Separation & Purification Reviews
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597294

High-Pressure Ion Exchange Chromatography as Applied to the Separation of Complex Biochemical Mixtures

Charles D. Scott[a]
[a] Oak Ridge National Laboratory, Oak Ridge, Tennessee

To cite this Article Scott, Charles D.(1974) 'High-Pressure Ion Exchange Chromatography as Applied to the Separation of Complex Biochemical Mixtures', Separation & Purification Reviews, 3: 2, 263 — 297
To link to this Article: DOI: 10.1080/03602547408066028
URL: http://dx.doi.org/10.1080/03602547408066028

SEPARATION AND PURIFICATION METHODS, 3(2), 263-297 (1974)

HIGH-PRESSURE ION EXCHANGE CHROMATOGRAPHY AS APPLIED TO THE SEPARATION OF COMPLEX BIOCHEMICAL MIXTURES

Charles D. Scott
Oak Ridge National Laboratory[1]
Oak Ridge, Tennessee 37830

INTRODUCTION

Interest in liquid column chromatography, including ion exchange chromatography, as a separation method has increased markedly in the past few years. Numerous new automated analytical techniques, as well as applications for preparative or production-scale separations, have been developed. This has been particularly true in the areas pertaining to separation and analysis of biochemical mixtures such as physiologic fluids. The recent trend in ion exchange chromatography has been toward achieving two goals: high-resolution analysis in the case of complex mixtures, and high-speed separation when simpler mixtures are involved. In either case, the use of small-diameter ion exchange resin particles coupled with high flow rates (and, in some cases, long columns) requires operation at relatively high column inlet pressures, since the pressure drop through the ion exchange column is dependent on factors such as resin particle size, flow rate, and column length.

The primary emphasis in this presentation will be on ion exchange liquid chromatographic (LC) systems that have recently been developed to provide high-resolution separations necessary for the analysis of complex biological mixtures. Such systems can be valuable tools in the medical sciences where they are used to

analyze for literally hundreds of the molecular constituents in physiologic fluids. Similarly, progressively greater attention is being focussed on the use of such analytical systems for studying dissolved organic pollutants in water on a molecular basis, particularly in the effluents of sanitary sewage plants.

A whole family of such systems is now available. These include analyzers for UV-absorbing constituents,[2-5] amino acids and related compounds,[6,7] carbohydrates,[2,4,8] organic acids,[9,10] and others even more highly specialized.[11,12] These analyzers utilize high-pressure LC separation systems; in fact, much of the early development work in the field of high-pressure LC was done in this area.

ANALYTICAL SYSTEMS

Automated and semiautomated liquid chromatographic systems utilizing ion exchange separations have been widely used at relatively low pressures (less than 100 psi) for many years; these include amino acid analyzers, nucleotide analyzers,[13,14] and others. However, high-pressure operation (up to 5000 psi) is now also becoming common.

In general, automated chromatographs of both types contain the following major components: (a) the separations section, which consists of a closed tubular column packed with the solid sorbent; (b) a section for storing the eluent and preparing the gradient solution; (c) a system equipped to deliver the eluent to, and force it through, the separation column; (d) a means for introducing the sample or feed to the column; and (e) a means for detecting and quantifying the separated constituents in the column eluate (see Figure 1). Automated data acquisition and processing may also be used. Variations in the choice of separation media, operating conditions, and eluate monitoring techniques can result in different analytical systems that, although specific for a variety of molecular constituents, are basically similar.

