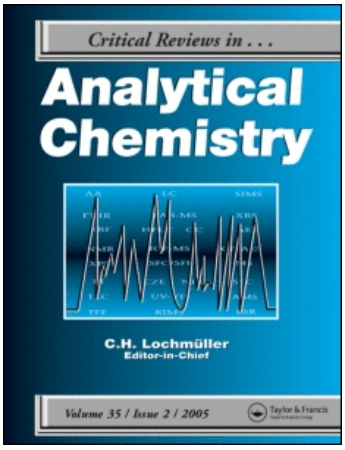

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

High Resolution Liquid Chromatography

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To cite this Article Veening, Hans and Burtis, Carl A.(1975) 'High Resolution Liquid Chromatography', Critical Reviews in Analytical Chemistry, 5: 2, 165 — 200
To link to this Article: DOI: 10.1080/10408347508542683
URL: http://dx.doi.org/10.1080/10408347508542683

HIGH RESOLUTION LIQUID CHROMATOGRAPHY

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I. INTRODUCTION

High resolution liquid chromatography (LC) is a relatively new analytical technique which has experienced an enormous increase in research activity during the last 10 years. The reason that this method, the discovery of which preceded gas chromatography (GC) by many years, was not used extensively until relatively recently was due to the lack of suitable hardware needed to accomplish fast and efficient separations. The development of sophisticated LC instrumentation during recent years, however, has made LC a powerful complement to GC.

High resolution LC is experimentally analogous to GC in that one makes use of small sample sizes (microliter quantities), narrow-bore columns, fast-flowing liquids, and continuous and sensitive detection devices. The term "liquid chromatography" includes several distinct types of interaction, i.e., (1) *liquid-liquid,* in which the components are separated by partitioning between an immiscible mobile and stationary liquid; (2) *liquid-bonded phase,* whereby the components interact with a stationary phase which is chemically bonded to the solid support; (3) *liquid-solid,* in which the components are selectively adsorbed on an active solid surface; (4) *ion exchange,* in which ionic components of the sample are separated by selective exchange with counter-ions of the support; (5) *permeation,* in which separations occur on a permeable gel by sieving action according to molecular size; and (6) *affinity chromatography,* in which biochemically active molecules are separated on the basis of selective biospecific interaction with the stationary phase. Affinity chromatography has been reviewed by Weetall.[1] Liquid-liquid, liquid-bonded phase, liquid-solid, ion exchange, and permeation chromatography have been reviewed by Snyder and Kirkland.[2,3]

The advantage of LC is that thermally unstable, nonvolatile compounds which cannot be eluted by GC can often be separated by LC since columns are operated at or near room temperature. Application are therefore possible for such high molecular weight compounds as proteins and polymers. Also, the interchange of solvents can provide special selectivity effects in LC since the relative retention of two solutes is strongly influenced by the nature of the eluent used. It is this latter feature which makes the technique of gradient elution possible and sometimes necessary.

In high resolution LC, the instrumentation consists of (1) a solvent reservoir, (2) a pump, (3) a damping unit to remove undesirable pump pulsations, (4) a pre-column, (5) a sampling or injection device to introduce the sample into the column, (6) the separation column, (7) a detector, and (8) a recorder. A temperature circulator can be used to thermostat the solvent reservoir, the pre-column, and the separation column. A schematic diagram of the component parts is shown in Figure 1. The purpose of the pre-column is to protect the column proper. It is generally identical to the separation column, except that its internal diameter is usually larger. In liquid-liquid chromatography its function is to presaturate the moving phase with the stationary phase prior to the entry of eluent into the separation column. In ion exchange chromatography, pre-columns can be used to prevent contamination of the separation column.

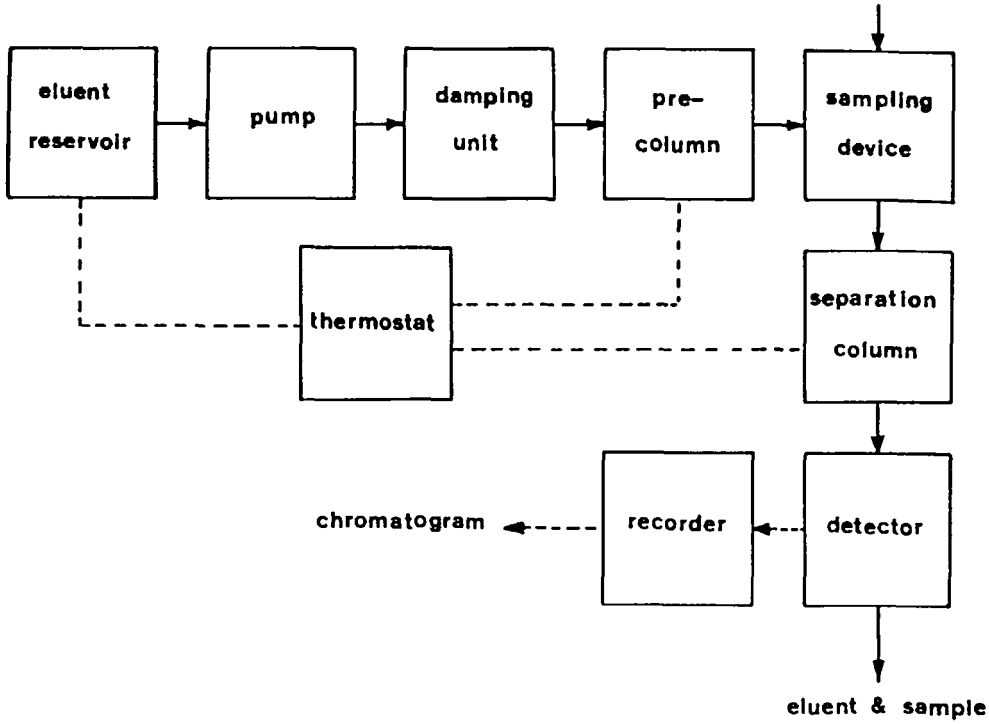


FIGURE 1. Schematic diagram of a liquid chromatograph. (From Huber, J. F. K., Kraak, J. C., and Veening, H., *Anal. Chem.*, 44, 1554 (1972). With permission.)

The initial part of this review will cover the latest developments which have taken place in such areas as column packings and column technology. Aspects of LC instrumentation will also be covered; these will include gradient elution and gradient elution devices, solvent delivery systems, and LC detectors. Applications of liquid-liquid, liquid-bonded phase, liquid-solid, and ion exchange chromatography to various chemical problems such as separation of metal complexes and the use of LC in clinical analysis will be described by using examples from the author's own work and other citations from the literature. No attempt will be made in this review to cover all of the many published applications of LC.

II. LC COLUMNS

A. Packings

In classical (gravity feed) liquid chromatography, columns are generally of large internal diameter (1 to several cm) and packing materials consist of large size, irregularly shaped particles (100 to 200 μm, average diameter). Such columns, however, are relatively inefficient and contain low plate numbers because the irregular particle shapes and wide particle diameter ranges of these substances produce packed beds which lack homogeneity and cause band spreading of the components to be separated. Also, in classical LC, column packing materials are completely porous. Thus, the molecules to be separated must diffuse within the pores in order to achieve equilibration. Since the diffusion rates in the liquid state are slow, a porous material will enhance band spreading. In modern, high resolution LC, columns are relatively small in internal diameter (1 to 3 mm) and are packed with uniformly small spherically shaped particles (5 to 10 μm). Nonspherical particles of small diameter can also be utilized in high resolution LC, although the packing technique is more difficult.

Huber[2,3] first showed that efficient columns and fast separations (comparable to GC) can be achieved by the use of finely divided, homogeneous packings. It should be pointed out, however, that such packings *necessitate* the use of high column inlet pressures in order to force the mobile phase through the column. Below 5,000 lb/in^2, pressure itself has little effect on resolu-

tion; the partition coefficient, or the separation factor. The widely used term *high pressure* LC, describing separations carried out within pressure ranges normally used (1,000 to 5,000 lb/in^2), is therefore misleading since it implies that *pressure* is responsible for high resolution, when in effect it is the *smallness* and *uniformity* of the packing material. A more accurate description is *high resolution LC*.

There are generally two types of packing materials for high resolution LC: superficially porous (or pellicular particles) and totally porous particles. In the pellicular material, an impervious, siliceous core is covered by a porous layer. The pores in such a material are therefore shallower, and band spreading due to excess diffusion is decreased. The porous layer in pellicular packings can be used for adsorptive interactions in liquid-solid chromatography, coated with stationary liquid for liquid-liquid chromatography, covered with a resin for ion exchange separations, or a stationary phase can be chemically bonded to the porous surface. Superficially porous packing materials are available under the commercial names of Zipax, Corasil I, Corasil II, Vydac, Perisorb, and Pellidon. One pronounced disadvantage of the superficially porous types of materials is the low capacity for handling large sample sizes. This is caused by the much smaller internal surface areas of these packings (10 to 15 m^2/g). For example, pellicular resins have a capacity of only 3 to 12 μEq/g. The resolution for these packings is excellent, but very sensitive detectors are required. Porous materials, on the other hand, have total internal and external surface areas on the order of 100 to 400 m^2/g, and these packings can be used for obtaining high resolution and efficiency for larger samples. Porous materials include silica gels, alumina, diatonaceous earths, and ion exchange resins.

No attempt will be made here to give a detailed classification of all available column packings since excellent reviews and detailed studies have been written on modern high resolution LC packings and column performance.[4-20]

B. Column Technology

An integral part of the column is the injector system. LC column injectors are located immediately prior to the column and must be of low lead volume in order to prevent loss of resolution. These devices permit direct "on-column" injection, which means that the sample is introduced directly on to the packing. Several different types of injection systems can be used. The first of these is the classical syringe injector, in which the sample is injected into the column through a septum with a microsyringe. This is a convenient and simple technique, although it cannot be used above a column inlet pressure of 1,500 lb/in^2, as the septum will rupture.

At higher pressures, a "sample-loop" injector can be used advantageously. This device illustrated in Figure 2 is mounted directly at the head of the column. It consists of a cylindrical stainless steel ring which contains six ports. A movable Teflon® cone located in the ring has three open segments, each of which connects a pair of external ports. An external sample loop of known volume connects two of the ports. In one of two possible configurations, the cone permits direct flow of eluent from the pump into the column, thus permitting the operator to fill the loop. Rotation of the cone by 30° makes the sample loop part of the moving stream and sweeps the sample into the column. These devices are used extensively in LC and provide trouble-free operation without leakage at pressures up to 5,000 lb/in^2.

Sie and van den Hoed[21] have studied the dependence of column performance on trans-column velocity differences caused by nonuniform permeability of the packed bed. They demonstrated that in normal techniques of column preparation, size segregation of the particles and uneven packing are likely to occur, and they presented methods to pack columns in which these problems are avoided. Majors and MacDonald[22] have studied the practical implications of LC column performance. They showed that faster separations may be achieved with shorter columns at lower pressures using smaller diameter particles. They also showed that the performance of columns packed with porous particles (diameter < 20 μm) exceeds that of pellicular beads. Baker et al.[23] have presented the requirements for scaling up from a small-diameter analytical column to a large-diameter preparative column. Snyder[11] has described a simple and rapid approach to allow the rapid selection of experimental conditions for achieving required changes in resolution in LC columns. No calculations are required, and the entire procedure from the inspection of the initial chromatogram to the final prediction of conditions takes 1 min.

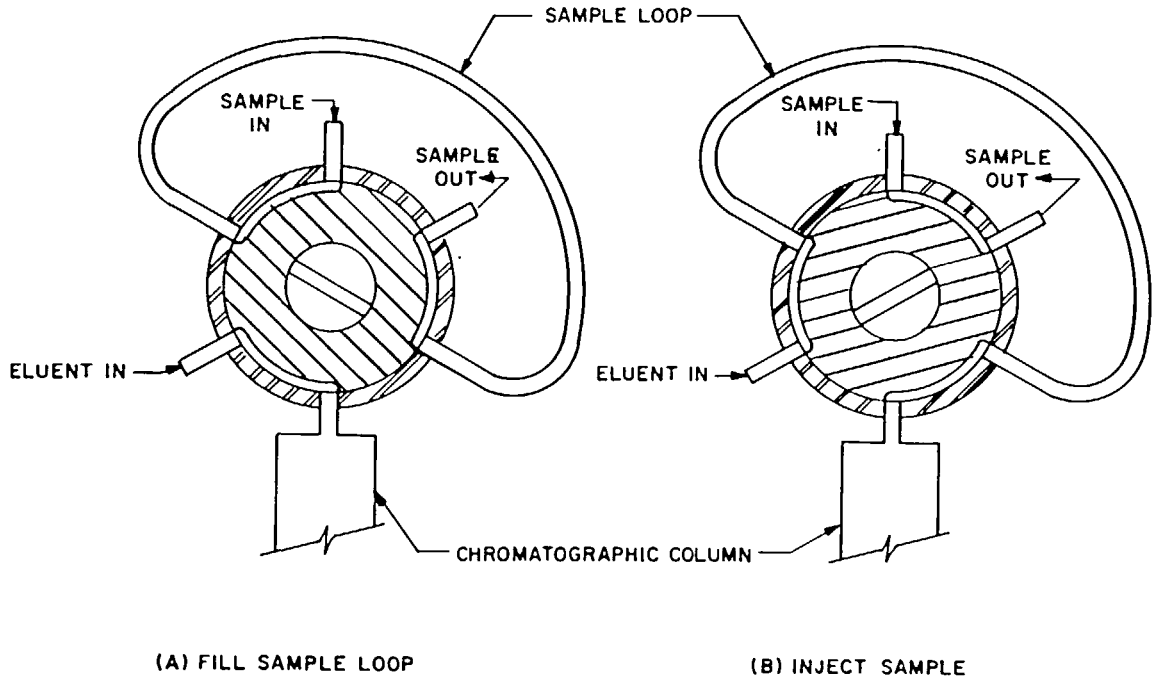


FIGURE 2. Sample loop injector. (From Scott, C. D., in *Modern Practice of Liquid Chromatography*, Kirkland, J. J., Ed., Interscience, New York, 1971. With permission.)

Cassidy, LeGay, and Frei[24] have investigated different slurry packing techniques for small irregularly shaped silica gels (10 μm). They studied an incremental addition technique and a balanced-density slurry technique. The effects of various parameters such as pump type, solvent density, and column length are reported. They recommend a reproducible and fast packing technique which yields HETP values of 0.18 mm at fluid velocities of 2 cm/sec. Asshauer and Halász[25] have studied the reproducibility and efficiency of LC columns packed with 10 μm silica. Stainless steel tubes were drilled and then packed with 10 μm silica. They found that the inner wall of undrilled stainless tubes is porous with longitudinal grooves and the inner diameter is variable, whereas the drilled tubes have a "clean" surface with radial grooves and a constant inner diameter. These columns were packed with the use of dispersing agents with high viscosities (40 to 60 cP) and densities of 1 g/ml. With a linear velocity of 2 cm/sec, up to 100 plates can be generated per second. Engelhardt, Asshauer, Neue, and Weigand[26] have described an *in situ* coating procedure to prepare heavy loaded liquid-liquid chromatography columns containing a prepacked silica support (5 to 10 μm). The support can be coated with from 0.2 to 0.8 g liquid/g silica, and the amount of stationary liquid in the column can be determined from subsequent chromatographic data. Up to 11 plates per second were generated, and the versatility of the method was demonstrated by illustrative separations of steroids, corticosteroids, and dansyl amino acids.

Huber, van der Linden, Ecker, and Oreans[27] have constructed and evaluated a high-pressure micro multiport column switching valve. The principle of operation of column switching in LC is to transfer selectively fractions of the mobile phase from the outlet of one column into the inlet of another column. The transferred volume can correspond to a group of peaks, a single peak, or a fractional peak. The design of the switching device is crucial since it must be capable of operating at high pressure and it must have a low lead volume to minimize peak broadening. A schematic diagram of the column switching valve is shown in Figure 3. The valve consists of a pneumatically

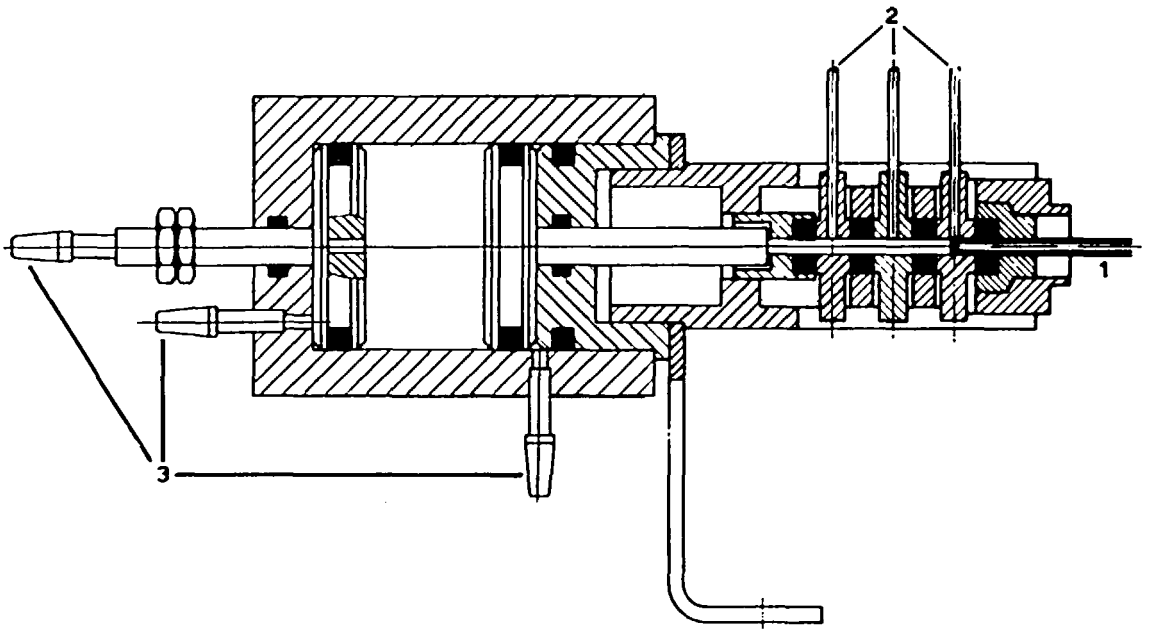


FIGURE 3. Schematic diagram of the switching valve. (From Huber, J. F. K., van der Linden, R., Ecker, E., and Oreans, M., *J. Chromatogr.*, 83, 267 (1973). With permission.)

operated piston, with one inlet which can be connected in turn to one of two or three outlets. The authors have demonstrated different modes of operation such as stripping part of the sample using a sectional column system, i.e. using a short first and a longer second column of the same type. Figure 4A shows a chromatogram obtained with both column sections together, and Figure 4B shows a chromatogram in which the peak-free space in the first chromatogram is eliminated by column switching. The first group of components is separated on both column sections, the second group only on the short first section. This method illustrates how the total analysis time can be drastically reduced (in this case from 45 to 8 min).

