum is particularly important in this case since matrix effects can be quite pronounced (*8*).

Substances can be changed to derivatives (e.g., trimethylsilylated carbohydrates) prior to chromatography to facilitate detection, identification or analysis.

Samples are usually applied as 0.1–1% solutions with a 1–25 μl micropipette or capillary tube. The spot size should be 1–3 mm d., and spots are kept 1–2 cm apart. Spotting templates are available as an aid in placing samples. For analytical separations, 1 μg to 0.5 mg of sample is generally applied, depending upon the layer material and the sensitivity of the detection method. The initial spots should be 15 mm from the lower end of the plate. Commercial sample applicators are available for the semi-automatic deposition of up to 12 similar or different samples onto a standard TLC plate [e.g., from Kensington Scientific Corp.; see also References (*9*), (*10*)].

Sample application can be directly made after the volatilization separation of substances from solid materials: The organic or inorganic mixture is put in a glass cartridge with a conical tip and heated in a furnace at a pre-set temperature. The emerging vapors, which can be pure substances or decomposition products (pyrolysis TLC), are deposited directly on the plate which is held one mm from the tip. GC fractions may be spotted directly on TLC plates in a similar manner.

2. *Choice of Solvent Systems*

Depending upon the sorbent, its activity, and the class of the solute compounds, a wide range of solvents can be used. Single solvents of the elutropic series, mixtures of solvents, totally aqueous, totally organic, aqueous-organic, and ionic solvents have all been used. The use of azeotropic mixtures reportedly (*11*) aids in the attainment of constant R_F values, for example methanol-chloroform-methyl acetate

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(180 : 257 : 135 v/v) for the separation of sulfonamides or alkaloids on silica-gel layers. Molten salts have been used as developers for the separation of inorganic ions on silica gel. Table 1 gives a limited, selective listing of solvent systems successfully used for the TLC separation of various compounds.

TABLE 1

SELECTED SOLVENT-SORBENT SYSTEMS FOR THE TLC SEPARATION OF VARIOUS COMPOUNDS

1. *Chloroplast Pigments*
   a. Isooctane-acetone-ether (3 : 1 : 1)—silica gel and polyamide
   b. Petroleum ether-benzene-$CHCl_3$-acetone-isopropanol (50 : 35 : 10 : 5 : 0.17)—cellulose
   c. Petroleum ether-*n*-propanol (99.2 : 0.8) followed by 20% $CHCl_3$ in petroleum ether (2 dimensional)—sucrose
   d. Petroleum ether-acetone (4 : 6)—alumina
   e. Petroleum ether-*n*-propanol (199 : 1)—starch
   f. Petroleum ether-acetone (7 : 3)—magnesia-celite (1 : 1 w/w) and hydroxyapatite
   g. Petroleum ether-acetone (8 : 2)—calcium carbonate
   h. Methanol satd. with paraffin—Silica gel G—$Ca(OH)_2$ (1 : 4) impreg. with paraffin
2. *2,4-Dinitrophenylhydrazones of Aldehydes and Ketones*
   a. Hexane—ethyl acetate (4 : 1 or 3 : 2)—silica gel
   b. Benzene or $CHCl_3$ or ether or benzene-hexane (1 : 1)—alumina
   c. Petroleum ether-benzene mixtures containing small amounts of pyridine—$ZnCO_3$
3. *Alkaloids*
   a. Benzene-ethanol (9 : 1) or $CHCl_3$-acetone-diethylamine (5 : 4 : 1)—silical gel
   b. $CHCl_3$ or ethanol or cyclohexane-$CHCl_3$ (3 : 7) plus 0.05% diethylamine—alumina
   c. Benzene-heptane-$CHCl_3$-diethylamine (6 : 5 : 1 : 0.02)—cellulose impreg. with formamide
4. *Amines*
   a. Ethanol (95%)-NH_3 (25%) (4 : 1)—silical gel
   b. Acetone-heptane (1 : 1)—alumina
   c. Acetone-H_2O (99 : 1)—kieselguhr G
5. *Sugars*
   a. Benzene-acetic acid-methanol (1 : 1 : 3)—silica gel buffered with boric acid
   b. *n*-propanol-conc. NH_3-H_2O (6 : 2 : 1)—silica gel G
   c. Butanol-pyridine-H_2O (6 : 4 : 3) or ethyl acetate-pyridine-H_2O (2 : 1 : 2)—cellulose
   d. Ethyl acetate-isopropanol-H_2O (65 : 24 : 12 or 5 : 2 : 0.5)—kieselguhr G buffered with 0.02 *N* sodium acetate
   e. Ethyl acetate-benzene (3 : 7) (for sugar acetates)—starch-bound silicic acid
6. *Carboxylic Acids*
   a. Benzene-methanol-acetic acid (45 : 8 : 8)—silica gel
   b. Methanol or ethanol or ether—polyamide
   c. Isopropyl ether-formic acid-H_2O (90 : 7 : 3)—kieselguhr G-polyethylene glycol (M-1000) (2 : 1)
7. *Sulfonamides*
   a. $CHCl_3$-ethanol-heptane (1 : 1 : 1)—silica gel G
8. *Food Dyes*
   a. Methyl ethyl ketone-acetic acid-methanol (40 : 5 : 5)—silica gel G
   b. Butanol-ethanol-H_2O (9 : 1 : 1, 8 : 2 : 1, 7 : 3 : 3, 6 : 4 : 4 or 5 : 5 : 5)—alumina
   c. Aq. sodium citrate (2.5%)-NH_3 (25%) (4 : 1)—cellulose
9. *Essential Oils*
   a. Hexane—starch-bound silicic acid
   b. Benzene-$CHCl_3$ (1 : 1)—silical gel G
10. *Flavonoids and Coumarins*
    a. Ethyl acetate-Skellysolve B—starch-bound silicic acid
    b. Methanol-H_2O (8 : 2 or 6 : 4)—polyamide
    c. Toluene-ethyl formate-formic acid (5 : 4 : 1)—silica gel G + sodium acetate
    d. Petroleum ether-ethyl acetate (2 : 1)—silica gel G
11. *Metal Ions*
    a. Dilute HCl—starch-bound alumina—Celite
    b. Acetone-conc. HCl-2,5-hexanedione (100 : 1 : 0.5)—silical gel G
    c. 1 *M* aq. $NaNO_3$—Dowex 1 + cellulose
    d. Methanol—alumina
12. *Insecticides*
    a. Cyclohexane-hexane (1 : 1) or CCl_4-ethyl acetate (8 : 2)—silica gel G
    b. Hexane—alumina
    c. Heptane saturated with acetic acid—starch-bound silicic acid
    d. Chloroform—silica gel G + oxalic acid