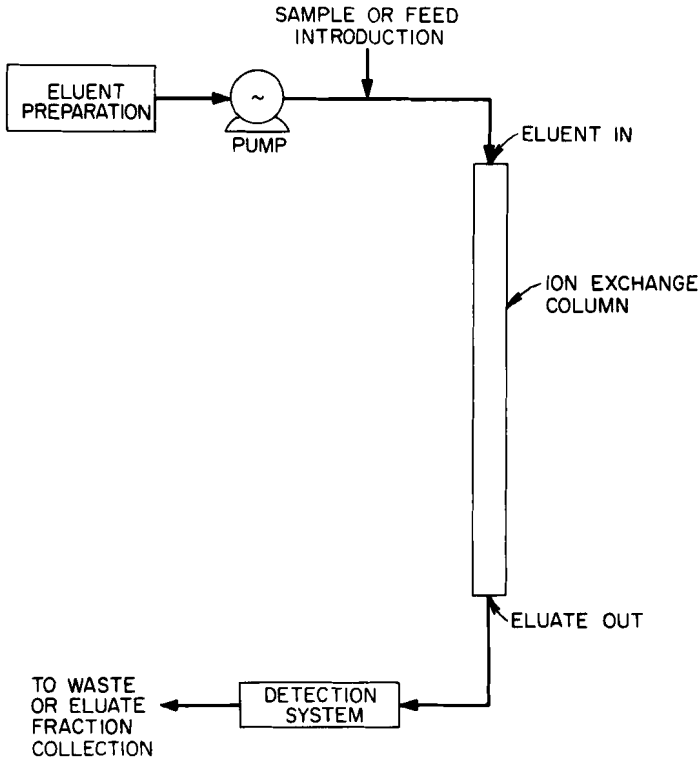


FIGURE 1

Components of an Ion Exchange Elution Chromatograph
(From Scott (4) with permission from Academic Press, Inc.)

Ion Exchange Separation Systems

The heart of any automated chromatographic system is the separation column. Recent advances in ion exchange chromatography have included the development of several new types of ion exchange resins. The aim in these developments has been to produce resins in which the solid-phase mass transport resistances are reduced. A reduction in these resistances will allow the chromatographic system to operate much closer to equilibrium conditions, and should result in more rapid as well as more effective separations.

Types of Ion Exchange Materials

All the systems under consideration here achieve high resolution by using relatively small particles of anion or cation exchange resin as the stationary sorption phase in chromatographic columns up to about 150 cm long. Most work has been done with microreticular resins, in which the sorption sites are located throughout the particle. However, of increasing importance has been the development of pellicular ion exchange resin in which a thin film of ion exchange material is bonded to the outer surface of an inert solid core. This material has the potential for achieving very rapid separations, especially in cases where sorption capacity is not important.

At present, many analytical ion exchange systems use resin particles with diameters of less than 40-μ, while the use of ~10-μ-diam resin is rapidly becoming commonplace. A diameter of about 2 or 3 μ probably represents the lower limit of useful size since particles smaller than this are almost certain to exist as colloidal suspensions.

Until recently, the smallest spherical ion exchange resin available was a nominal minus 400-mesh fraction containing particles in the 5- to 60-μ-diam range. To obtain quantities of the smallest resin, large portions of the as-received material had to be size-separated by elutriation.[15] Now, however, several manufacturers supply ion exchange resin in any desired size range down to a diameter of about 10 μ.

Structural Strength of Ion Exchange Resin

The structural strength of a particular type of ion exchange resin is an important consideration since high eluent flow rates may cause distortion or collapse of the particles, which can, in turn, lead to large pressure drops in the bed. Collapse of the particles is caused by the shear stress resulting from the force of the flowing liquid. These effects have not been observed for

pellicular resins or for resins that have a very high degree of cross-linkage.

As an example of such effects, one can consider the strongly basic anion exchange resins utilizing a polystyrene-divinlybenzene (DVB) matrix (Dowex 1). The degree of cross-linking is usually expressed as weight percent DVB in the polymerization mixture, and this characteristic has been used as the basis for studying the structural stabilities of these resins. Preliminary results with resin particles that were a nominal -400 mesh show that most resins can be used at low flow velocities; however, as the flow rate is increased, a point is reached where the individual resin particles begin to be distorted and the bed begins to collapse. When the flow rate is increased further, the collapse of the bed continues almost exponentially until it becomes catastrophic. Bed collapse, which can be qualitatively determined by measuring the pressure drop as a function of flow rate, is considered to have begun when the pressure drop becomes significantly greater than that which would be observed if there was a linear increase of pressure with flow velocity. This condition can also be correlated with the bed compression or decrease in resin volume (Figure 2).

If bed compression is small, the collapse is reversible, and the column can be reused. However, if the bed is allowed to become compressed to the extent that the pressure drop is more than about 10% higher than the extrapolated linear value, distortion continues and the bed becomes unusable. A desired flow velocity, then, would be a velocity that causes a deviation of 10% or less in the pressure drop. Suitable ranges of flow velocity for several Dowex 1 resins are shown in Table I. These data indicate that ion exchange resins with a cross-linkage of 10% or greater have sufficient structural strength for most chromatographic uses; those with less cross-linkage can also be used, but only at decreased eluent flow. No attempt was made to study the effect of particle size on collapse; however, qualitatively it was noted