Scott and Lee[15] have shown that separation of metabolites in physiological fluids can be achieved by sequential columns of microreticular and pellicular ion exchange resins. The first column (microreticular resin) contributes the necessary capacity for a preliminary separation, and the final column containing the pellicular resin provides a rapid, final separation. A schematic diagram of the coupled column chromatograph is shown in Figure 5. The authors showed that coupling a short microreticular and a longer pellicular column produced a more rapid separation and improved resolution of components than the pellicular column alone.

III. GRADIENT ELUTION

Gradient elution or *solvent programming* is a technique which is used in LC whereby the composition of the moving phase is changed with time or elution volume. (When the solvent composition is kept constant throughout a chromatographic run, the mode of operation is known as *isocratic elution.*) In gradient elution, the composition of the moving phase is continually changed by increasing the concentration of a second liquid and decreasing that of the first. Gradient elution can provide powerful selectivity effects and improved resolution in LC since the partition coefficients are strongly influenced by the nature of the eluent used. The technique is comparable to temperature programming in GC, and it is especially useful when the components in a mixture have widely differing partition coefficients. A number of different devices have been used for producing gradients in LC.

In the multifunctional gradient device described by Byrne, Schmit, and Johnson,[28] a number of gradient shapes and time durations can be produced during the course of chromatographic elution. This gradient generator consists of a time-proportioning electromechanical system whereby liquids from two reservoirs are mixed automatically by two electronically controlled

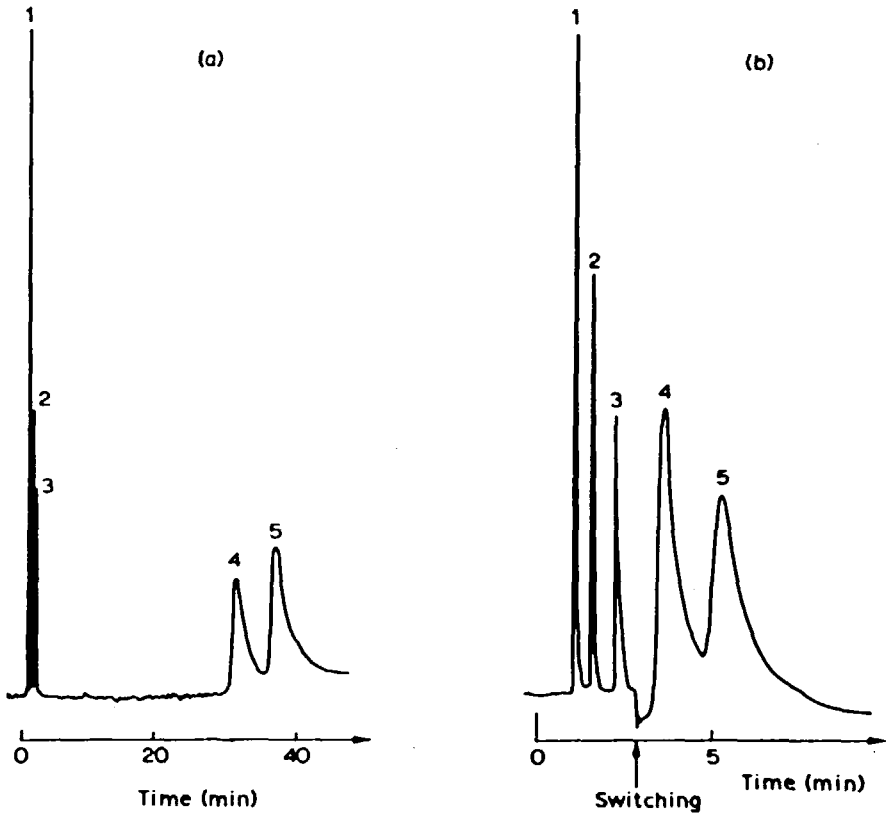


FIGURE 4. Column stripping. Sample: 1 = toluene, 2 = azobenzene, 3 = 2-nitrobenzene, 4 = p-cresol, 5 = phenol; injection volume, 10 μl. Column: packing, 10% (w/w) 1,2,3-tris(cyanoethoxy) propane on silica support, 4 to 8 μm; eluent, 2,2,4-trimethylpentane. Detector: UV, 270 ± 10 nm. (a) Columns 1 and 2 for total sample. (b) Columns 1 and 2 for components 1 to 3; column 1 alone for components 4 and 5. Column 1, 30 × 3 mm; column 2, 290 × 3 mm. Flow rate: column 1, 2.27 ml/min; columns 1 and 2, 1.33 ml/min. (From Huber, J. F. K., van der Linden, R., Ecker, E., and Oreans, M., *J. Chromatogr.*, 83, 267 (1973). With permission.)

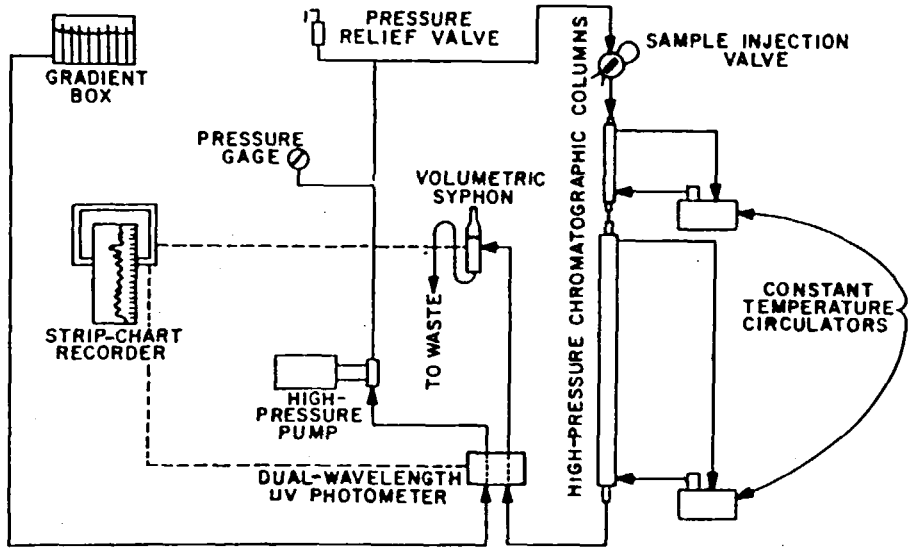


FIGURE 5. Coupled column chromatography with sequential columns of microreticular and pellicular anion exchange resins for the analysis of the UV-absorbing constituents in physiological fluids. (From Scott, C. D. and Lee, N. E., *J. Chromatogr.*, 83, 383 (1973). With permission.)

proportioning valves. The valves are alternately cycled by electronic ramp generators to allow a prescribed amount of each liquid to flow into a mixing chamber. Both linear and exponential as well as isocratic and step elution programs can be generated. The time duration, starting concentration, and final concentration can be controlled and a single pump is used. Figure 6 shows the nine basic gradient shapes which are available. Exponential gradients have the advantage of changing the rate of the gradient during the run. The advantage of using a gradient is illustrated in Figure 7. In this separation, the authors separated a 62% chlorinated mixture of biphenyls isocratically, as shown in Figure 7A. They then eluted the same mixture using an exponential gradient, as shown in Figure 7B. With the gradient, the total analysis time is shorter, and the peaks are sharper.

In many commercially available liquid chromatographs, gradients are produced by utilizing two pumps. An example is the gradient system utilized in the Waters Associates liquid chromatograph in which each pump supplies the respective flows from liquids A and B. The flow rate for each pump continually changes during the course of the run and is controlled by a programmer. The combined flows are then fed to a mixer and to the column. The schematic flow diagram is shown in Figure 8.

There are far less expensive and simpler methods to produce gradients in LC. One of the most widely used is the versatile nine-chamber Varigrad,[29] which is particularly useful for ion exchange gradients where it is necessary to use an ionic strength or pH gradient for separations. This device consists of a box subdivided into a number of chambers. Each chamber is filled with equal weights (not volumes) of buffers, the concentration (or pH) of which slowly changes. The chambers are interconnected and individually stirred when the run begins, and the eluent is pumped out of the first chamber. The disadvant-

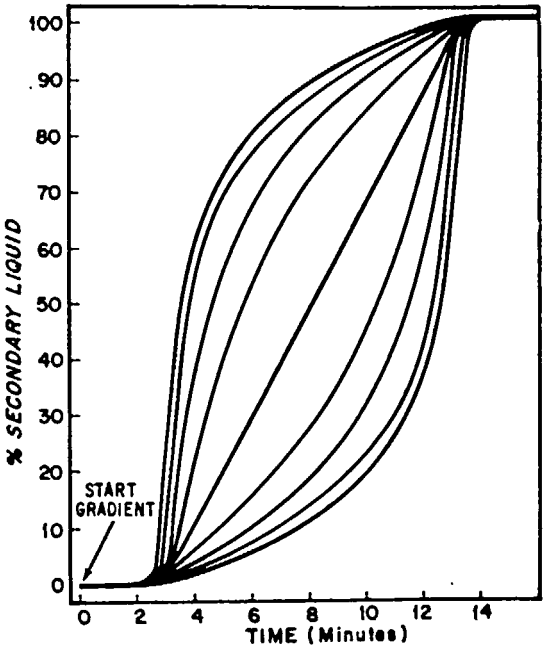


FIGURE 6. Gradient elution profiles; 0 to 100% at 10%/min. (From Byrne, S. H., Schmit, J. A., and Johnson, P. E., *J. Chromatogr. Sci.*, 9, 592 (1971). With permission.)

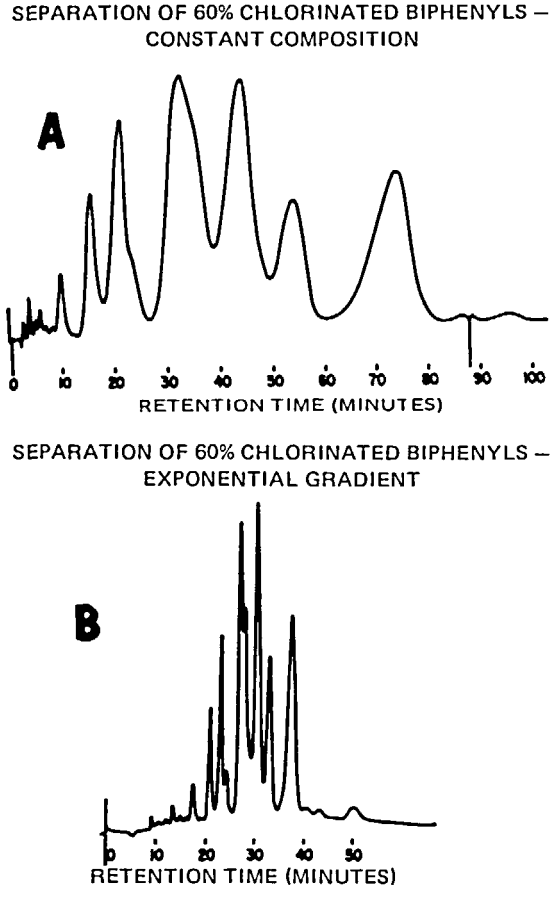


FIGURE 7. (A) Isocratic separation of a mixture of 60% chlorinated biphenyls. Column: ODS, Permaphase; mobile phase: 60% methanol/40% water; column temperature: 50°C; flow rate: 2 ml/min; column pressure: 1,200 lb/in^2. (B) Chlorinated biphenyls separated with an exponential gradient. Operating conditions same as (A) except for gradient. Gradient from 20% methanol/25% water at 3%/min. (From Byrne, S. H., Schmit, J. A., and Johnson, P. E., *J. Chromatogr. Sci.*, 9, 592 (1971). With permission.)

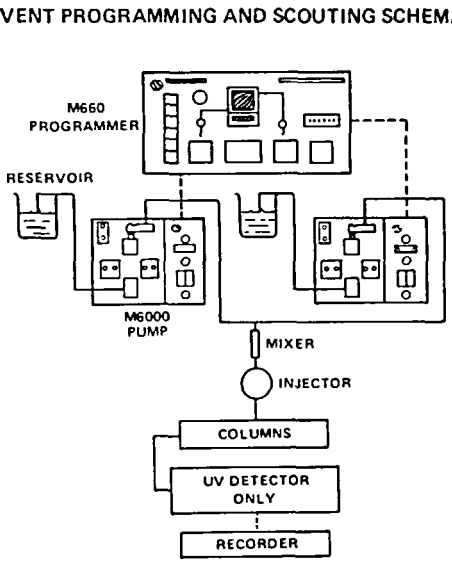


FIGURE 8. Schematic flow diagram for solvent programming and gradient elution. (From Veening, H., *J. Chem. Educ.*, 50 A429 (1973) and Waters associates. With permission.)

age is that devices such as this are often constructed out of acrylic plastic; they can therefore not be used with organic solvents. Glass gradient boxes are available, but they are more expensive.

Karlsson, Ohman, and Mills[30] have designed and reported a gradient accessory which can mix simultaneously two or three liquids, and it can be used to repeat, automatically, the complete chromatographic processes of equilibration, gradient production, and column rinsing. This instrument, known as the "Ultrograd," is a combination of an electronic control unit and a photoelectric scanner. The electronic control unit controls either one- or two-way valves, which are connected between the solution vessels and the mixing cell. The scanner consists of a combined light beam and photocell which immediately senses any changes in the light-reflecting quality of the area being scanned. The scanning is done over a rectangular window in which the gradient profile chart is placed. The photoelectric unit begins scanning from left to right until the run is completed. The actual mixing is controlled by signals which are relayed from the scanner. Figure 9 illustrates both the electrical connections and the liquid flow for producing a gradient with this instrument.

An automated two-chamber gradient device has been developed by Chilcote, Scott, and Pitt.[31] This unit has been mathematically designed to duplicate simply and reproducibly the performance of more complicated devices. This gradient generator utilizes two wedge-shaped containers for liquids *A* and *B*, as shown in Figure 10. The two eluents flow simultaneously from the respective chambers into a common mixer. The cross-

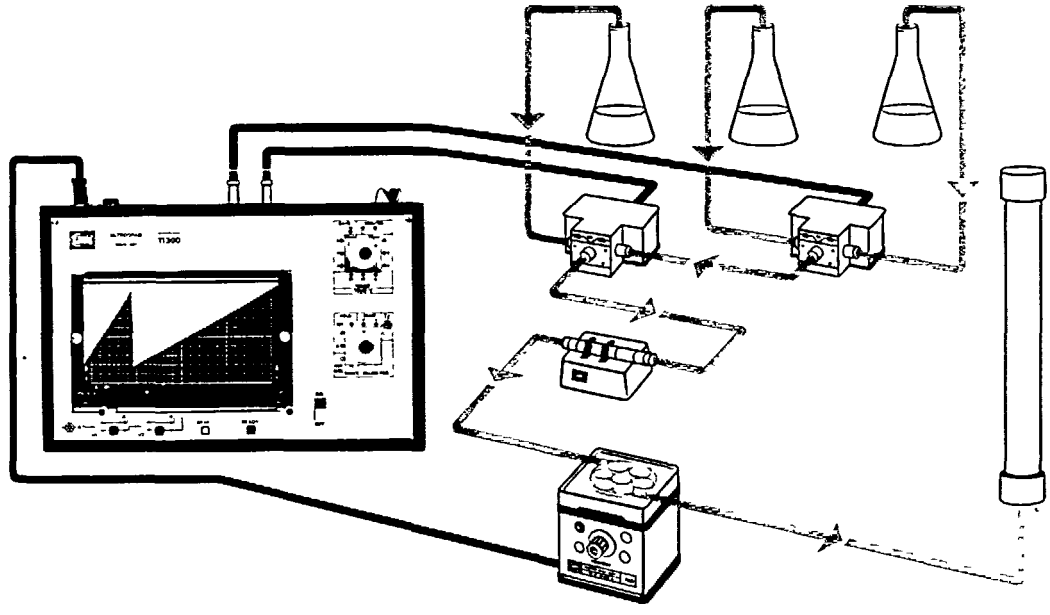


FIGURE 9. Ultrograd. Electrical (black lines) and flow (gray lines) connections between the gradient mixer, both two-way valves v1 and v2, and pump P. The solutions are mixed in the mixing chamber M and the gradient formed is fed to column C. (From Karlsson, C., Ohman, J., and Mills, P., *Am. Lab.*, 4, 41 (1972) and LKB Instruments, Inc. With permission.)