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TABLE 1—(Continued)

13. *Lipids*
   a. Petroleum ether-diethyl ether-acetic acid (90 : 10 : 1 or 70 : 20 : 4)—silica gel G
   b. Petroleum ether-diethyl ether (95 : 5)—alumina
   c. $CHCl_3$-methanol-H_2O (80 : 25 : 3)—silicic acid
14. *Fatty Acids*
   a. Petroleum ether-diethyl ether-acetic acid (70 : 30 : 1 or 2)—silica gel G
   b. Acetic acid-CH_3CN (1 : 1)—kieselguhr impreg. with undecane
   c. Benzene-diethyl ether (75 : 25 or 1 : 1)—starch-bound silicic acid
   d. CH_3CN-acetic acid-H_2O (70 : 10 : 25)—silica gel G impreg. with silicone oil
15. *Glycerides*
   a. $CHCl_3$-acetic acid (99.5 : 0.5)—silica gel G impreg. with $AgNO_3$
   b. $CHCl_3$-benzene (7 : 3)—silica gel G
   c. $CHCl_3$-methanol-H_2O (5 : 15 : 1)—silica gel G impreg. with undecane
   d. Petroleum ether-diethyl ether (9 : 1 to 4 : 6)—plaster-bound silicic acid
   e. Methyl isobutyl ketone—hydroxyapatite
   f. Acetone-CH_3CN (8 : 2 or 7 : 4)—kieselguhr G impreg. with petroleum
16. *Glycolipids*
   a. Propanol-12% NH_3 (4 : 1)—silica gel G
17. *Phospholipids*
   a. $CHCl_3$-methanol-H_2O (60 : 35 : 8 or 65 : 25 : 4)—silica gel G
18. *Nucleotides*
   a. 0.15 *M* NaCl or 0.01–0.06 *N* HCl—Ecteola cellulose
   b. Sat. aq. $(NH_4)_2SO_4$-1 *M* sodium acetate-2-propanol (80 : 18 : 2)—cellulose
   c. 0.02–0.04 *N* aq. HCl—DEAE cellulose
   d. Gradient elution: start with 1 *N* formic acid and add 10 *N* formic acid which is 2 *M* in ammonium formate—DEAE Sephadex A-25
   e. 1.0–1.6 *M* LiCl—cellulose PEI
19. *Phenols*
   a. Xylene, $CHCl_3$ or xylene-$CHCl_3$ (1 : 1, 3 : 1, 1 : 3)—starch-bound silicic acid or silicic acid-kieselguhr (1 : 1)
   b. Benzene—alumina plus acetic acid
   c. Benzene-1,4-dioxane-acetic acid (90 : 25 : 4)—silica gel G
   d. Diethyl ether—alumina
   e. Hexane-ethyl acetate (4 : 1 or 3 : 2)—silica gel plus oxalic acid
   f. Hexane or cyclohexane or benzene—δ-polycaprolactam
   g. Ethanol-H_2O (8 : 3) containing 4% boric acid and 2% sodium acetate—silica gel G plus boric acid
   h. CCl_4-acetic acid (9 : 1) or cyclohexane-acetic acid (93 : 7)—polyamide 6
20. *Amino Acids*
   a. Butanol-acetic acid-H_2O (3 or 4 : 1 : 1) or phenol-H_2O (75 : 25) or propanol-34% NH_3 (67 : 33)—silica gel G
   b. Butanol-acetic acid-H_2O (4 : 1 : 1)—cellulose
   c. Butanol-acetic acid-H_2O (3 : 1 : 1) or pyridine-H_2O (1 : 1 or 80 : 54)—alumina
   d. Ethanol-NH_3 (conc.)-H_2O (7 : 1 : 2)—silica gel G buffered with equal portions of 0.2 *M* KH_2PO_4 and 0.2 *M* Na_2HPO_4
   e. *n*-Butanol-acetone-NH_3-H_2O (10 : 10 : 5 : 2) followed by isopropanol-formic acid-H_2O (20 : 1 : 5) (2 dimensional)—cellulose
21. *Polypeptides and Proteins*
   a. $CHCl_3$-methanol or acetone (9 : 1)—silica gel G
   b. Potassium phosphate buffers, pH 6.5—polyamide-bound hydroxyapatite
   c. H_2O or 0.05 *M* NH_3—Sephadex G-25
   d. Phosphate buffers—DEAE Sephadex A-25
22. *Steroids and Sterols*
   a. Benzene or benzene-ethyl acetate (9 : 1 or 2 : 1)—silica gel G
   b. $CHCl_3$-ethanol (96 : 4)—alumina
   c. Ethyl acetate-cyclohexane mixtures—starch-bound silicic acid
   d. Benzene-isopropanol—silica gel plus NaOH
   e. Methanol-H_2O (95 : 5)—Celite impreg. with paraffin oil
   f. Cyclohexane-heptane (1 : 1)—silica gel G-kieselguhr G (1 : 1)
   g. Cyclohexane-ethyl acetate (99.5 : 0.5)—kieselguhr G
   h. Acetic acid-H_2O (92 : 8 or 90 : 10)—kieselguhr G impreg. with undecane
23. *Terpenoids*
   a. Hexane or hexane-ethyl acetate (85 : 15)—starch-bound silicic acid
   b. Benzene or benzene-petroleum ether or -ethanol mixtures—alumina
   c. Isopropyl ether or isopropyl ether-acetone (5 : 2 or 19 : 1)—silica gel G