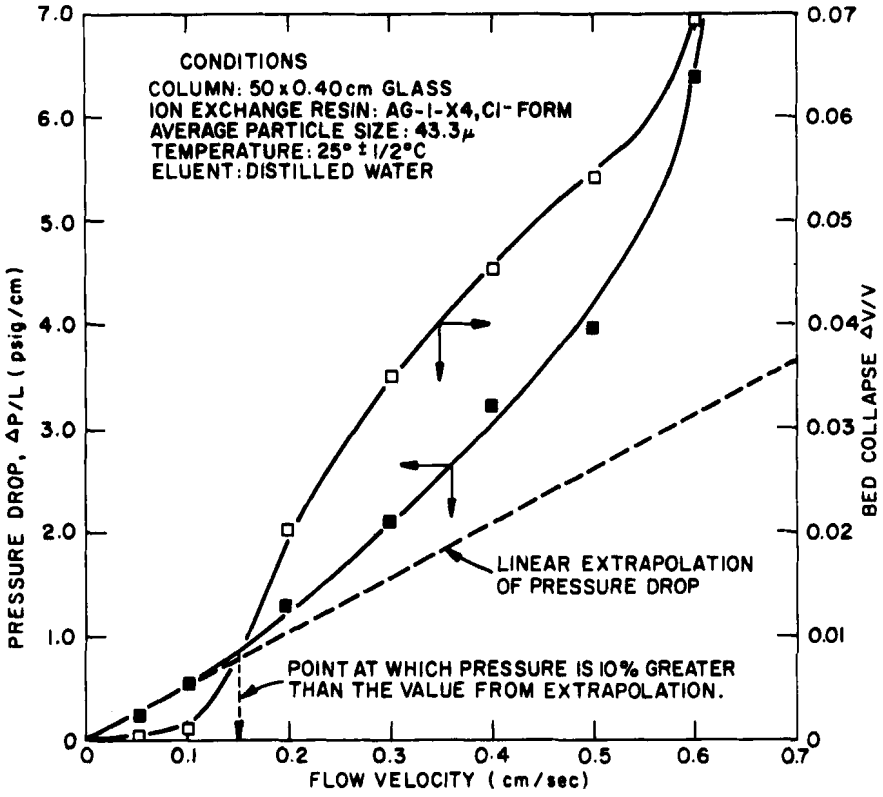


FIGURE 2

Effect of Eluent Flow Rate on Ion Exchange Resin Bed Collapse and Pressure Drop for a Typical Low-Cross-Linked Anion Exchange Resin

that one can operate with ion exchange beads of smaller particle size at greater flow rates before collapse.

Pressure Drop

The effects of column and operating parameters on the pressure drop of ion exchange chromatographic columns designed to operate at pressures less than 1000 psi can essentially be disregarded; however, such effects are very important in high-pressure chroma-

TABLE I

Maximum Flow Velocity for Several Dowex-1 Resins* Prior to Irreversible Bed Collapse

Nominal DVB Cross-linkage %	Maximum Flow Velocity** cm/sec
2	0.05
4	0.15
8	0.45
10	0.95

*Nominal minus 400-mesh resin.

**Eluent velocity at which the pressure drop is 10% higher than the linearly extrapolated value.

tography. For a particular type of ion exchange resin, the major parameters that influence the pressure drop across the column are: particle diameter, flow rate, column length, and fluid properties such as density and viscosity. These effects have not been thoroughly studied for small resin particles; on the other hand, previous data have shown that the pressure drop across an ion exchange column is inversely dependent on the square of the mean diameter of the ion exchange resin particles and linearly dependent on the flow velocity and the length of the column.[16] Obviously, a more general correlation, similar to the correlations derived in engineering studies of pressure drop in fixed beds of larger particles, would be useful. Typical of these is a relationship developed by Ergun,[17] who followed earlier workers in correlating a dimensionless flow parameter called the modified Reynolds number, N_{RE}, as follows:

$$f = 150 \frac{1 - \epsilon}{N_{RE}} + 1.75,$$

where

$$f = \frac{\Delta P}{L} \frac{g_c D_p}{\rho U^2} \frac{\epsilon^3}{1 - \epsilon},$$

$$N_{RE} = \frac{D_p U \rho}{\mu} ,$$

ΔP = pressure drop across the column,

L = length of the column,

g_c = gravitational constant,

D_p = average diameter of the spheres in the column,

ρ = density of the mobile phase,

U = superficial fluid velocity based on an empty column and an average pressure,

ϵ = void fraction = $\frac{\text{void volume}}{\text{geometric volume}}$,

μ = viscosity of the eluent.