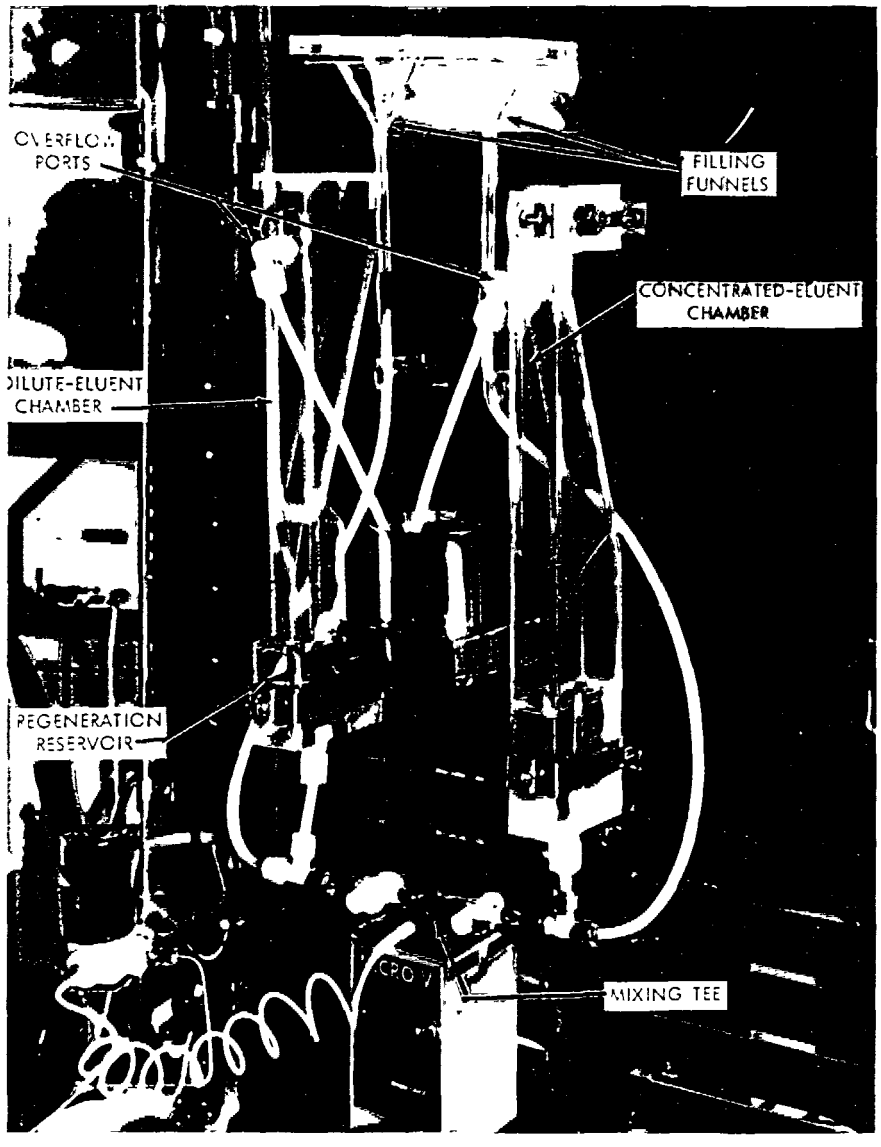


FIGURE 10. Automated two-chamber gradient device. (From Chilcote, D. D., Scott, C. D., and Pitt, W. W., Jr., *J. Chromatogr.*, 75, 175 (1973). With permission.)

sectional area at the top of the dilute eluent chamber (1) is large compared with that of the concentrated eluent chamber (2); however, the cross-sectional area at the bottom of chamber 1 is small compared with that of chamber 2. Thus, if chamber 1 is filled with A and chamber 2 with B, the concentration of A in the eluent will be large at the start of the chromatographic run and small at the end. Chamber shape determines the gradient profile. While one loses flexibility in not being able to change the gradient profile, the main advantage is the reproducibility which can be achieved for routing operations involving repetitive runs of the same separation. This device has been used successfully for the gradient separation of carbohydrates (as their borate complexes) in physiological fluids.[32]

In ion exchange chromatography, buffered salt solutions of variable ionic strength and constant pH are often used in order to separate complex mixtures. It has been found by Katz[33] that the initial buffering capacity in anion exchange chromatographic runs may be insufficient to maintain a system pH within desired limits, and, as

a result, the column eluate pH may vary with time. The exchange of resin hydroxide ions with eluent acetate ions results in a temporary increase in eluate pH during the initial stages of a run when an increasing buffer concentration gradient is employed. It was also found that there were temporary decreases in pH when the eluent buffer concentration was decreased. Excursions in pH caused by these concentration gradients were observed for eluted urine samples.

IV. SOLVENT DELIVERY SYSTEMS

A great deal of effort in LC has been directed during the last few years toward obtaining high resolution and faster speeds. This has been accomplished by using smaller-diameter columns and very small particle packings, thus necessitating higher pressures at the column inlet. Such columns require pressures of ca. 5,000 lb/in^2, and accordingly it has become necessary to build pumps which can meet these requirements. Also, since LC detectors are sensitive to variations in flow, there has been a great deal of interest in producing pulseless and constant flow pumps. LC pumps must also be equipped with controls which enable the operator to change the flow rate.

Two different types of pumps can be used in LC: *constant pressure* (direct pressurization of the mobile phase with an inert gas) and *constant flow.* In constant flow pumps, the flow is held constant and the pressure is allowed to vary as column permeability changes. These pumps possess as their main advantage the fact that a change in pressure has little effect on the chromatographic separation, whereas a change in flow affects retention time, resolution, and baseline characteristics. Constant flow pumps can be further subdivided into *reciprocating, diaphragm,* and *single displacement* types. In the reciprocating pump, pressure changes due to viscosity changes cause leak rates in the check valves and piston packing materials to result in complex flow behavior. One interesting type of constant pressure pump utilizes a *pneumatic amplifier* system. Reviews and discussions of the principles of solvent delivery systems have been published recently.[18,19,34-37]

A. Reciprocating Pumps

These pumps supply liquid to the chromatographic system by placing the piston in direct contact with the moving liquid. The intake stroke of the piston withdraws liquid from the reservoir to fill the pump head; the discharge stroke forces liquid into the column. Check valves control the direction of flow of liquid. A disadvantage of these pumps is the fact that they supply a pulsating flow of liquid to the chromatographic system, resulting in severe detector noise. These pulsations can be removed by a suitable damping system, such as a long (6 m), coiled section of stainless steel hypodermic tubing. Flexing of the coil will absorb the bulk of these pulsations. An advantage of reciprocating pumps is that their delivery is not limited by a certain volume (as in single displacement pumps) and they can operate continuously. Another advantage is that it is possible to recycle the eluted sample back through the pump easily.

Several commercially available pumps operate by means of motor-driven dual reciprocating pistons (Spectra-Physics and Waters). The 180° phase difference and special cam shape result in a steady flow, but slight fluctuations are eliminated by a unique flow-feedback system. A flow-through transducer in the pump outlet line continually measures the flow rate (not pressure) and sends the information to the control module, where it is compared with the flow rate setting. Flow rate fluctuations create an error signal, which changes the motor speed accordingly. This "closed-loop" system is illustrated in Figure 11. Such a pumping system will produce a *metered,* pulseless flow of *unlimited* volume.

B. Reciprocating Diaphragm Pumps

The Orlita pump is an example of a reciprocating type utilizing a diaphragm. This pump is used in the Siemens and Hewlett-Packard liquid chromatographs. A working piston and a floating piston oppose each other in an oil chamber which is separated from the solvent chamber by a stainless steel membrane. The working piston operates at a constant stroke of 15 mm at 100 strokes per minute. If the stroke of the opposing piston is the same as that of the working piston, they move in harmony and no pressure is developed. Delivery rate of the pump is adjusted by limiting the travel of the opposing piston to some fraction (0 to 100%) of the working piston. Maximum pressure capability is 325 atm; maximum flow is 600 ml/hr. The pump comes equipped wth an in-line pulsation damper. The principle of operation of this type of pump is illustrated in Figure 12.

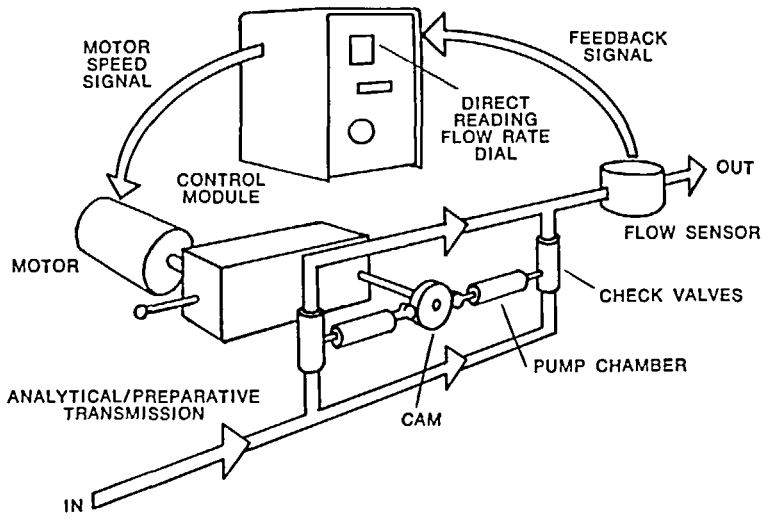


FIGURE 11. Flow-feedback schematic. (From Veening, H., *J. Chem. Educ.*, 50, A429 (1973) and Spectra-Physics, Inc. With permission.)

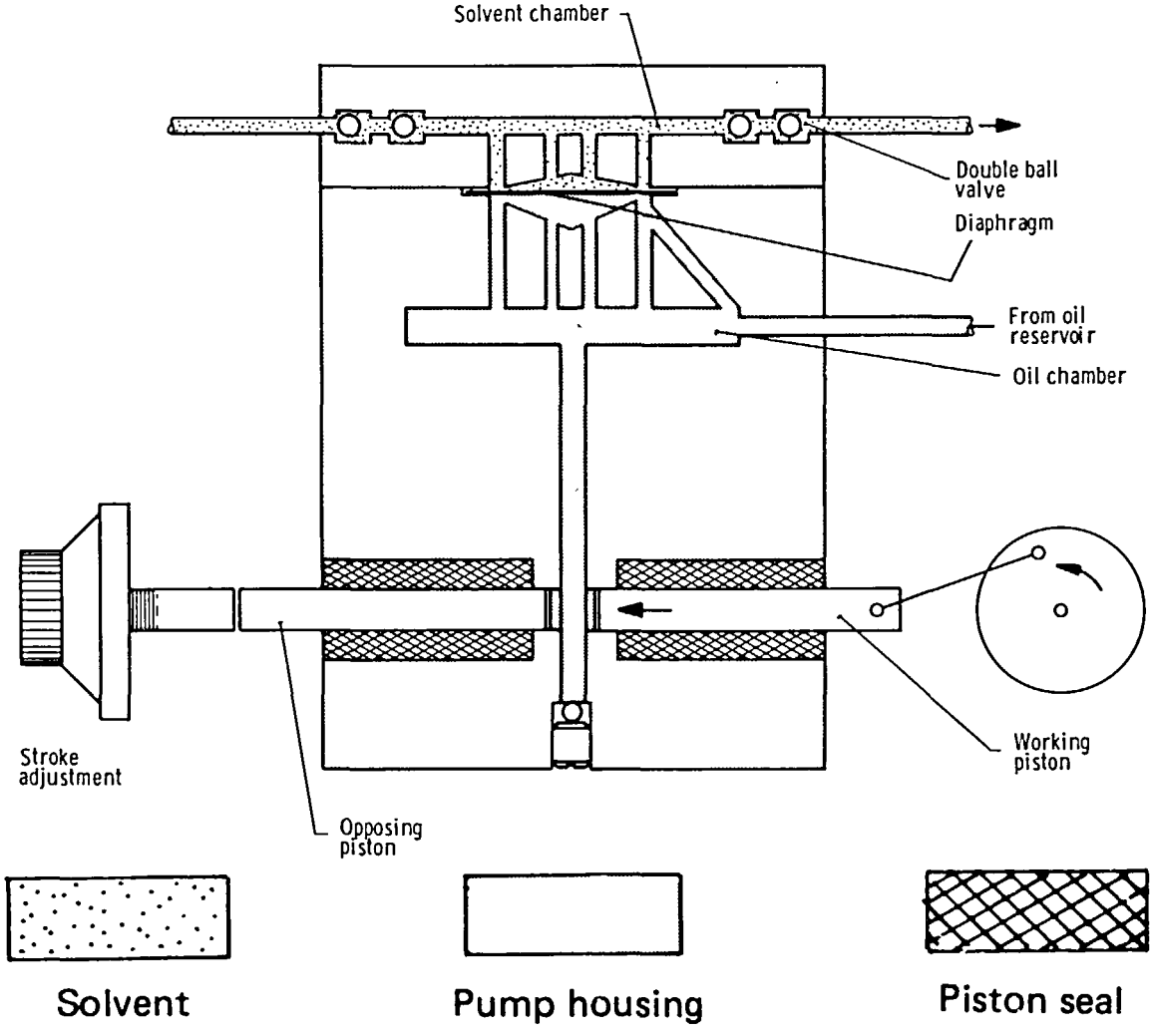


FIGURE 12. Schematic diagram for a dual piston diaphragm pump. (From Veening, H., *J. Chem. Educ.*, 50, A429 (1973) and Hewlett-Packard. With permission.)

C. Single Displacement Pumps

Mechanical pumps which operate with a screw-driven syringe have the advantages that they are pulse-free and capable of very high pressures. Several commercial pumps (Instrumentation Specialties Co., Perkin-Elmer, and Varian) incorporate this principle. The disadvantage of the single displacement principle is that the pump has a limited volume capacity and must be refilled at the end (or sometimes in the middle) or a run.

D. Pneumatic Pumps

Pneumatic constant pressure pumps operate by applying gas pressure to a collapsible container or a movable piston. One of the earliest high-pressure LC pumps which operated on the pneumatic principle was described by Jentoft and Gouw;[38] it utilized high-pressure air as the source of pressure, and mercury was used as the displacing agent for the solvent. This pump was capable of delivering pressures up to 1,000 lb/in^2.

In pneumatic amplifier pumps (DuPont and Tracor), air or nitrogen at 100 lb/in^2 is delivered to a hydraulic piston of much smaller surface area, thus providing a 50-fold pressure amplification.[36] This principle is illustrated in Figure 13. Pneumatic amplification pumps are pulseless, deliver constant flow rates, and are capable of high pressures, but they must be refilled once the pumping cycle has been completed.

E. Other Pumping Systems

A novel pumping system involving a continuous gas displacement pump (CDP) has been reported by Karger and Berry.[39] The system pressure in this pump is maintained constant with a gas regulator, and a pulsating piston pump supplies the flow. The flow is split between the main flow-stream and a controlled leak section which recycles the excess solvent to a reservoir. The CDP controls pressure to ±0.5%. The advantages of the classical gas displacement pump, i.e., no pulsations, inexpensive construction, and an equivalent detector noise level, are realized with this system.

Rogers and his co-workers have studied ultra-high pressure liquid chromatography in the range of 5,000 to 50,000 lb/in^2.[40-43] A high-pressure pump (S. C. Hydraulic, Los Angeles, CA) was used and could produce pressures in excess of 50,000 lb/in^2. This pumping system utilized a small-diameter hydraulic piston connected to a large-diameter air piston producing a 660-fold hydraulic to air amplification in pressure. The stainless steel column used in the chromatograph was ¼ in. (inside diameter) X 1¼ in. (outside diameter) X 12 in.