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TABLE 1—(Continued)

24. *Vitamins*
    a. Methanol, CCl_4, xylene, $CHCl_3$ or petroleum ether—alumina
    b. Methanol, propanol, or $CHCl_3$—silica gel G
    c. Acetone-paraffin (H_2O sat.) (9 : 1)—silica gel G impreg. with paraffin
25. *Barbiturates*
    a. $CHCl_3$-*n*-butanol-25% NH_3 (70 : 40 : 5)—silica gel
26. *Digitalis Compounds*
    a. $CHCl_3$-pyridine (6 : 1)—silica gel
27. *Polycyclic Hydrocarbons*
    a. CCl_4—alumina
28. *Purines*
    a. Acetone-$CHCl_3$-*n*-butanol-25% NH_3 (3 : 3 : 4 : 1)—silica gel

[This table was prepared with the help of information supplied by Dr. Fred Rabel, H. Reeve Angel & Co.]

3. *Development Methods*

Chromatograms are normally developed by the ascending method over a distance of 10-18 cm in glass tanks lined with filter paper to assure good vapor saturation (N or NS chamber) or in a sandwich chamber (S chamber) (Figure 51). The latter requires very little solvent and permits development under vapor-saturated conditions by using a pre-soaked cellulose-coated saturation plate (b) to achieve results which closely approximate those in the N chamber with most solvents. If the coated saturation plate is not used (c), saturation is not complete, and modestly improved separations may result due to the effects of solvent evaporation as the system approaches a steady state situation (*12*). The thin layer is inserted into the solvent to a depth of approximately 5 mm in all cases. It is difficult to predict in advance which chamber (N or S) will yield the best separation in any given case, and trial and error is usually necessary to find the best chamber environment. The mechanism of separations obtained with single and multicomponent solvents in various kinds of chambers with different environments is not clearly understood. See, for example, Reference (*13*).

Descending development is more difficult to achieve because the solvent must be fed to the top of an inclined layer through a wick arrangement. This method is seldom used except with Dextran gel layers or for eluting zones off the layer prior to analysis.

Horizontal development can be used with regular layers or with nonadherent (loose) layers. If spots of sample are applied at the end and solvent is fed to the edge of the horizontal plate through a wick, resolved spots will result. If the mixture is applied as a spot near a hole in the center of the plate and solvent is fed to the layer from below through the hole, ring-shaped zones result (circular method).

Continuous development is achieved by allowing the upper end of the plate to remain uncovered in the horizontal method (BN-chamber) or to protrude out the end of a slot in the cover of the chamber in the ascending method. In either case the solvent flows continually and evaporates from the uncovered area (which can be warmed).

It has been found that some separations can be improved by vapor not originating from the developing solvent. Two new chambers have been described for providing vapor control over the entire plate ("vapor-programmed TLC"). These are the Vario-KS-Chamber (Camag, Inc.) and the vapor programming chamber designed by de Zeeuw (*14*). Procedures have also been reported for producing gradient layers (with a systematic change in the composition of the adsorbent along the plate, either parallel or at right angles to the solvent flow) or gradient elution (a continuous change in the composition of the solvent). Although improved resolutions have been reported, these procedures detract from the inherent simplicity of normal TLC.