Preliminary studies show that such a correlation is consistent within the same packed bed; the resulting plot of log f vs log N_{RE} gives a relatively straight line with a slope similar to that of the extrapolated Ergun relationship (Figure 3). Nevertheless, particle size seems to have some effect on the absolute values of the friction factor. Thus, in the case of very small particles, it may be necessary to consider particle size in the correlation.

Columns

Metal columns, which can be easily fabricated from seamless metal tubing, can be used for high-pressure techniques. Conventional compression tubing fittings can be used for the fluid entrance and exit, and for holding a porous metal support for the resin bed.[2] Although the use of precision-bore tubing may be slightly more advantageous, good results have been obtained with common seamless tubing. Temperature control can be achieved by enclosing the tubing in a heated oven or by using a heating jacket around the column with a controlled-temperature fluid circulator. The former method has been chosen by most of the commercial manufacturers of LC systems.

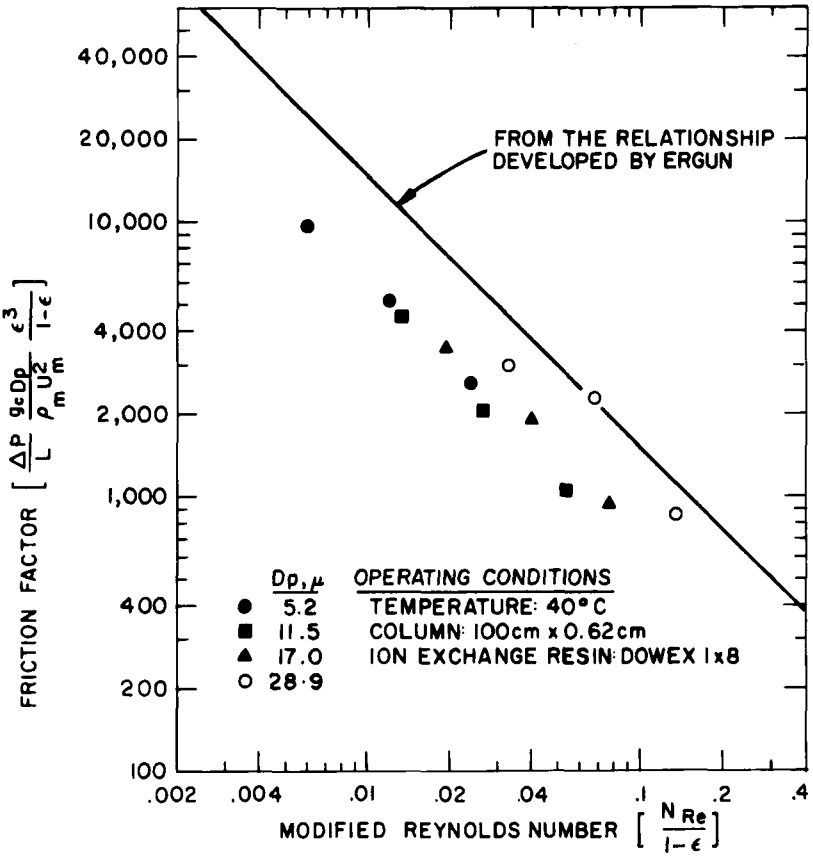


FIGURE 3

Pressure Drop in Fixed Beds of Small Ion Exchange Resin Particles

Column Geometry

The geometry of an ion exchange column has a significant effect on the resolution that is achieved. As the length of a column is increased, the separation of two components becomes more effective; however, the width of the chromatograph peaks is also increased. The diameter of the column should not have a great effect on resolution (assuming that comparable flow velocities and a proportionally scaled sample size are used) as long as it is

sufficiently small to prevent radial variations in fluid properties but not small enough to require a sample of such limited volume that the separated solutes cannot be detected by the column monitoring system. Columns for analytical systems usually have diameters in the range of 1 to 6 mm and lengths of 50 to 150 cm.

Packing Columns with Small-Diameter Resin

Although packing a chromatographic column with ion exchange resin requires little skill, it should be done with a reasonable amount of care. The major steps in the conventional method for packing a column with relatively large, closely sized ion exchange resin particles are: (a) preparing a slurry of the resin particles; (b) introducing the slurry into the column or a chamber attached to the column; and (c) allowing the resin particles in the slurry to settle via gravity. The supernate is removed when necessary, and additional slurry is added until the column is completely filled with resin.