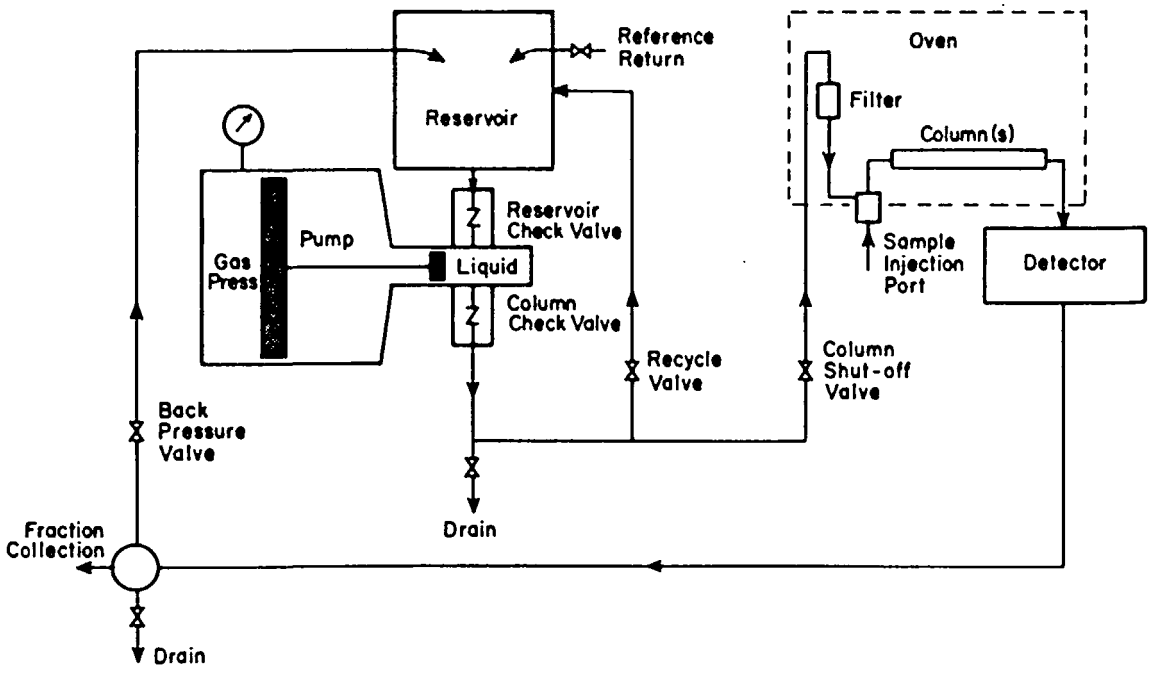


FIGURE 13. Pneumatic amplification pump, shown as part of a complete liquid chromatograph. (From Veening, H., *J. Chem. Educ.*, 50, A429 (1973) and E. I. DuPont de Nemours and Co., Inc. With permission.)

V. LC DETECTORS

The detector is one of the most critical component parts of a liquid chromatograph. There is no truly "universal" LC detector which compares with the GC thermal conductivity cell in sensitivity and utility. This feature can be an advantage or a disadvantage depending on circumstances. The subject of LC detectors has received a great deal of attention during recent years.[34,35,44-49] These reports deal specifically with theory, performance evaluation, sensitivity, and applications.

An LC detector is a device which measures a physical property such as light absorption or refractive index of the column effluent and converts changes in these properties into usable signals by the production of a current or a potential which can be amplified and fed to a recorder. Chromatographic detectors generally operate differentially, responding to either the concentration or the mass flow rate. Differential detectors responding to the concentration (photometric, refractometric, fluorimetric, conductance) yield a signal (Y) which is proportional to the concentration (C) which traverses the detector.

$$Y = K_1 C$$

If Y is plotted against time (t), an elution peak results. It can be shown that for such detectors, the area (A) under the peak is proportional to the mass (m) of component and inversely proportional to the flow rate (F).

$$A = K_1 \frac{m}{F}$$

It is therefore crucially important that flow of mobile phase be kept constant for such detectors, if quantitative analysis is to be carried out.

In differential detectors which respond to the mass flow rate, dm/dt (e.g., flame ionization), it can be shown that the area is directly proportional to the total mass

$$A = K_2 \int \frac{dm}{dt}\, dt = K_2 m,$$

and there is no dependency on flow rate. Requirements for a suitable high resolution LC detector are high sensitivity, low noise levels, a wide linear dynamic range, and a small dead volume of the sample cell. The latter feature is very critical due to the large amount of convective mixing which can occur in the cell itself and thus lead to undesirable peak broadening and subsequent loss of resolution. The *linear dynamic range* of a detector represents the concentration range within which the detector operates linearly with respect to concentration. The linear dynamic range is defined as the ratio of the upper and lower linearity limits.

In general, LC detectors can be classified according to the type of physical property measured. These properties include: absorption of radiant energy, refractive index, phase transformation, fluorescence, radioactivity, polarography, coulometry, and others.

Photometric and refractive index detectors account for the majority of devices which are utilized at the present time. Fluorescence detectors are also rapidly gaining in importance for many biochemical applications. Phase transformation (moving wire) detectors are used in certain commercial instruments. Radioactivity, polarographic, coulometric, Christiansen effect, interferometric, mass, and spray detectors are relatively new and are presently in the developmental stage.

A. Photometric Single and Multi Wavelength Detectors

Photometric LC flow detectors have a very wide range of applications. These detectors are used mainly in the ultraviolet (UV) region of the spectrum since numerous systems of chemical and biological interest are strong absorbers. In photometric detectors, the eluted stream passes through a flow cell across which monochromatic energy is transmitted. The increase in absorbance or decrease in transmittance of the stream is recorded at constant wavelength. An acceptable photometric detector useful in high resolution LC should possess certain features including a small cell volume (a few microliters), readout in absorbance units enabling direct Beer's Law calibrations of peak area, and a high intensity of primary energy providing for high sensitivity.

The optical path for a typical constant wavelength detector is shown in Figure 14. Light beams from a low pressure mercury source S are collimated by lens L and passed through the flow cell chambers C_1 and C_2, a plane

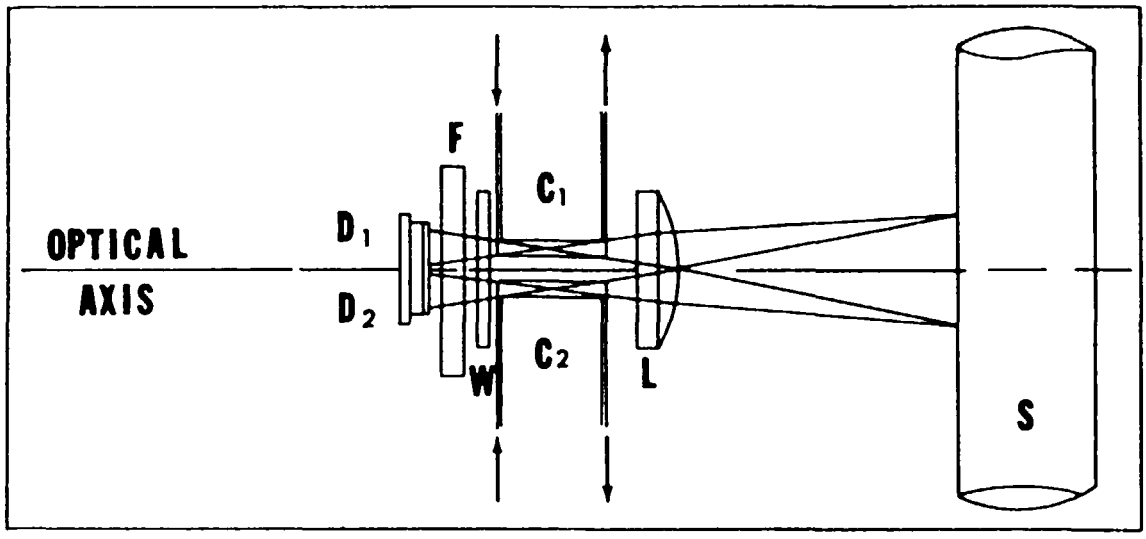


FIGURE 14. Optical path in a constant wavelength UV photometric LC detector. S = mercury source; L = collimating lens and cell window; C_1 and C_2 = flow cell chambers; W = plane window; F = filter; D_1 and D_2 = detectors. (From Veening, H., *J. Chem. Educ.*, 47, A549 (1970) and Laboratory Data Control. With permission.)

window W, and a filter F. The beams impinge on dual transducers D_1 and D_2. Typically, the flow cells for such detectors are 10 mm in path length and 8 to 10 μl in volume.

Dual wavelength detectors usually operate simultaneously and differentially and can monitor one or two streams at 254 and 280 nm. A mercury lamp illuminates a screen partially coated with a phosphor capable of converting 254 nm radiation to 280 nm. The screen thus acts as a composite light source. In the Laboratory Data Control duoMonitor, the dual flow cell has two slot-shaped absorption chambers sealed by lens windows. Each of the two wavelengths are separated into two beams (one for each chamber), and by means of a unique optical system, the two completely separated beams are then sensed by dual detectors.

A simple and sensitive dual wavelength UV detector for monitoring carbohydrates in physiological fluids has been developed by Katz and Thacker.[50] Dynamic mixing of the column effluent with sulfuric acid results in the rapid production of chromophores with absorption maxima in the 290- to 310-nm region. In order to provide better discrimination from other constituents in body fluids which absorb strongly below 300 nm, this UV photometer was provided with an analytical channel at 306 nm rather than the 296-nm optimum wavelength, due to the availability of a suitable phosphor. The 254-nm channel was retained. The analytical channel consisted of a phosphor-rod light source, a quartz flow cell with a 0.27-cm optical path, a Corning 7-54 filter, a Sylvania phosphor type 2382, and a photoconductor. The phosphor-rod is activated by 254-nm energy from a mercury lamp and emits the broad spectrum shown in Figure 15. The UV portion passes through the filter and is absorbed by the Sylvania phosphor with the efficiency also shown in Figure 15. A maximum at 306 nm is thus produced. The dual wavelength UV photometer is shown in Figure 16. This detector was found to be far more sensitive for monitoring carbohydrates than the earlier detector which operated at 254 nm only.

One interesting degree of selectivity possessed by photometric detectors of variable wavelength, such as recording spectrophotometers, is that two poorly resolved components eluting from the column can potentially be determined separately by repetitive runs at two different wavelengths. Also, with a variable wavelength detector one is able to discriminate against compounds which are of no interest, such as the solvent.

A number of spectrophotometers have been used successfully as LC detectors. The Beckman DB spectrophotometer has been used to monitor arene metal carbonyls[51] and to detect UV-absorbing components in physiological fluids.[52] In the latter application, the spectrophotometer was modified by coupling a servo motor with the wavelength mechanism so that the detector could

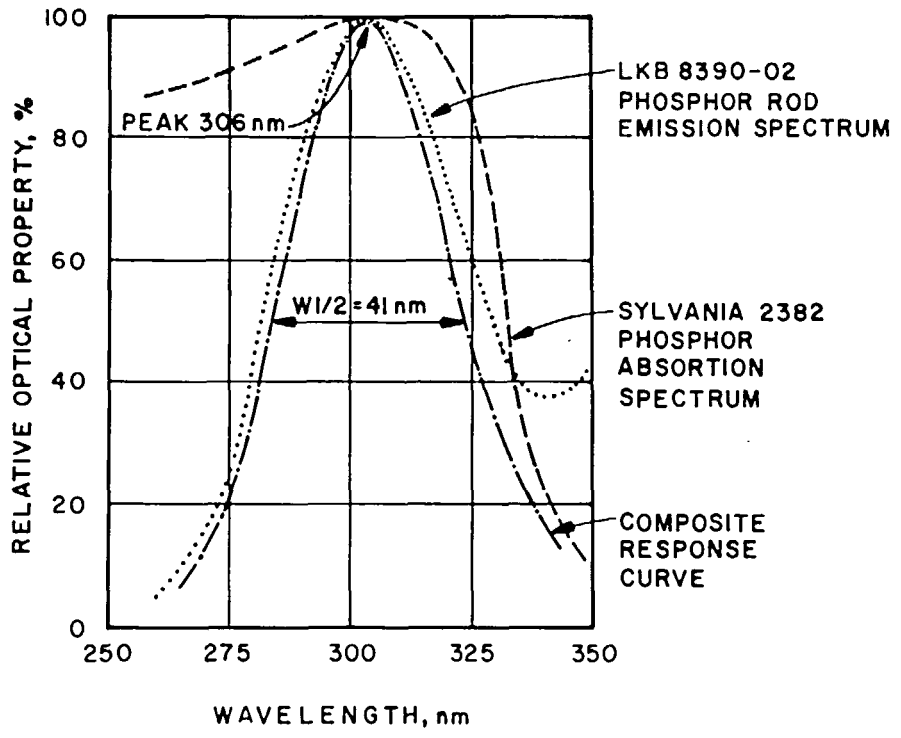


FIGURE 15. Combination of emission and absorption spectra utilized in UV detection system. (From Katz, S. and Thacker, L. H., *J. Chromatogr.*, 64, 247 (1972). With permission.)

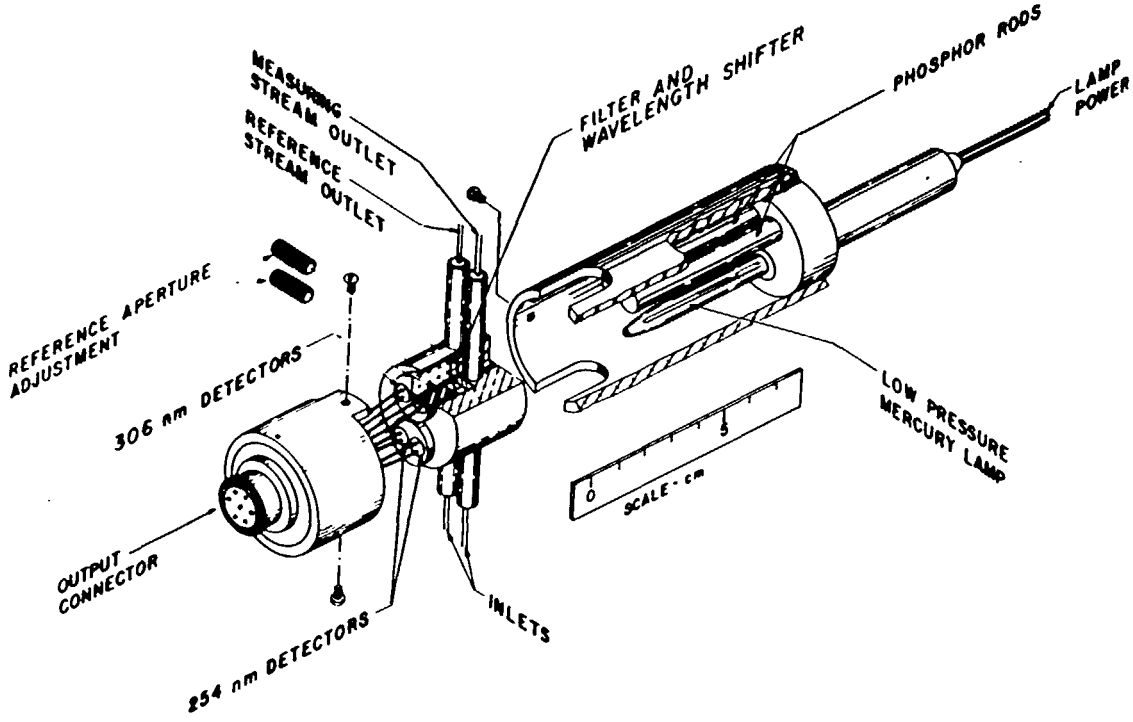


FIGURE 16. Two wavelength UV photometric detector. (From Katz, S. and Thacker, L. H., *J. Chromatogr.*, 64, 247 (1972). With permission.)

operate alternately at four different wavelengths in the UV (250, 260, 280, and 290 nm). Other UV spectrophotometers have also been used for LC separations.[34,46,53,54] A number of commercial LC instruments now utilize variable wavelength detectors (Tracor, Spectra-Physics, DuPont, Altex, Laboratory Data Control, Micromeritics, Packard, Perkin-Elmer, and Varian).

Cell design is a very important feature of a photometric detector. Most cells are typically one cm in path length and are of minimal volume (8 to 10 μl) in order to prevent band spreading and loss of resolution. Some cells have been designed to operate at high pressure in order to prevent the formation of bubbles. Also, a number of different internal geometries such as an "L," "U," "Z," or split stream flow paths have been used in order to optimize flow characteristics and to minimize sensitivity to flow fluctuations in the cell.[55,44]

B. Refractometric Detectors

Differential refractometry is particularly useful for LC detection if the solvent is UV absorbing. However, since virtually any change in composition of the moving stream can be detected, this mode of detection is normally only used with isocratic elution.

Refractometric LC detectors operate on either one of two principles. In the first type, the measurement is based on an optical displacement of the beam. The optical path for such a refractive index detector is illustrated in Figure 17. The light beam from the source passes through a mask, is collimated by the lens, and proceeds through the liquid-filled sample and reference prisms. The beam is then reflected back through the sample and is focused by the lens on the photodetector. If the refractive index of the moving phase changes due to the elution of a sample component, the beam is slightly deflected. The photodetector produces a signal which is proportional to the intensity (and thus the deflection) of the light beam.

The second method utilizes the "Fresnel" principle, which relates reflectance intensity of a dielectric interface to the refractive indices of the interface materials.[44] Such an interface may be formed between a glass prism of selected optical properties and the liquid whose refractive index is to be measured. The optical path of such a refractometer is shown in Figure 18. Two collimated beams strike the glass-liquid interfaces within the cell prism. Rotation of the projector assembly permits adjustment of the angle of incidence on the interfaces. The sample and reference flow cells consist of oblong openings in a thin Teflon gasket clamped between the cell prism and the back plate. Since the ratio of reflected light to transmitted light is a function of the refractive index of the two liquids, the illumination of the cell back-up plate is a direct measure of the refractive index of the liquid in each chamber.

Refractive index detectors are generally very reliable and are capable of detecting any type of component in isocratic elution. The Fresnel and the deflective type of refractive index detectors are roughly equivalent in performance. The disadvantages of refractive index detectors are their relatively low sensitivity and their inability to function satisfactorily in gradient LC.