Sealed disposable plastic bags have been used in place of conventional chambers for the TLC of radioactive substances.

Development at temperatures higher or lower than ambient will change R_F values and may lead to improved resolution. Multiple development with one or more solvents and two-dimensional development with different solvents are also used. If mixtures of solvents are employed and the solvents are properly chosen, two dimensional separations governed by different mechanisms in each direction may be achieved.

Humidity variations can have a marked influence on R_F values obtained in TLC, and constant humidity is essential for reproducible results. Ideally, spots should be applied in a room with constant humidity. For alumina, silica gel and magnesium silicate, increasing humidity results in higher R_F values with nonpolar solvents; for cellulose, increasing humidity results in lower R_F values.

A new development technique is termed drum TLC by Saunders and Snyder (*15*). This method allows unlimited migration of a pair of sample bands along a TLC bed at constant, relatively high solvent flow rates, so that total resolution is increased relative to other TLC techniques by an order of magnitude, or equivalent separations can be carried out in a much shorter time. In its basic form, only a few bands of very limited

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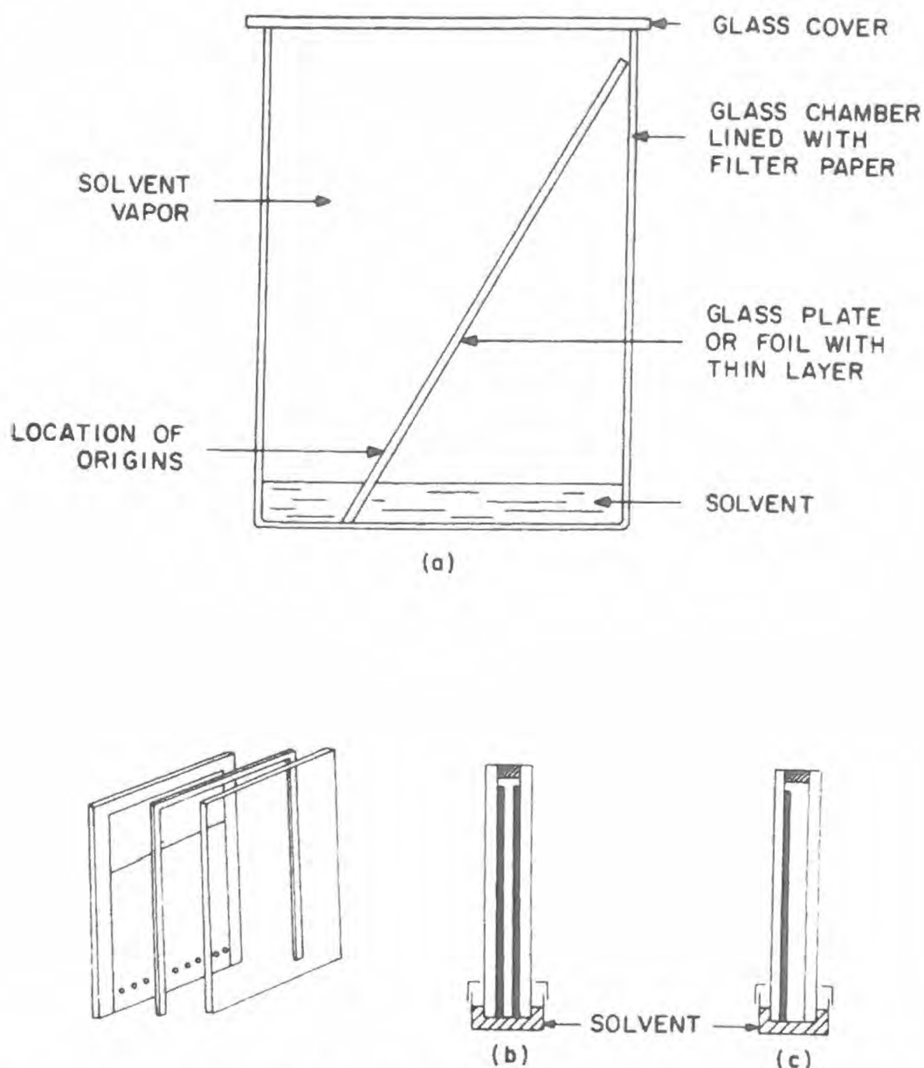

Figure 51. Developing chambers for ascending TLC: (a) rectangular developing tank; (b) sandwich chamber, saturated; (c) sandwich chamber, unsaturated. To use the sandwich plate, a TLC plate is prepared in the usual way. A strip of the adsorbent layer 10 mm wide is scraped off along the sides and upper edge of the plate. The ⊓-shaped cardboard frame is placed on the cleared strip and the cover plate (coated or uncoated) is placed on top of the frame. The sandwich thus formed is held together with clamps, two of which are firmly connected to the stand. Then the sandwich assembly, open end down, is placed through the slot in the cover into the solvent trough. Only 15 ml of solvent are required for development [available from Camag, Inc.]. Reproduced with permission from Camag, Inc., New Berlin, Wisc.