This technique has distinct disadvantages when the sizes of the resin particles vary over a large range. The larger particles settle more rapidly, and the resulting bed is composed of longitudinal zones containing particles of various sizes. Also, radial variation in particle size occurs if the column is not held precisely vertical during the packing procedure. Such variations affect the resolution of a column and make it difficult to pack columns having reproducible characteristics. An additional problem exists when a chromatographic column is being packed with the small resin particles used in most high-resolution systems. If gravity sedimentation is used, the settling velocity of the particles is very low and the time required to prepare a given column is prohibitively long. In such cases, the technique of dynamic packing has been successfully used.[18]

In dynamic packing, the ion exchange resin particles, which are contained in a flowing slurry, are forced into the packed bed

at a velocity much greater than their settling velocity. Dynamic packing can be done in either of two ways: (a) by displacing a thick slurry, or (b) by extruding a prepacked bed. Packing by slurry displacement is accomplished by connecting a chamber (or reservoir) to the chromatographic column, filling the chamber with a thick slurry of the ion exchange resin, and then displacing the slurry into the column with a liquid that is pumped into the top of the slurry chamber. If the linear velocity of the displacement fluid in the slurry chamber is substantially greater than the settling velocity of the largest particle, size segregation will not occur in the resulting bed. During the packing of the resin, the flow rate of the liquid should be greater than the anticipated flow rate of the eluent.

When small-diameter columns of considerable length are packed with finely divided ion exchange resin, difficulty is encountered in pumping liquid through the slurry chamber and into the column at a sufficiently rapid rate to prevent size segregation. This difficulty is caused by a high pressure drop (in the long column), which prevents rapid displacement of the slurry from the slurry chamber into the column. However, as an alternative, a fixed bed can be packed into a reservoir, or cartridge, of larger diameter; then it can be extruded into the small-diameter chromatographic column by displacement with liquid. This extrusion technique can even be used to pack a coiled column, and it has also proved to be useful for preparing column charges that must be transported from one laboratory to another.

Eluent Delivery System

Two basic types of eluent delivery systems are used: constant flow and pulsating flow. Examples of the former include constant-drive syringes and reservoirs with gas overpressure; reciprocating piston pumps are typical of the latter. Both types can be used in high-pressure chromatographic systems; however, each has specific advantages in certain applications.

In general, pulsating pumps are less expensive and simpler to use. They are particularly advantageous when gradient elution (i.e., an eluent composition that changes with time or elution volume) is used since the gradient can be developed prior to contact with the high-pressure environment (e.g., by interconnection of multiple reservoirs with eluents of varying properties)[19] (Figure 4). If it becomes necessary to use gradient elution with the constant-flow devices, multiple delivery systems with high-pressure coupling are required; in addition, a complicated system of flow programming may be required.

The pulsations that occur in pulsating pumps may be undesirable; however, it is doubtful that they will affect the sorption process in high-pressure systems since the flow rate through the column typically does not vary more than 10% during the pulsation. On the other hand, some on-line detection systems (e.g., refractive index monitors) are very vulnerable to variations in flow rate and the pulsations contribute to significant background noise. Although such pulsations can be damped to some degree by using in-line flow resistances coupled with surge chambers, the constant-flow type of eluent delivery is superior in instances where "flow noise" is a problem.

Many different types of pulsating pump and constant-flow devices are now available commercially with pressure ratings up to 10,000 psi. In general, the constant-flow devices cost a factor of 5 to 10 more than the pulsating systems.

Sample Introduction

The most effective method for introducing a sample into an automated chromatographic system is to feed it directly into the eluent line just before the latter contacts the ion exchange resin. A hypodermic syringe with introduction through a septum may be used to accomplish this; however, at pressures greater than about 1000 psi, the syringe technique will usually necessitate stopping