A third type of refractometric detector which has been proposed by Gow-Mac Instrument Co. is the so-called "Christiansen Effect Detector" (CED). The CED utilizes the developments described in 1884 by Christiansen in his work with crystal filters.[55] A sample cell, provided with an inlet and outlet connection, is packed with a solid having the same refractive index as the moving phase eluting from the column. Visible light is transmitted through the cell as long as the refractive indices of the solid and liquid remain the

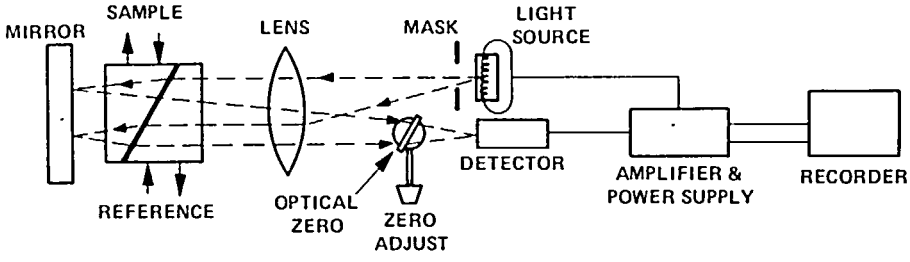


FIGURE 17. Optical diagram for the displacement refractive index detector. (Courtesy of Waters Associates, Inc.)

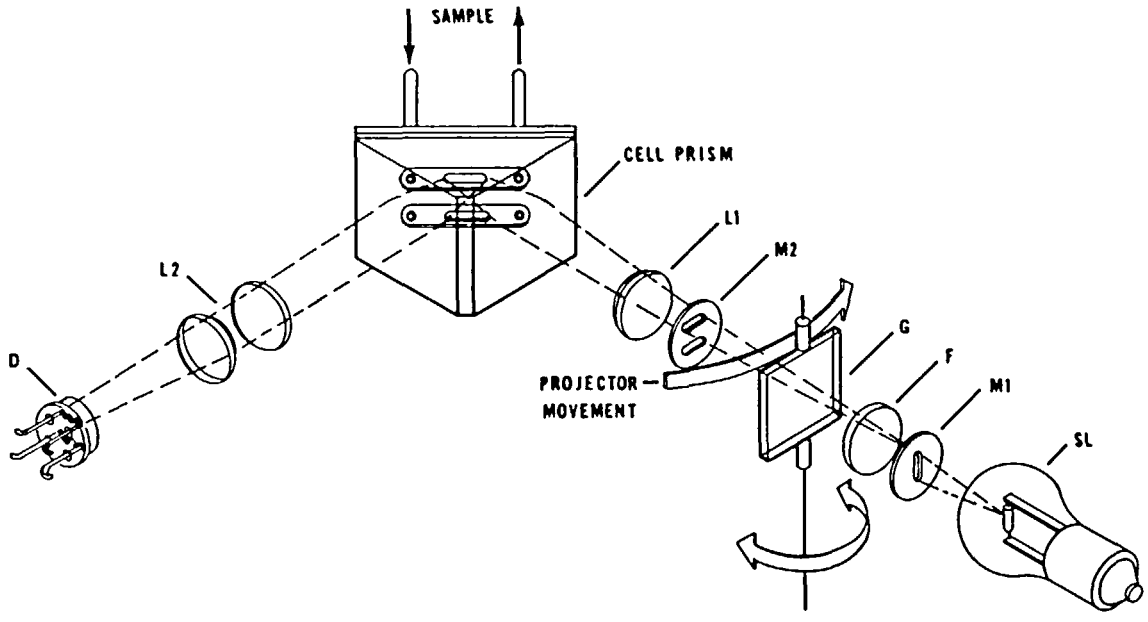


FIGURE 18. Optical diagram for the Fresnel-type refractive index detector. (From Veening, H., *J. Chem. Educ.*, 47, A549 (1970) and Laboratory Data Control. With permission.)

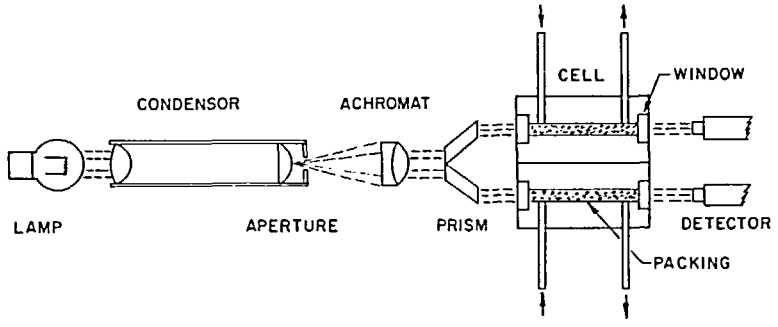


FIGURE 19. Schematic diagram of the Christiansen effect refractive index detector. (From Veening, H., *J. Chem. Educ.*, 50, A429 (1973) and Gow-Mac Instrument Co. With permission.)

same. When a sample is eluted from the column and carried through the cell, the refractive index changes and no longer matches that of the solid. This is indicated by a change in the amount of light transmitted and is measured by the photodetectors. An optical schematic of the CED is shown in Figure 19.

If polychromatic energy is employed, the central part of the transmitted beam will exhibit the color corresponding to the wavelength at which both substances have the same refractive index. The remaining wavelengths will be refracted in other directions, causing a "halo" effect. When a sample appears in the liquid stream, its refractive index changes and the wavelength in the center of the beam shifts, causing a change which can be measured by photodetectors. Gow-Mac has developed a wide range of solids (1.3 to 1.6 RI) to match the various solvents used in LC. The minimum detectable RI difference is 10^{-6} RI units. The CED should have a wide range of applications in isocratic LC, although it has not yet been utilized on a regular basis.

C. Phase Transformation Detectors

The phase transformation detector (also known as the "sample transport" or "moving wire") detector has been commercially available for several years. The transport principle has been described by Scott and Lawrence[56] and is il-

lustrated in Figure 20. The column effluent is passed over a continuously moving platinum wire, which is delivered from a feed spool. Both solvent and solute from the column are thus coated on the wire in the coating block. The wire then transports solvent and solute to an evaporator oven, which selectively removes the solvent. The solute remaining on the wire is then oxidized in a high-temperature oxidizer oven (850°C) to carbon dioxide. The CO_2 is mixed with H_2 and passed over a nickel catalyst at high temperature, where reduction to methane takes place. The methane is detected by means of a conventional GC flame ionization detector.

The phase transformation detector is excellent for gradient elution since its operation is independent of the nature of the moving phase. It is also an excellent detector for quantitative work since all organic compounds are detected as one substance (methane). This detector also has been reported to have a very wide linear dynamic range.[56] The disadvantages are its high cost, its complexity, and its low sensitivity. This detector has the obvious potential for achieving LC-mass spectrometry interfacing, and it will no doubt undergo many improvements in the near future.

D. Fluorescence Detectors

The use of fluorescence as a technique for monitoring high resolution LC columns is rapidly gaining in popularity. The fluorescence detector has advantages, namely, selectivity for certain classes of compounds and the capability to detect traces of materials (about 10^{-9} g).[57] Fluorescence suffers from vulnerability to interfering processes such as quenching, solvent fluorescence, and turbidity. This method of detection, however, seems particularly well suited for monitoring such biologically active materials as proteins, porphorins, vitamins, plant pigments, and fluorescent derivatives of amines and amino acids. Several manufacturers now supply flow fluorimeters. These instruments are designed similarly to filter fluorimeters and usually are equipped with a reference cell to correct for background fluorescence. Cell volumes are very small (ca. 10 μl), and the excitation and fluorescence wavelengths can be selected by the use of appropriate filters.

Fluorescence detection in LC has been used very successfully for monitoring fluorescent derivatives formed in post-column reactors. The concept of forming a reaction product immediately after column elution and prior to the detector has been used primarily in the separation and determination of biologically active components in physiological fluids.

An oxidative-fluorescence LC detection system based on the production of cerium (III) fluorescence has been reported by Katz and Pitt[58] and has been evaluated for monitoring aromatic acids by Katz, Pitt, and Jones.[59] In this method the column effluent is mixed with 10^{-4} *M* Ce(IV) dissolved in 1 *M* sulfuric acid. The mixed stream is then passed through a heated reaction bath and

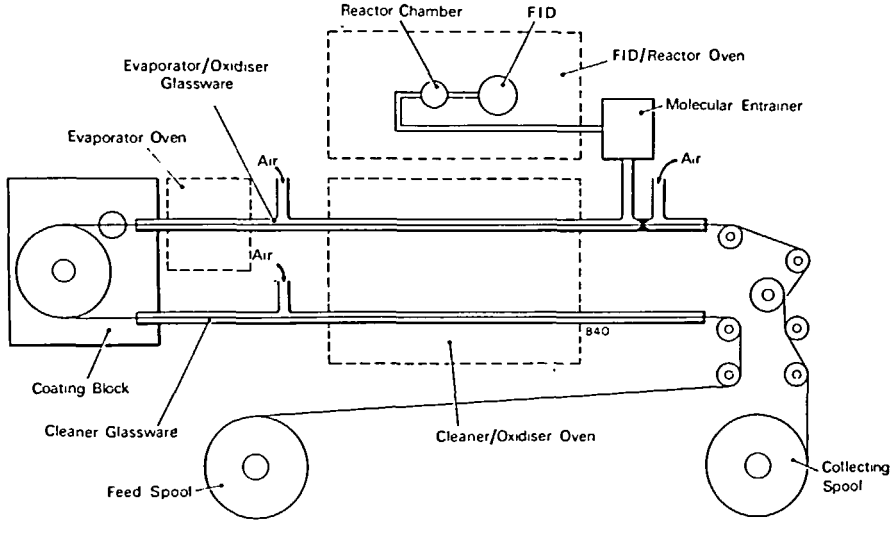
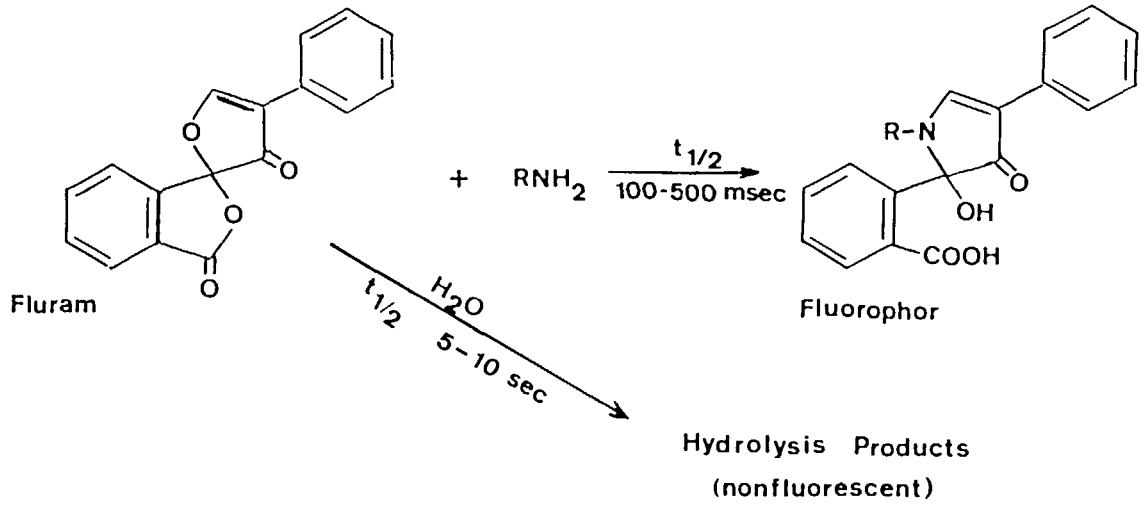


FIGURE 20. Phase transformation (moving wire) detector. (From Veening, H., *J. Chem. Educ.*, 50, A481 (1973) and Pye Unicam Ltd. With permission.)

then to the fluorimeter, where the fluorescence of cerium (III) is monitored. The flow system for this detector is shown in Figure 21. This "reaction detector" is very versatile and responds to any organic compounds which are oxidizable by Ce(IV) under the given conditions. This detector is much more sensitive to many aromatic acids, and it has been used in series with a UV detector for monitoring such acids in urine. A typical UV-fluorescence liquid chromatogram for urine is shown in Figure 22. It can be seen that the two detectors complement each other in detecting these sample components.

A relatively new reagent, "fluorescamine" (or Fluram®), has been used successfully for producing fluorescent derivatives with primary amines and amino acids. This reagent, which was first reported by Udenfriend et al.,[60] is nonfluorescent but reacts very rapidly at pH 9 with primary amines and amino acids, separated by ion exchange chromatography, to form highly fluorescent derivatives (390 nm excitation, 475 nm emission), while the excess reagent is hydrolyzed to give nonfluorescent products. The reaction between fluorescamine and primary amines is shown below.



The reagent has been demonstrated to be two orders of magnitude more sensitive for the detection of picomole quantities of amino acids than the standard ninhydrin procedure.[61] Fluorescamine has also been used for separating and detecting polyamines in physiological fluids by gradient cation exchange chromatography.[62] In this case the aqueous column effluent (pH 11 to 12) was first mixed with a continuous stream of 0.1 *M* boric acid in order to buffer the pH to values between 9 and 10. This mixture was then fed to a mixer where a solution of reagent (Fluram in acetone) was introduced. The resulting fluorophors, formed from the reaction between the polyamines and the reagent, were then detected with a flow fluorimeter. For several of the polyamines, the lowest detectable quantity ranged from 50 to 100 ng.

Samejima has used fluorescamine as a derivatizing agent for several polyamines *prior* to liquid chromatography.[63] The reaction was carried out in aqueous borate solution at a pH of 8.0. The derivatized sample was injected directly into a reverse-phase LC column. The moving phase was 0.1 *M* sodium borate buffer at pH = 8.0, and a Vydac reverse-phase packing was used in the column. The eluted fluorophors were detected by means of a spectrofluorimeter. Excellent separations of various derivatized polyamines were obtained using this method. This method is more efficient and faster than the ion exchange procedure, although it was not applied to physiological fluids.

While the fluorescence detection system utilizing the fluorescamine method is very sensitive for many of the amino acids and amines, the sensitivity appears to decrease as the number of amine functional groups in the molecule increases.[62]

E. Other Detectors

In addition to the detectors already described, a number of others have been reported and are presently in the developmental stage.

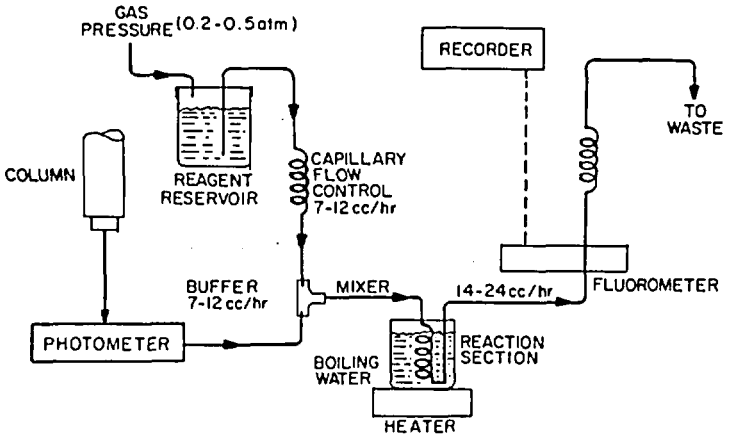


FIGURE 21. Flow system for the cerate oxidative fluorescence detection system. (From Katz, S. and Pitt, W. W., Jr. *Anal. Lett.*, 5, 177 (1972). With permission.)

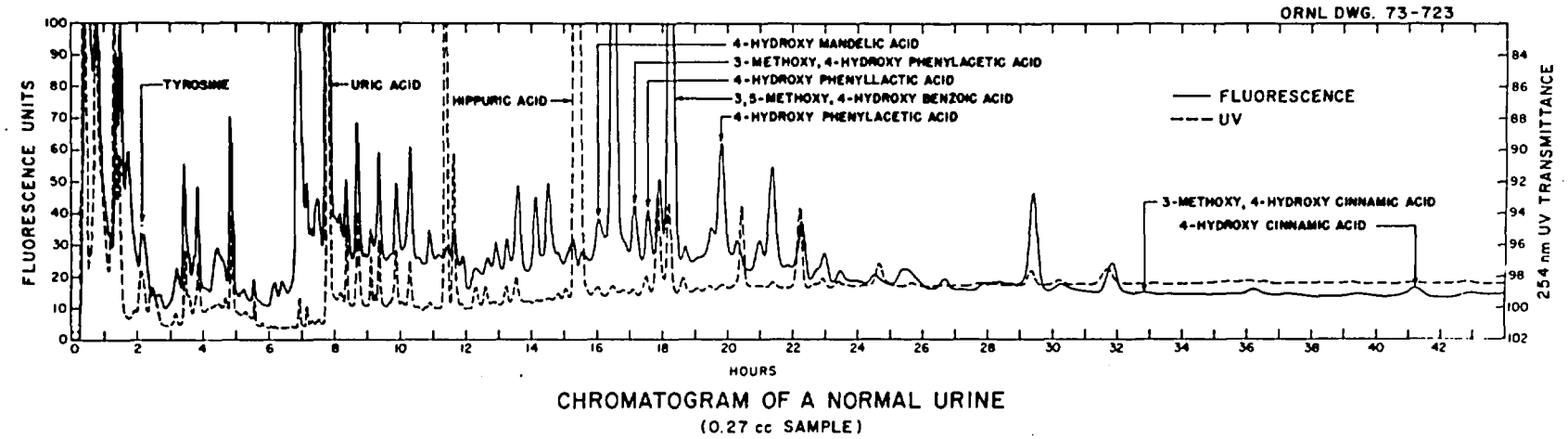


FIGURE 22. Liquid chromatogram of human urine using a UV detector and the cerate oxidative fluorescence detector in series. (From Katz, S., Pitt, W. W., Jr., and Jones, G., Jr., *Clin. Chem.*, 19, 817 (1973). With permission.)