R_F range can be simultaneously resolved by this technique, but it is well suited for the analysis of two difficultly separable bands. Combined with inverse solvent programming, the separation of complex, multicomponent samples at high separation efficiency may be possible.

D. ZONE DETECTION, IDENTIFICATION AND DOCUMENTATION

1. *Detection*

After development, which may take from 20 minutes to over one hour depending upon the system, the plates are removed from the tank and dried after marking the front. Colorless substances are detected using the same types of color-forming reagents as for paper chromatography (Section II.I). In addition,

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corrosive and charring reagents detect nonvolatile, organic compounds. These reagents are, of course, destructive, and are used after nondestructive tests. Detection methods are generally more sensitive in TLC compared to PC.

Another charring detection procedure is the hot-line technique: An electrically heated glowing nichrome wire brought to within 1 mm of the plate chars organic material in a 1–2 mm wide strip.

Compounds which fluoresce are located by inspection of the layer under UV light. Nonfluorescent spots appear as dark areas against a fluorescent background when layers incorporating a fluorescing phosphor are employed.

Radioactive spots are located by scanning with a radiation detector or by autoradiography. Layers heavily loaded with a powdered scintillator (e.g., anthracene or zinc silicate) have been used for the detection of weak-energy radionuclides (e.g., tritium) by β-radioluminescence (scintillation fluorography on photographic film or direct photoelectric detection); interference with the separation is not necessarily caused by the presence of the scintillator (*16*, *17*). Bioautography is applicable to antibiotics.

2. *Identification*

Spots are identified in the usual ways as described in sections above, for example by comparison of migration with authentic reference compounds or by spectroscopy. A unique inlet system has been designed so that compounds separated by TLC can be directly exposed to a mass spectrometer source for rapid identification. Separated spots may be eluted from the support into KBr powder on the TLC plate with 100–150 μl of a dry, nonpolar solvent; the KBr powder is added to a KBr microdisk and pelletized for IR spectroscopy (*18*).

3. *Documentation*

TLC separations are recorded in various ways: The separated spots can be sketched onto tracing paper placed over the plate, or a photograph in black and white or color can be made upon illumination with visible light (transmitted or reflected) or long or shortwave UV radiation (a commercial TLC camera stand is supplied by Camag, Inc.). The thin-layer chromatogram can be copied on an office copying machine after treatment of the layer with paraffin to make it translucent. The layer can sometimes be pulled from glass intact with transparent adhesive tape; if the plate is sprayed with a plastic dispersion [e.g., Neatan (Merck)], the layer forms a flexible film which can be pulled from the glass and retained. Photodensitometer curves, which are used for quantitative analysis (below), also serve as a record of peak locations. Flexible layers on plastic or aluminum backing can be covered with Saran wrap and taped into a notebook for a permanent record.

E. QUANTITATIVE ANALYSIS

Methods of quantitative analysis are similar to those in paper chromatography, and readers interested in details are referred to the book edited by Shellard on this topic.

Photodensitometry of the layers (or alternatively of a photographic print, negative or autoradiograph) in transmitted or reflected light is often used. The methods of thin-layer densitometry have been reviewed (*19*). The flying spot densitometer for TLC has been described by Koopmans and Bouwmeester (*20*). This instrument attempts to eliminate the problem of uneven distribution of material in the spot by oscillating the TLC plate while it is being scanned.

Scanning of fluorescence is used where applicable and radiochromatograms are evaluated with radioscanners. In all of these *in situ* methods, quantitation is accomplished by measuring the areas under appropriate peaks, and comparisons are made between standards and unknowns treated in an identical manner.

Zones can be removed from glass plates by scraping and from flexible sheets by cutting prior to elution from the sorbent for quantitative analysis by weight determination, instrumental methods, chemical methods liquid scintillation counting, etc. Figure 52 shows a simple technique for zone extraction in TLC employing elution columns prepared from commercially available disposable capillary pipettes. A plug of glass wool is inserted into the top of a Pasteur disposable pipette, 5¾ in. long, until it completely covers the tip. Acetone is used to rinse the pipette and plug which dry before the tip is inserted inside vacuum tubing leading to a water aspirator. A plain dissecting probe allows finer outlining of the zone to be eluted and more exact scraping of the adsorbent powder from the supporting glass plate. Particles are sucked into the pipette and caught on the glass wool. The pipette is removed from the vacuum tubing; the tip is rinsed with acetone on the outside and dried; and then it is placed through a hole in a cork. The cork has been inserted into the top of a 15-ml conical centrifuge tube, as shown in Figure 52b. Solvent is slowly dropped into the pipette over the powdered adsorbent in the glass wool, and the compound is eluted through the capillary end into the centrifuge tube.

Elution is with a solvent polar enough to remove the compound of interest completely. To remove a very polar compound from a polar adsorbent, pyridine or another strongly adsorbed compound may be added to facilitate elution. Washing the layer with the elution solvent prior to activation and sample application will remove impurities which would also be eluted along with the separated zones (*8*).

The accuracy of the spot elution-spectrophotometry method will be affected by light scattering if silica gel particles which are too small to be removed by normal filtration are suspended in the eluate. The

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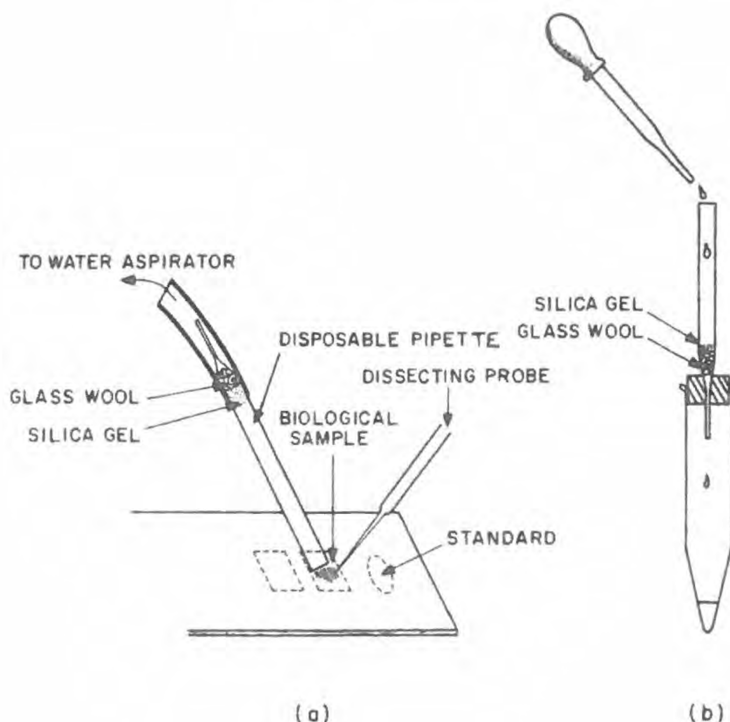

Figure 52. (a) Extraction of a zone from a thin-layer plate; (b) elution of the sample from the sorbent [after (*24*)]. Figure on elution and extraction from TLC plates, page 110, *J. Chem. Educ.*, **44** (1967). Permission to reproduce from Division of Chemical Education, American Chemical Society.

problem is particularly prevalent when polar solvents such as ketones, alcohols or water are used for elution. The contaminating particles can be removed by membrane filtration or by evaporating the polar solvents and redissolving the residue in a less-polar solvent, so that the silica gel particles remain precipitated and can be filtered off (see "The Brinkman Notebook", Fall issue, 1970, p. 5).

Novel methods recently applied to quantitative TLC include ultramicro carbon analysis, *in situ* X-ray emission spectrometry, the flame-ionization detector and neutral activation analysis.

The application of the sample is especially critical in both quantitative PC and TLC, and studies have been made of the variation between different techniques of application (*21*), and the variation between different workers using the same technique (*22*). Use of internal standards can remove the problem of quantitative sample application, but raises other problems, such as contamination of the sample.

F. PREPARATIVE TLC

Preparative separations are carried out on either regular 8 × 8 in. chromatoplates or on larger plates (e.g., 8 × 16 in.) coated with thick layers (0.5–2 mm or thicker) of an adsorbent. Many workers use normal slurries for preparing preparative layers while others recommend the use of slurries with less water (especially if layers >2 mm are used) and a longer period of air-drying before activation in an oven.

Samples up to 10 ml are manually applied with a streaking pipette (Shandon Scientific Co.). Automatic sample streakers are available for depositing long, narrow bands of solution quickly and reproducibly on preparative plates (e.g., from Applied Science Laboratories, or the Chromatocharger from Camag, Inc.).

Single plates are developed in large developing tanks, or up to five plates can be developed simultaneously in special chromatotanks (Shandon, Inc.) or in a regular tank by use of a multi-sandwich assembly (Camag, Inc.). Resolution on thick layers is sometimes not as good as on the thinner analytical layers so that multiple or stepwise development may be advantageous. Also, spots may be more diffuse on thicker layers. In many cases, however, solvent systems determined by TLC are directly applicable to preparative TLC with similar results. Preparative TLC is often faster and requires less solvent than would comparable separations by column chromatography. Development times are usually less than 3 hr.

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At the end of the development, the locations of the bands of interest are determined by spraying guide strips (Figure 47) with an appropriate reagent. The adsorbent containing each zone is then scraped off, collected and eluted with an appropriate solvent. The solvent is evaporated for recovery of the solute.

Analytical TLC separations can be directly scaled-up for the separation of multigram amounts by use of dry-column chromatography in pre-packed columns (Quantum Industries, Inc.).

G. APPLICATIONS

TLC has been applied to the separation and analysis of many classes of compounds as indicated by the earlier table in this section and the data presented later in this Handbook. It is especially useful for substances which are either nonvolatile or too labile for GC analysis. As typical examples, Figures 53 and 54 show separations of vitamins and chloroplast pigments by TLC.