Radiometric detectors have not been used extensively. Their useful features include adaptability to gradient elution and the fact that they are nondestructive. Their applicability is, however, limited. Utilization of beta radiation using suspended scintillators in continuous flow measurement has been reported.[64] The theory and application of radiometric detectors have also been evaluated by van Urk-Schoen and Huber.[65] The detector was tested with respect to peak broadening, sensitivity, linearity, precision, and detection limit, and it was shown to be very suitable for high speed LC.

Polarographic detectors are not presently commercially available, although they have been used and reported. The design of a high speed LC polarographic detector has been described in a paper by Huber et al.[66] The detector consists of a fixed dropping mercury electrode (DME) in a cylindrical space (1 mm in length and 1 mm in diameter) through which the column effluent flows. A mercury pool was used as the non-polarizable electrode. Downstream from the DME there is a vertical side channel which facilitates the removal of gas bubbles from the detector cell. Since current varies widely over the lifetime of a drop, it is necessary to damp the signal by means of RC-circuits. A diagram of the polarographic detector is shown in Figure 23. This detector is particularly useful for the detection of reducible substances such as metal ions, pesticides, alkaloids, aldehydes, and ketones. This detector was used successfully for the LC determination of traces of pesticides such as parathion and methylparathion in agricultural crops.[67] Joynes and Maggs developed a polarographic detector, the design of which was based on the properties of a carbon-impregnated silicone rubber membrane as an electrical conducting medium. The detector responds to a range of inorganic and organic compounds.[68]

Coulometry is gaining in interest as a potential method for LC detection. Takata and Muto[69] have developed a constant potential coulometric LC detector capable of detecting metal ions, halides, amino acids, carboxylic acids, phenols, and sugars separated by ion exchange chromatography. Primary, secondary, and indirect coulometry was employed for the detection of 5×10^{-7} to 5×10^{-10} mol of these materials. Flow rates as large as 6 ml/min could be used with a detector efficiency of more than 99.5%. Also,

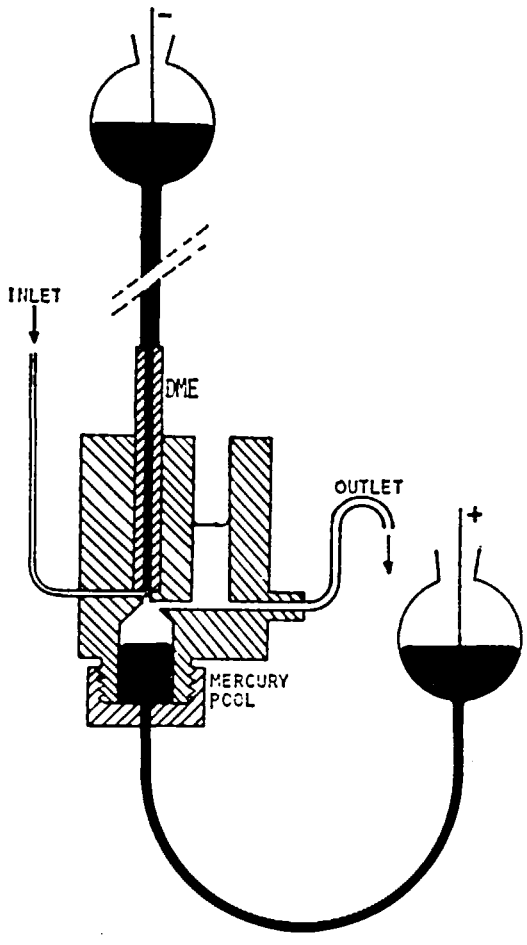


FIGURE 23. Polarographic LC detector. (From Koen, J. G., Huber, J. F. K., Poppe, H., and den Boef, G., *J. Chromatogr. Sci.*, 8, 192 (1970). With permission.)

Johnson and Larochelle[70] have developed a coulometric LC detector consisting of a tubular platinum electrode packed with small chips of platinum. This detector has been shown to operate with 100% efficiency and was applied to copper and iron determinations. Taylor and Johnson[71] have also used the tubular platinum coulometric detector for the LC determination of antimony. Davenport and Johnson have used a cadmium tubular electrode detector in a forced flow LC determination of nitrite and nitrate.[72]

Kissinger, Felice, Riggin, Pachla, and Wenke[73] have reported a new thin-layer electrochemical cell which can be used as a high resolution LC detector. The surface of the thin layer serves as the working electrode and the other two electrodes are an auxiliary and a reference (Ag/AgCl). This detector has been successfully applied to the

detection of traces of several pharmaceuticals and electroactive drugs in physiological fluids.

A new type of detector based on the volume change accompanying the sorption and desorption of ions on a strip of ion exchange membrane has been reported by Gilbert and Dobbs.[74] A DuPont Model 941 Thermomechanical Analyzer was used in conjunction with a Model 900 Thermal Analyzer to measure changes in linear dimension of the membrane. The device was evaluated for the detection of alkali metals and inorganic anions separated by ion exchange chromatography.

Bakken and Stenberg[75] have adapted an interferometer as a detector for LC. The detector was designed and constructed to focus the central portion of the fringe pattern into a photocell; the signal so produced was transferred into an electronic recorder. The recorder trace is cyclical and the number of cycles is proportional to the quantity of compound eluted. Ideal solvents for optimum results are those whose refractive indices are well removed from those of most organic compounds. The detector was used for the separation of several organic compounds. The authors noted that the detector cannot be used with gradient elution.

A unique mass detector for LC was reported by Schulz and King.[76] In this detector the column effluent is sprayed on a pizoelectric quartz crystal surface, the solvent is evaporated, and the mass of residual solute is determined from the change in crystal frequency. Sampling of the liquid stream and solute deposition occur at rapid intervals. The sensitivity of this detector was observed to be similar to other commonly used LC detectors. The mass detector was used in the separation of polymers separated by gel permeation chromatography. This detector would appear to be potentially useful and universally applicable. It is nondestructive and independent of flow and pressure, and it is potentially useful for gradient elution. A limitation is that solvents used must be farily volatile.

Recently a "Spray Impact" detector was reported by Mowery and Juvet.[77] The principle of this detector is based on the fact that violent rupturing of a stream of mobile phase at a target electrode creates a baseline current which changes upon elution of an organic or inorganic compound. A number of compounds such as fatty acids, bile acid salts, metal chelates, alkylsulfonates, amino acids, amines, and inorganic salts were detectable in the 10^{-9} to 10^{-11} g/sec range. The detector's operation during reverse-phase chromatography (using water as solvent) has also been studied. This very sensitive detector also offers many potential uses in LC.

VI. APPLICATIONS

There are hundreds of applications of liquid chromatography to many different types of chemical separations which have been published in the literature during the last few years. No attempt will be made to include a complete coverage of all of these applications; however, several examples of high resolution liquid-liquid, liquid-bonded phase, liquid-solid, and ion exchange chromatography will be cited. Specific uses of ion exchange chromatography in clinical chemistry will be presented in some detail.

A. Liquid-Liquid Chromatography

In liquid-liquid chromatography (LLC), the stationary liquid which is impregnated on the solid support must be immiscible or slightly miscible with the eluent. The polar liquid is usually coated on the support, and the nonpolar liquid is used as the eluent. If the roles of the two are reversed (i.e., the nonpolar liquid is stationary), then the separation is known as reverse-phase LLC.

Huber has described the criteria for the choice of binary and ternary liquid-liquid systems.[7] Martire and Locke have described the thermodynamic basis for selectivity in LLC.[78] Binary systems which can be used include water/pentanol, water/4-methyl-2-pentanone, water/aniline, water/butanol, 1,2-ethanediol/4-methyl-2-pentanone, and many others. For these solvents the mutual solubility can be taken as a measure of the polarity difference between the phases. The polarity difference between the two phases determines the partition coefficient, and if this difference is too large, the sample components will distribute unevenly. If the polar phase is chosen to be stationary, a hydrophilic solid support such as diatomaceous earth must be used. On the other hand, a hydrophobic solid support such as silanized diatomaceous earth must be used if the stationary phase is nonpolar.

Ternary liquid-liquid systems are obtained from binary liquid-liquid systems by adding a third component which is completely miscible with both phases of the binary system. One ternary

two-phase system which has been found to be extremely useful is water, ethanol, and 2,2,4-trimethylpentane. The three-component phase diagram for this ternary system is shown in Figure 24. The shaded area on the diagram represents the two-phase region. The area above the equilibrium line represents a one-phase homogeneous solution. The equilibrium line is composed of two branches which represent the equilibrium composition of the two phases. The branches meet one another at the *critical mixing point (plait point)*, at which the entire system becomes homogeneous. At this point the partition coefficient for sample components becomes 1. If this equilibrium line is known, it is convenient to prepare the two phases separately in order to minimize time of preparation of the system. Partition coefficients in a ternary system can be varied over a wide range by changing the overall composition of the system; however, the more the two phases become similar, the greater the mutual solubility of the two phases and the greater the chance for removal of the stationary phase from the column. If, on the other hand, the partition coefficient becomes very large, the detection of sample components becomes very difficult. Selectivity factors for a number of different types of components have been determined in this ternary two-phase system. Huber, Meijers, and Hulsman[79] and Menheere et al.[80] have reported a method for predicting partition coefficients in six ternary liquid-liquid systems (water, ethanol, and 2,2,4-trimethylpentane).

Huber, Hulsman, and Meijers have shown that the ternary liquid-liquid system water, ethanol, and 2,2,4-trimethylpentane can be utilized for the rapid column liquid-liquid chromatographic determination of estrogens in urine.[81] Chromatograms for a 50-ml pregnancy urine and for a test mixture of estrone, estradiol, and estriol are shown in Figure 25. Huber, Kolder, and Miller[82] studied a number of binary and ternary liquid-liquid systems (polyethylene glycol/2,2,4-trimethylpentane; polyethylene glycol/2,2,4-trimethylpentane and CCl_4; and water, ethanol, and 2,2,4-trimethylpentane) for the LLC separation and determination with UV detection of nonionic surfactants in water

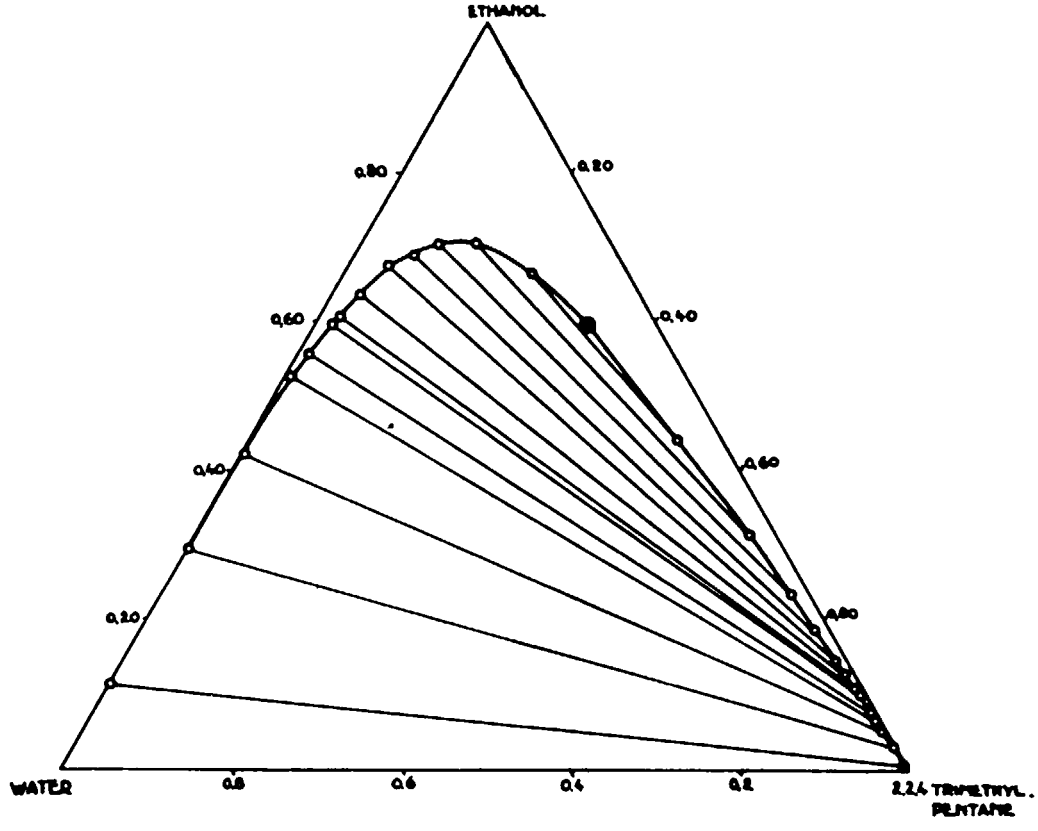


FIGURE 24. Liquid-liquid equilibrium phase diagram of the ternary system water, ethanol, and 2,2,4-trimethylpentane. (From Huber, J. F. K., *J. Chromatogr. Sci.*, 9, 72 (1971). With permission.)

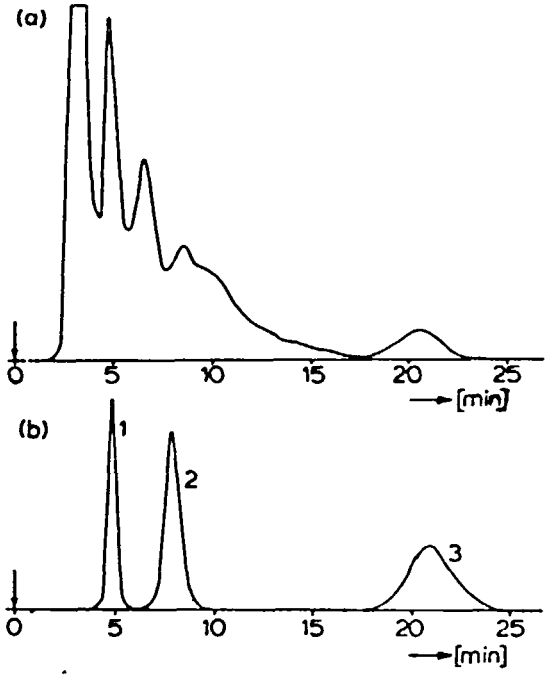


FIGURE 25. Chromatograms (a) from 50 ml of pregnancy urine after hydrolysis and extraction and (b) from a test mixture of estrone (1), estradiol (2), and estriol (3). Column: 50 x 0.27 cm; liquid-liquid system water, ethanol, 2,2,4-trimethylpentane. Sample volume 50 μl. (From Huber, J. F. K., Hulsman, J. A. R. J., and Meijers, C.A.M., *J. Chromatogr.*, 62, 79 (1971). With permission.)

pollution studies. Adducts varying in chain length from 1 to 20 ethylene oxide units were separated, and a series of commercial products were characterized.