H. TLC *vs* PC

Many of the basic techniques in PC and TLC are essentially the same, for example sample spotting, ascending development, detection of the spots and quantification, especially directly on the chromatogram. Some techniques are simpler in PC than in TLC, namely circular, horizontal and descending solvent development methods (due to the ability to fold the paper) and methods for removing the spots from the

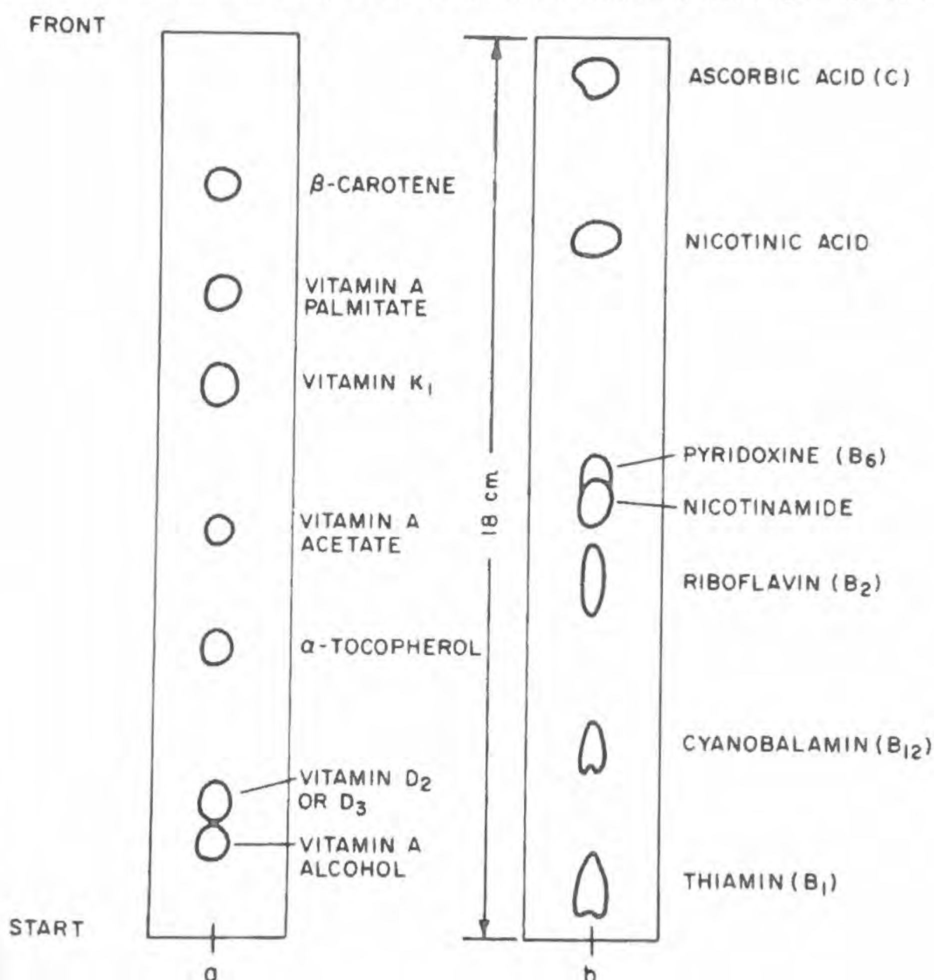

Figure 53. (a) Separation of 20–30 μg each of fat-soluble vitamins on a silica gel G layer containing a fluorescent indicator with cyclohexane-diethyl ether, 8 : 20. Time of run 60 min. (b) Separation of 3–30 μg each of water-soluble vitamins on silica gel G layer containing a fluorescent indicator with water as wash liquid. Time of run 40 min. [After (*25*)]. Part of Figure 115 and part of Figure 102 from Stahl, Thin Layer Chromatography, 1965. Permission received to reproduce from Springer-Verlag, Berlin, Germany.

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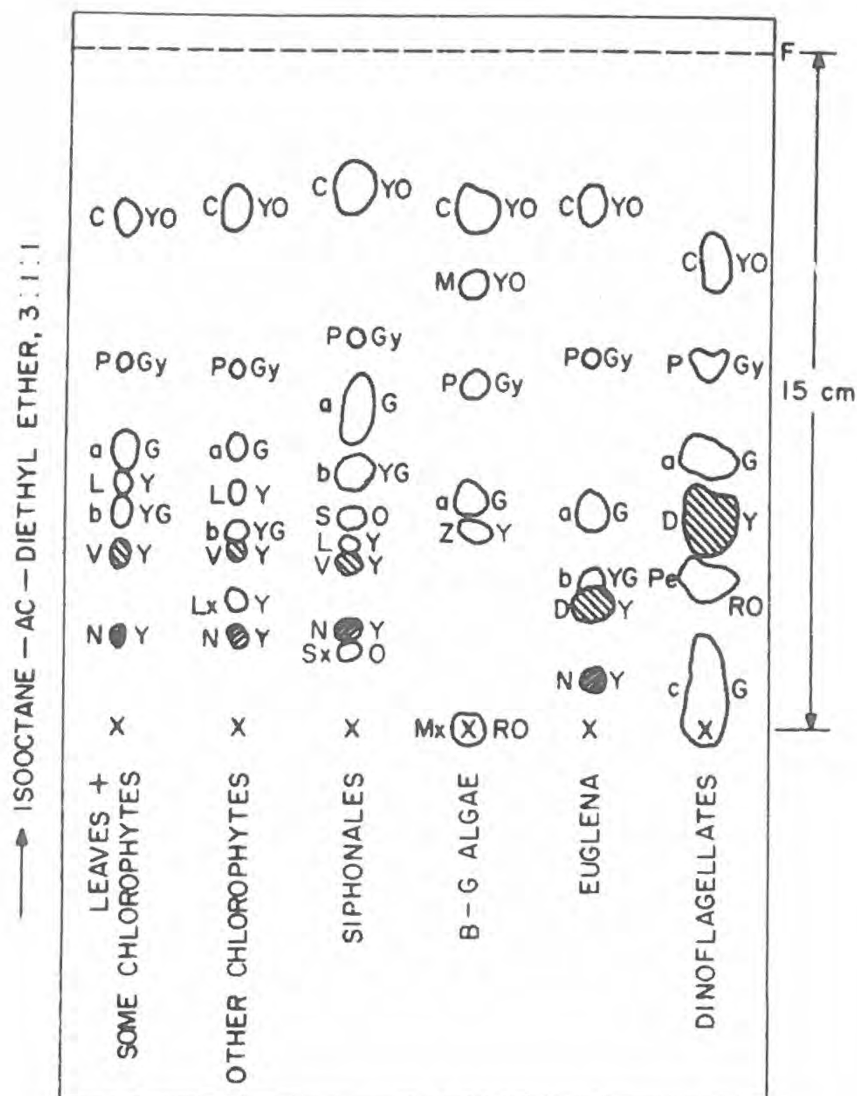