The ternary liquid-liquid system (water, ethanol, and 2,2,4-trimethylpentane) was also used successfully for the LLC separation of a series of metal-β-diketonates by Huber, Kraak, and Veening.[53] The metal chelates were monitored with UV detection and included the acetylacetonates and trifluoroacetylacetonates of Be(II), Al(III), Cr(III), Fe(III), Co(II), Co(III), Ni(II), Cu(II), Zn(II), Zr(IV), and Ru(III). A number of equilibrium compositions of the ternary liquid-liquid system were investigated. The one found to be the most satisfactory contained 34.3% water, 64.1% ethanol, and 1.6% 2,2,4-trimethylpentane by weight in the more polar (stationary) phase, and 0.7% water, 2.2% ethanol, and 97.7% 2,2,4-trimethylpentane in the less polar (moving) phase. Interesting but anomalous results were obtained for the aluminum acetylacetonate, Al $(AA)_3$, chelate as shown in Figure 26. Curve 1 represents the peak produced when a freshly prepared solution of $Al(AA)_3$ in the stationary phase was injected into the column. Curves 2 and 3 represent the elution profiles for 1-week and 2-week-old solutions of $Al(AA)_3$, respectively. It was specu-

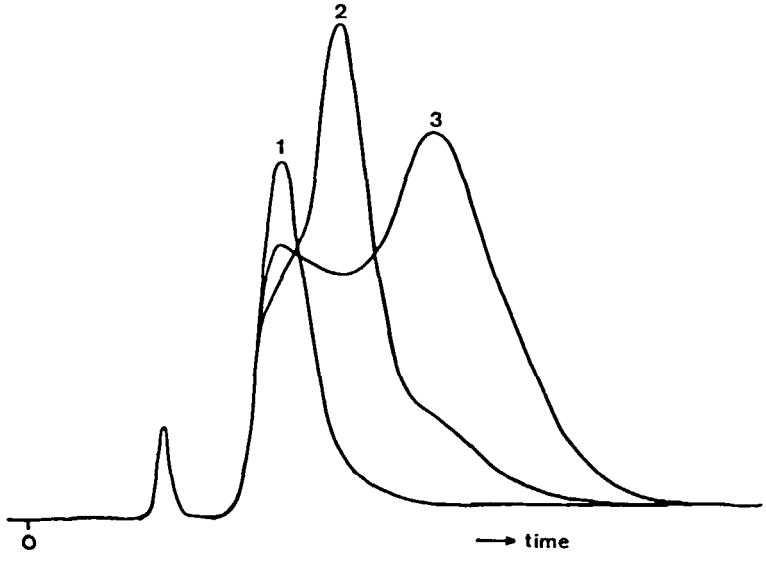


FIGURE 26. Successive elution profiles for $Al(AA)_3$. Curve 1: fresh solution of $Al(AA)_3$; Curve 2: 1-week-old solution of $Al(AA)_3$; Curve 3: 2-week-old solution of $Al(AA)_3$. (From Huber, J. F. K., Kraak, J. C., and Veening, H., *Anal. Chem.*, 44, 1554 (1972). With permission.)

lated that the aluminum chelate underwent hydrolysis in the stationary phase on standing, thus producing what were apparently three different species (perhaps mixed hydroxy-acetylacetonatoaluminum(III) complexes). These species were found to separate on the column. Dissociation and hydrolysis were apparently responsible for these results, and these effects became more severe as the water content of the stationary phase increased. Undesirable dissociation reactions were also observed for several other metal complexes. The suppression of these side reactions was accomplished by adding a trace of acetylacetone ligand to the moving phase. The amount of ligand was such that at equilibrium there was 0.8% in the moving phase and 0.2% in the stationary phase. A number of metal acetylacetonate separations were carried out successfully using the ternary liquid-liquid system plus a trace of ligand. Figure 27 shows a rapid LLC separation of six metal acetylacetonates achieved in 25 min.

Twenty-four different liquid-liquid systems (including two-phase systems composed of water/octanol, water/heptane, water/cyclopentane-2-aminopropane, water/ethanol, and 2,2,4-trimethylpentane-2-aminopropane and several acetonitrile two-phase systems) were evaluated by Muusze and Huber.[83] The distribution coefficients of these compounds were determined by a static method, and the influence of pH was studied.

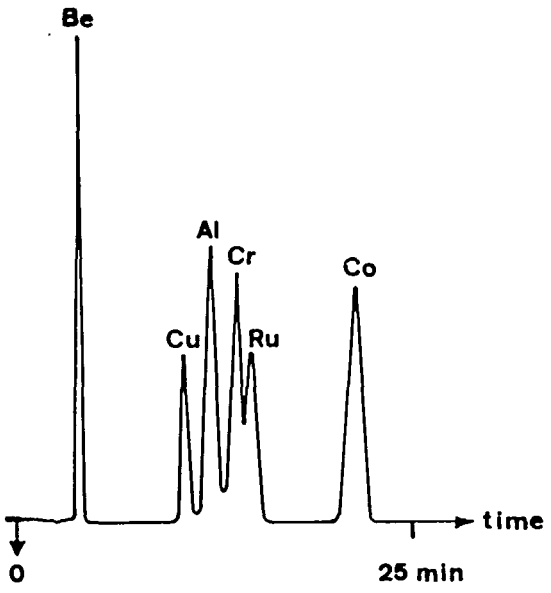


FIGURE 27. Separation of six metal acetylacetonates. *Column:* 500 × 2.7 mm; *particle size:* 5 to 10 μm; *fluid velocity:* 1.8 mm sec^{-1}; pressure drop: 17 atm; theoretical plate number for $Co(AA)_3$: 2,200. (From Huber, J. F. K., Kraak, J. C., and Veening, H., *Anal. Chem.*, 44, 1554 (1972). With permission.)

It has been shown by Eon, Novosel, and Guiochon[84] that the interfacial tension between the mobile and the stationary liquid-liquid phases reflects the partition properties of the two phases and can be used as a criterion in choosing phases for liquid-liquid chromatography. Several water/organic solvent liquid-liquid systems were studied. Organic liquid phases included amyl acetate, olive oil, nitrobenzene, 1:1 nitrobenzene-toluene, toluene, cyclooctane, 1:1 heptane-toluene, and heptane. Plots of the logarithm of the partition coefficient *versus* interfacial tension between the mobile and the stationary phases showed good linearity and permitted a judicious choice of the composition of the mobile phase for a given separation.

In conclusion, the choice of a suitable liquid-liquid system is still largely empirical; however, there are presently some guidelines available for the many different types of liquids which can be used. The number of possible applications for LLC appears to be unlimited. An advantage of LLC is that columns can be prepared in a very reproducible manner once the composition of the two phases is known. This is often not the case in liquid-solid chromatography, where the partition coefficients of the components depend on the surface activity of a chosen batch of solid support used as the packing. Another advantage of LLC is the inherent versatility of ternary two-phase systems. The polarity of either phase can usually be adjusted by changing the content of one of the three components. A major disadvantage of LLC is that column lifetime is relatively short because of the loss of stationary phase via dissolution in the eluent. A pre-column must therefore be used in order to minimize this problem.

B. Liquid-Bonded Phase Chromatography

In order to prevent the problems of LLC, i.e., stripping of the stationary liquid from the solid support by the eluent, stationary phases can be chemically bonded to the solid support. This type of liquid chromatography is neither liquid-liquid nor liquid-solid, but rather a composite of both. In liquid-bonded phase chromatography (LBC), separation takes place due to interactions between the sample components and the chemically bonded stationary liquid. Liquids can be bonded

to the solid support by reacting the stationary phase with the support material. Pryde has written an excellent review on LBC.[85]

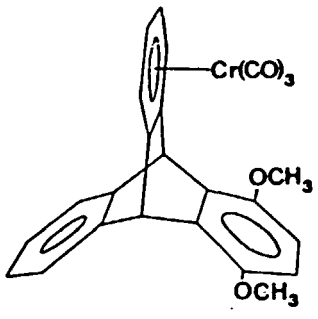
The first reported sample of LBC was reported by Halász and Sebestian.[86] Silica chloride was esterified with 3-hydroxypropionitrile, $HO(CH_2)_2CN$. This produced the bonded phase: $\geq SiO(CH_2)_2CN$. The cyano groups can thus interact with sample components. One of the problems with these initially esterified bonded phases was the fact that they readily hydrolyzed. More recently, Kirkland and DeStefano[87,88] have prepared a series of chemically bonded silicone packings. This was done by hydrolysis of alkoxysilanes, followed by partial polymerization of the silanols formed and bonding of the polymer to the support. Complete cross-linking was then achieved by heating. These phases were bonded to a "Zipax" controlled surface porosity material. A number of bonded-phase supports are presently commercially available; these include such bonded groups as β,β'-oxydipropionitrile, octanol, polyethylene glycols (carbowaxes), phenyl groups, $-NH_2$ groups, and cation and anion exchange resins. A discussion of the theory relating to chromatographic retention on bonded phases will not be included here. This topic has been covered in a number of papers.[16,89-92] LBC can be applied to almost any type of LC separation, and a variety of examples from the literature involving the separation of steroids, vitamins, estrogens, nucleotides, dyes, and metal complexes are cited by Pryde.[85]

In reviewing the literature it is clear that bonded-phase supports are very powerful and extremely useful LC column packings. There are many more examples in the literature involving LBC than LLC. The reasons for this are obvious: The chemically bonded supports can provide excellent resolution, they possess thermal and solvolytic stability, there is no need for preequilibration of two phases, the bonded phase is permanently attached to the solid (so that it cannot be stripped off), and it is not necessary to use a pre-column.

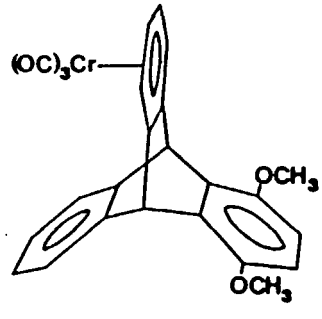
C. Liquid-Solid Chromatography

In liquid-solid chromatography (LSC) a chromatographic eluent flows through the column and past the surface of an active solid. Selective adsorption of the sample components on the active surface sites of the solid represents the basis for chromatographic separations. Generally LSC is very useful for many organic molecules containing a variety of functional groups. Olefins, aromatic hydrocarbons, aldehydes, ketones, esters, etc. can all be separated satisfactorily by LSC. Column packing materials can consist of silica, alumina, or charcoal. These materials can be either porous or pellicular. In the porous materials, the pores extend through the entire particle, whereas the pellicular packings contain a solid inlet core with a porous surface. Pellicular solids do not have as high a sample capacity as the porous materials; however, they provide excellent resolution. Many of the commercially available LSC packings have been listed by Snyder and Kirkland.[93]

One interesting application of LSC involves the separation of the isomeric chromium tricarbonyl complexes of 2,5-dimethoxytriptycene.[94] When 2,5-dimethoxytriptycene reacts with hexacarbonylchromium, two isomeric complexes are formed, as shown in structures I and II. The $Cr(CO)_3$ group always bonds on one of the nonsubstituted rings and can be oriented toward (I) or away (II) from the



I



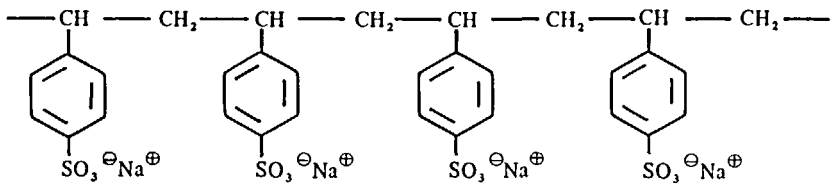
II

methoxylated ring. Figure 28 shows the separation and resolution achieved when a sample of the mixed isomers dissolved in benzene was eluted with 2,2,4-trimethylpentane on a 20-cm Corasil I (pellicular) column using UV detection at 235 nm. From preparative LC and NMR data it was shown that the first peak was due to isomer I and the second peak was isomer II. This same separation was also done using LBC (Corasil I – Carbowax-400), and it was found that the two isomers were well resolved; however, the analysis time was longer.

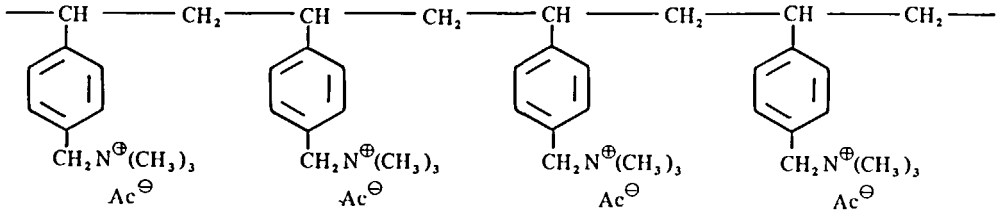
LSC is a very useful mode of operation; however, it is not applicable to very polar or ionic compounds due to severe tailing of such components. An advantage of the technique is the obvious elimination of the pre-column and the simplicity of packing and column preparation. Disadvantages are that identically packed columns do not always behave reproducibly, and sometimes it is possible to foul or poison a column by injecting substances which are permanently retained by the support. For a complete review of this topic, the reader is referred to a review by Snyder[95] on the role of the solvent in LSC and to the text by Snyder and Kirkland.[93]

D. Ion Exchange Chromatography

Ion exchange chromatography (IEC) represents an important mode of liquid chromatographic operation. Ion exchange packings are resins which contain fixed charges in an insoluble solid polymeric matrix. These charges are neutralized by a mobile counter-ion which can exchange with sample ions of like charge. The fixed charges may be anionic or cationic, and they are attached to a cross-linked polymeric backbone such as polystyrene. A typical strong acid cation exchange resin contains the polystyrene polymer with sulfonate groups attached to the aromatic rings. Strongly basic anion exchangers also contain the polystyrene structure; however, the functional group is a quaternary ammonium ion.



Strong acid cation exchange resin



Strong base anion exchange resin

In weakly acidic cation resins, the functional group is usually a carboxyl group attached to a cross-linked hydrocarbon chain. Weakly basic anion exchange resins are similar in structure to the strongly basic resins, except that the functional group consists of a primary or secondary amine, $-NH_2R^+Cl^-$ and $-NHR_2^+Cl^-$.

It is therefore possible to use acidic (cation) resins for the separation of cationic substances, for example, amino acids or amines in their protonated form. Conversely, it is possible to separate the anions of carboxylic acids and of phenolic compounds by basic (anion) exchange resins. In most ion exchange separations it is necessary to use gradient elution in order to resolve complex mixtures of organic or biochemical substances. An aqueous salt or pH gradient can be used to advantage in many instances.

Scott has reviewed ion exchange resins and their use in high resolution LC.[96] He has shown

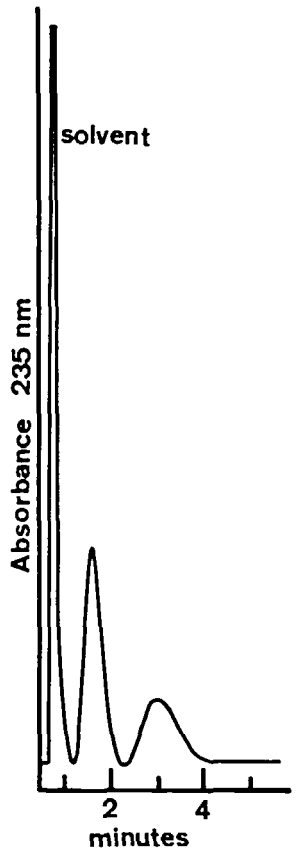


FIGURE 28. LC separation of the isomers of the chromium tricarbonyl complexes of 2,5-dimethoxytriptycene. Column: 200 X 2.3 mm Corasil I; temperature 28°C; sample size: 50 μg. (From Gardner, S. A., Seyler, R. J., Veening, H., and Willeford, B. R., *J. Organometal. Chem.*, 60, 271 (1973). With permission.)

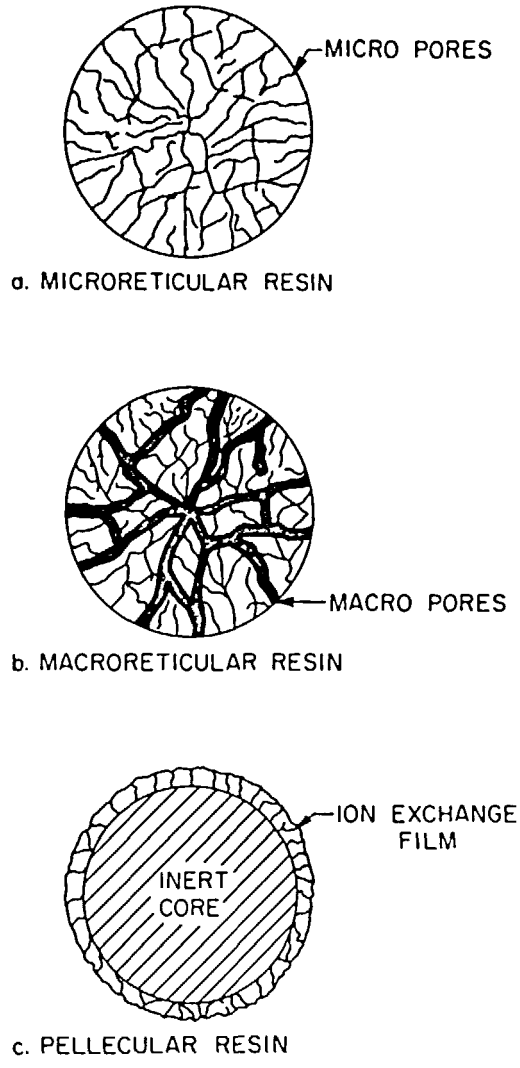


FIGURE 29. Structural types of ion exchange resins. (From Scott, C. D., in *Modern Practice of Liquid Chromatography*. Kirkland, J. J., Ed., Interscience, New York, 1971, 292. With permission.)

that structural differences are an important consideration in choosing a particular resin for a specific application. *Microreticular resins* contain cross-linked structural networks characterized by relatively small molecular size openings. They have a gel type of consistency. *Macroreticular resins* contain *micro* as well as *macro* pores; the latter can be several hundred angstroms in diameter. These resins have high porosities and render the particle accessible to large as well as small molecules. Finally, there are *pellicular resins,* where a solid inert core is surrounded by an ion exchange resin film. Pellicular resins are very useful for obtaining excellent resolution; however, their capacity is very low so that very small sample sizes must be used. The structures of these three different types are shown in Figure 29.

It has been shown by Burtis, Munk, and MacDonald[97] that it is also useful to use a pre-column in ion exchange LC in order to protect the resin in the column proper from becoming contaminated.

High resolution ion exchange liquid chromatography is now used routinely for the separation of biochemically active substances in physiological fluids. Much of this work has recently been reviewed by Scott.[98] The majority of the instruments which have been developed for this work are high resolution liquid chromatographs which can be used for the separation and determination

of acidic UV-absorbing components, carbohydrates, amino acids, and polyamines in physiological fluids. The advantage of using LC instead of GC in these separations is that the samples are aqueous and therefore no special sample preparation is required since the mode of operation (IEC) is also aqueous. A disadvantage is that the ion exchange method is slow; nonetheless, a wealth of data can be provided. A number of LC units, designed for specific separations of certain families of compounds, will now be described.