Figure 54. Pigments separated from 2–5 μl of extract of various plants by thin-layer chromatography in Eastman silica-gel Chromagram sheets. Abbreviations and symbols: a = chlorophyll *a*, AC = acetone, B = blue, b = chlorophyll *b*, C = carotene, c = chlorophyll *c*, D = diadinoxanthin, F = front, G = green, Gy = grey, L = lutein, Lx = loroxanthin, M = myxoxanthin, Mx = myxoxanthophyll, N = neoxanthin, O = orange, P = pheophytin, Pe = peridinin, R = red, S = siphonein, Sx = siphonaxanthin, V = violaxanthin, X = starting point, Y = yellow, Z = zeaxanthin, ▧ = blue by HCl vapors, ▨ = blue-green by HCl vapors [after (*26*)]. Figure 2, page 479, *J. Chem. Educ.*, **46** (1969). Permission to reproduce received from Division of Chemical Education, American Chemical Society.

chromatogram (the paper, as well as flexible thin layers, can be cut with scissors, while layers must be scraped from glass plates).

Chromatography paper is uniform and quite inexpensive; the latter is important if many experiments are to be performed. The casting of thin layers on glass plates is considered by some to be a chore, and it is not always possible to prepare uniform layers from day to day. Precoated glass plates and aluminum and plastic sheets are commercially available and are reasonably uniform, but they are expensive relative to paper. Not all adsorbents are commercially available as precoated layers (e.g., magnesia). Likewise, it is

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not especially convenient to prepare chromatography papers loaded with various materials when these are not commercially available.

Chromatographic theory predicts that TLC should be superior to PC both with respect to separation efficiency (resolution) and speed of solvent flow because the thin layers are composed of fine particles rather than fibers as in paper. The smaller particles of the layer material should lead to faster equilibria and sharper spots. Many studies have indicated such advantages for TLC but other work indicates that the differences in speed and resolution obtained in practice are sometimes only minor, for example when comparing certain separations on cellulose paper and thin layers. As another example, leaf pigments are separated with comparable speed and resolution on silica and alumina thin layers as compared with silica gel and aluminum hydroxide impregnated papers (*23*).

Another reason for increased speed and resolution in TLC is because TLC plates are thinner. Smaller spots are placed on them with smaller loadings, consequently the spots which are resolved are smaller and resolution is increased. This is related to the increased sensitivity often reported for TLC compared to PC. Turner and Redgwell reported, for example, that in one study the sensitivity of the ninhydrin reaction was 50 times greater on a mixed layer of cellulose and silica gel than on paper, and that the time required for autoradiography was about one-twentieth of that required for the same amount of extract separated by PC.

A large variety of sorbents have been used to prepare thin layers, but paper has been impregnated with an equally wide range of materials to provide selective separations. Many of these loaded papers are commercially available. In addition, glass fiber papers permit the use of corrosive charring solutions so popular for detecting spots on inorganic thin layers.

It is suggested that TLC is not likely to displace PC or column chromatography as suggested by some people even though it is a method of very great value. The various chromatographic methods are all important because each has its own peculiar advantages and limitations. Some separations can be performed with equal success by several separation methods, while in other situations one technique may stand out clearly as the method of choice. Careful comparative studies of different methods for the separation and analysis of all classes of compounds is a definite need. The different techniques of chromatography are in many cases complementary. For example, thin-layer and paper chromatography can be used to quickly scout for solvents which will provide successful separations on columns containing adsorbents or cellulose. Alternatively, one excellent way to monitor fractions collected during a liquid column separation is to develop a portion of each fraction by TLC or PC to check its purity.

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