1. UV Absorbing Components

The "UV-Analyzer," developed at the Oak Ridge National Laboratory,[99] is now used routinely in medical research facilities for the separation and determination of UV-absorbing constituents in physiological fluids. This system has been further developed to increase sample handling capacity by use of parallel columns.[100] The flow diagram for this instrument is shown in Figure 30. A special high-pressure multiple-column sample injection valve was designed for this instrument. A further development has been to operate the detectors in a differential mode to allow differential comparison between the separated constituents of two samples.[101] Differences between the urine of a normal subject versus that of a pathological subject are notable, as shown in Figure 31; furthermore, the effects of drug therapy can be studied by comparison of samples before and after treatment. In a modified version of the UV-analyzer, a UV detector is placed in series with the previously described cerate oxidative reaction detector, thus providing greater detection capability (see Figure 21).

2. Carbohydrates

An improved, automated, high resolution anion exchange analyzer has been developed for separating and determining carbohydrates in physiological fluids.[32] This liquid chromatograph is known as the "Carbohydrate Analzyer." The carbohydrates are separated on an anion exchange column as their borate complexes using a borate/boric acid buffer gradient. The eluted species can be detected photometrically by utilizing the color developed when the column effluent is mixed with

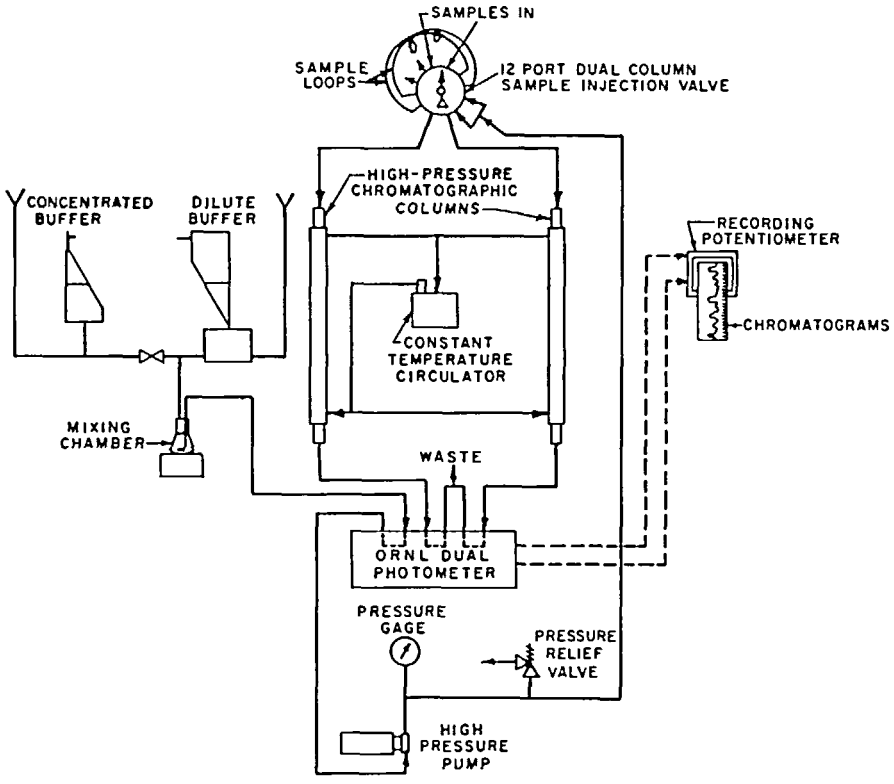


FIGURE 30. UV Analyzer utilizing parallel columns for differential LC. (From Scott, C. D. and Pitt, W. W., Jr., *J. Chromatogr. Sci.*, 10, 740 (1972). With permission.)

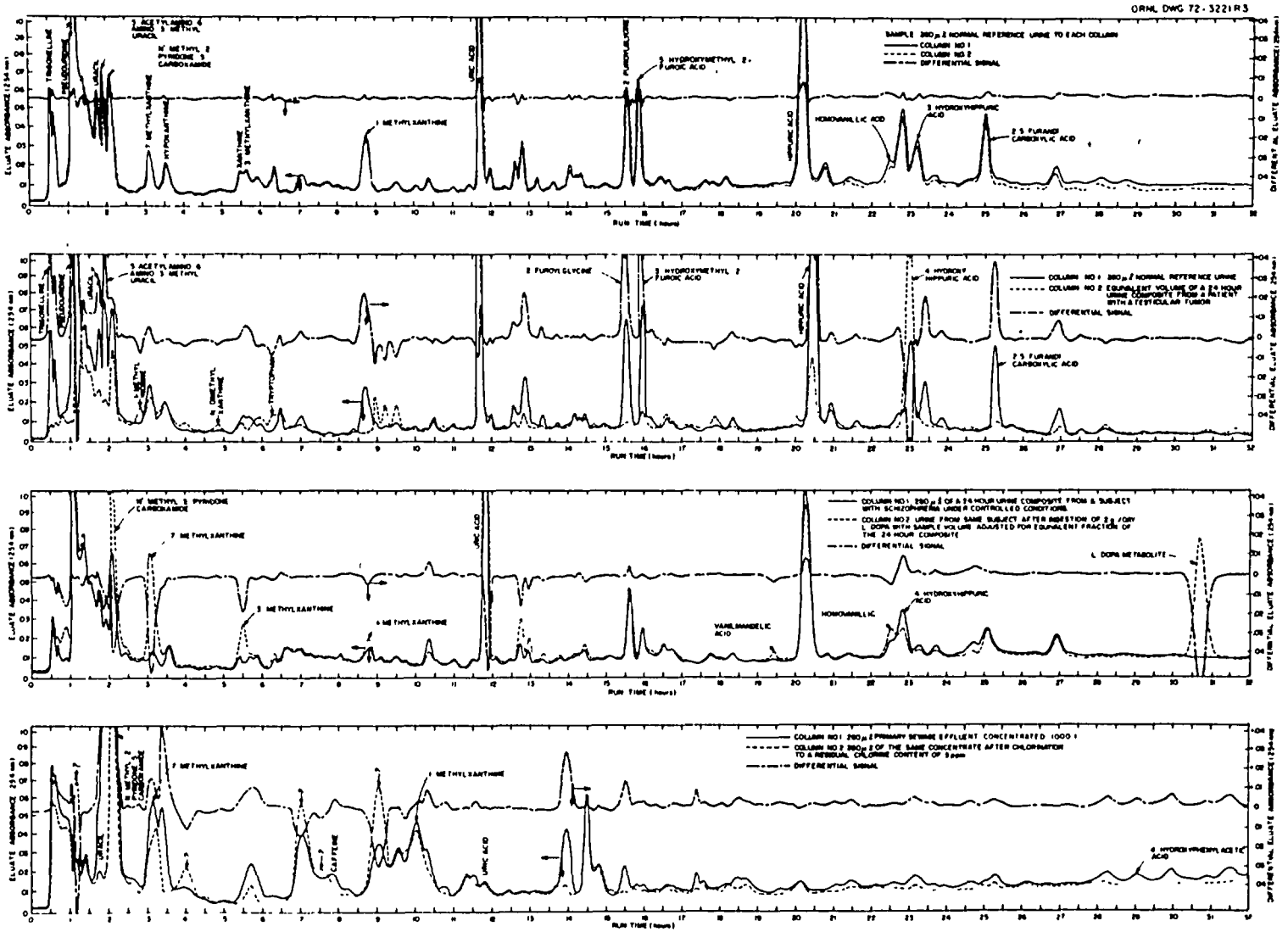


FIGURE 31. Differential liquid chromatograms of the UV absorbing molecular constituents in biochemical mixtures. (From Scott, C. D. and Pitt, W. W., Jr., *J. Chromatogr. Sci.*, 10, 740 (1972). With permission.)

phenol and sulfuric acid. Alternatively, the carbohydrates can be detected by utilizing the simplified UV photometric system described previously.[50] The flow system for this instrument is illustrated in Figure 32.

3. Amino Acids

High resolution ion exchange chromatographs can be designed specifically for performing the separation and detection of amino acids in physiological fluids. The amino acids are first separated on a cation exchange resin and are then chemically converted to chromophors or fluorophors which can be detected. The eluent usually used is sodium citrate buffer. Detailed descriptions of several available amino acid analyzers have been reported previously.[35]

4. Indoles

A liquid chromatographic system utilizing ion exchange resins has been described by Chilcote and Mrochek for the separation of nanogram quantities of indoles in physiological fluids.[102] A coupled column configuration was used in which an anion exchange column was directly connected to a cation exchange column. The indoles were eluted with an ammonium acetate-acetic acid buffer, and they were detected fluorimetrically

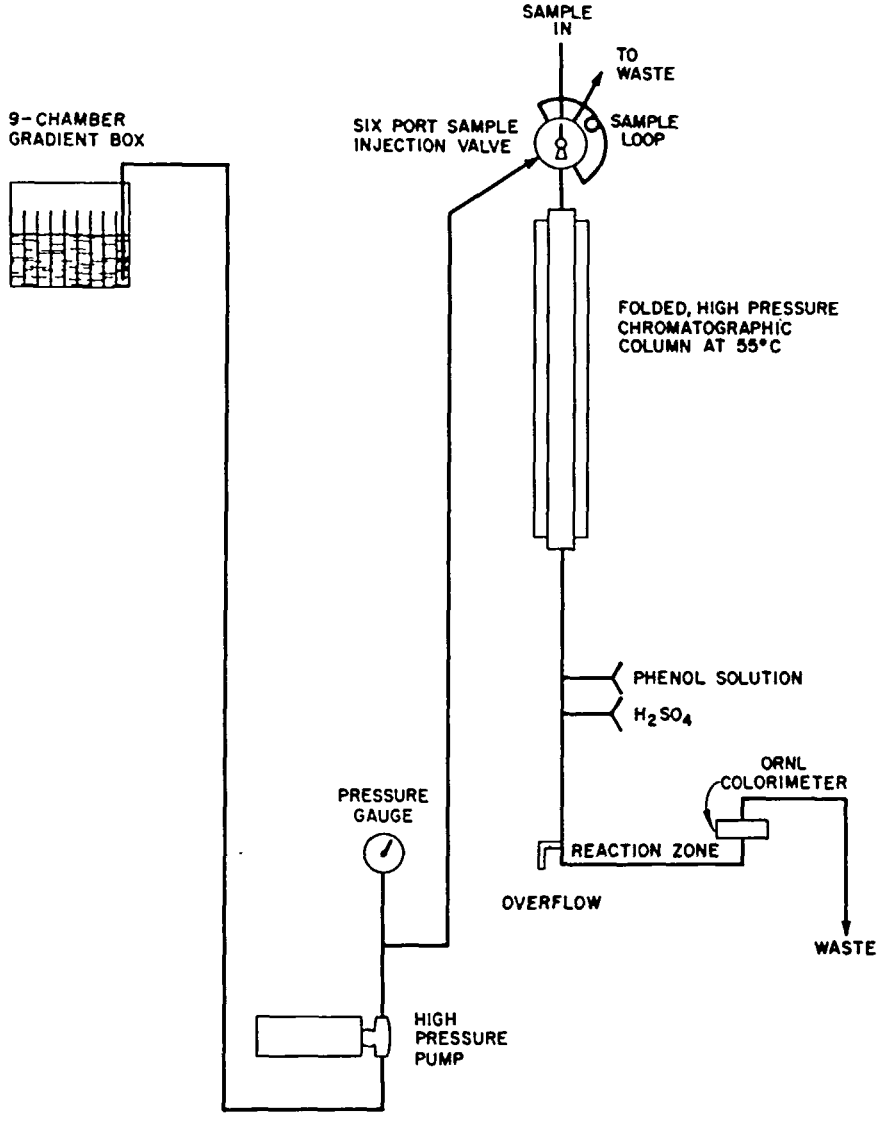


FIGURE 32. Flow diagram for the Carbohydrate Analyzer. (From Katz, S., Dinsmore, S. R., and Pitt, W. W., Jr., *Clin. Chem.*, 17, 731 (1971). With permission.)

using an excitation wavelength of 292 nm and an emission wavelength of 330 nm. Coupling the anion exchange column with the cation exchange column results in a much more effective separation in the front end of the chromatogram.

5. Polyamines

An ion exchange chromatographic procedure has been reported by Veening, Pitt, and Jones for the separation and fluorimetric detection of the urinary polyamines: 1,3-diaminopropane, putrescine, spermidine, cadaverine, and spermine.[62] Recent clinical experiments suggest that patients with active cancer may have elevated levels of these compounds in their urine or blood serum. The polyamines were separated on a 15- X 0.45-cm cation exchange column at 70°C using a combined pH-salt gradient. Separation conditions were designed to enable utilization of the previously described fluorophor-forming reagent, fluorescamine. The column effluent was first mixed with boric acid solution in order to buffer the stream to optimum pH levels for formation of the fluorescent derivatives. The buffered stream was then mixed with an acetone solution of fluorescamine reagent, and the fluorophors were detected by means of a fluorescence detector. The flow diagram for this liquid chromatograph is shown in Figure 33. Experiments were carried out to determine the effects of several variables such as pH, reagent concentration, and flow rate. The method was applied successfully to both normal and pathological samples of human urine.

VII. CONCLUDING COMMENTS

High resolution LC has been shown in this review to be a technique which offers a far greater choice of operating conditions, modes of operation, and methods of detection than GC. While high resolution LC is presently still a separation method which is complementary to GC, developments in the field indicate that LC will become more widely applicable than GC during the coming years. There are presently some 35 companies which either manufacture liquid chromatographs,

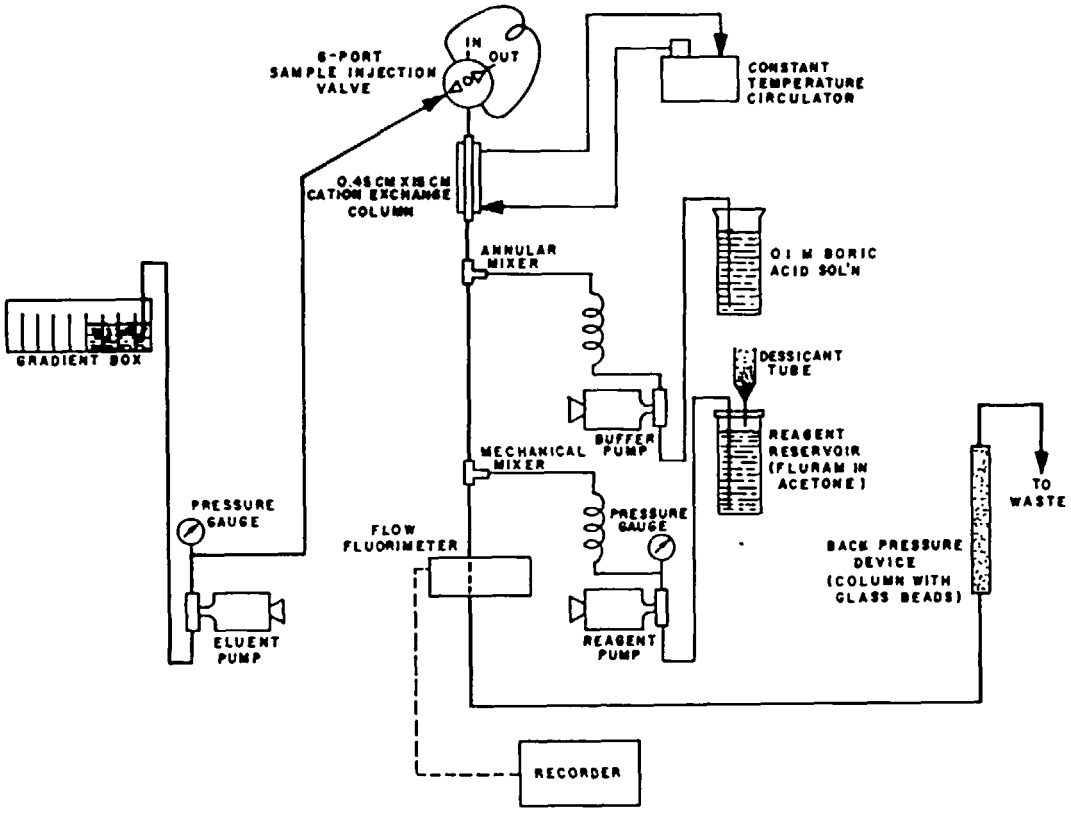


FIGURE 33. Ion exchange chromatograph for the separation and fluorescence detection of polyamines. (From Veening, H., Pitt, W. W., Jr., and Jones, G., Jr., *J. Chromatogr.*, 90, 129 (1974). With permission.)

column materials, detectors, or other component LC parts. This fact alone indicates the keen interest and activity which presently exists in LC. Detectors in LC have a tendency to be selective to certain classes or families of compounds. This is generally considered to be a disadvantage by many; however, the fact that many detectors can be used selectively and that they can discriminate against certain compounds may very well become a unique and useful feature. In the near future, LC will tend to be coupled with other instrumental methods, for example, mass spectrometry. While LC still will undergo many refinements and improvements in the coming years, the technique has already established itself as one of the most popular, versatile, and useful methods of separation.

VIII. ACKNOWLEDGMENT

The author wishes to thank the donors of the Petroleum Research Fund, administered by the American Chemical Society (PRF No. 3516-B), for partial financial support.

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