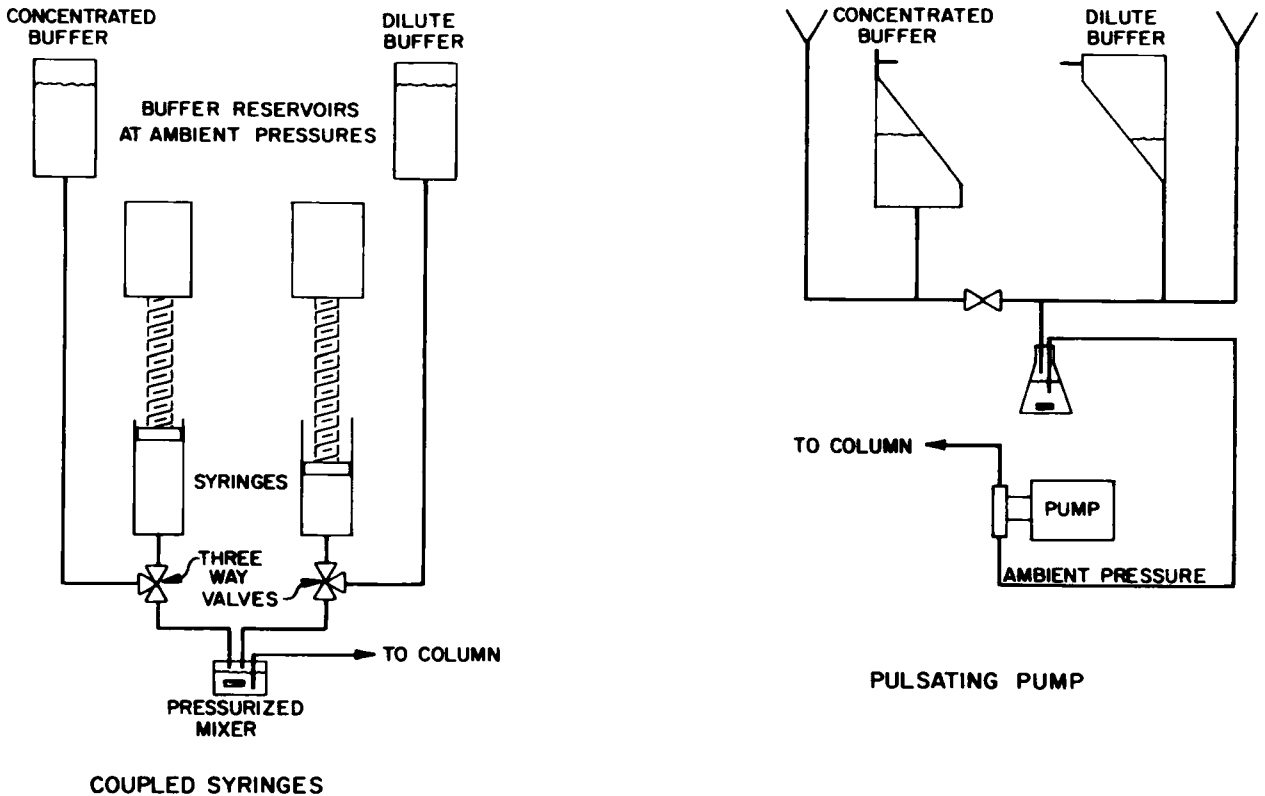


FIGURE 4

High-Pressure Eluent Delivery with Gradient Elution using Coupled Syringes or a Pulsating Pump
(From Scott (4) with permission from Academic Press, Inc.)

the eluent flow temporarily. Samples can also be introduced via an injection valve. Such a valve usually contains six ports, each pair of which is interconnected. In one orientation of the valve, a sample can be loaded into a sample loop, which becomes a part of the eluent line when the ports are reoriented by turning the valve handle (Figure 5). Valves that allow automated sample introduction at pressures up to 5000 psi without interrupting the system have been developed[20] and are now available commercially.

Eluate Detection Systems

An eluate detector or monitor is necessary to complete the automated analytical systems. This component continuously monitors a specific property of the eluate which makes it possible to detect and quantitate the separated solutes. Two types of detectors are commonly in use in monitoring the separation of biochemical mixtures: (a) flow photometers, and (b) photometric monitors in conjunction with reagent development methods. In the latter case, reagents are mixed continuously with the eluate stream and the resulting reaction mixture is continuously monitored.

When reagent development methods are used, additional process variables must be considered. These variables become important due to the necessity of introducing a metered stream or streams of reagent into the eluate stream, mixing the two streams thoroughly, allowing time for the necessary chemical reaction to occur, and continuously monitoring the reaction stream.

For systems in which large reagent flow rates (greater than 10 ml/hr) are used, the reagent flow can be effectively metered with positive displacement pumps. When pulsating pumps are used, the variation in flow rates must be reduced by suitable damping devices. For systems that require very low reagent flow rates, and even for some systems requiring larger flow rates, a successful reagent metering system can be designed to include a reagent reservoir with near-constant overpressure or hydrostatic head

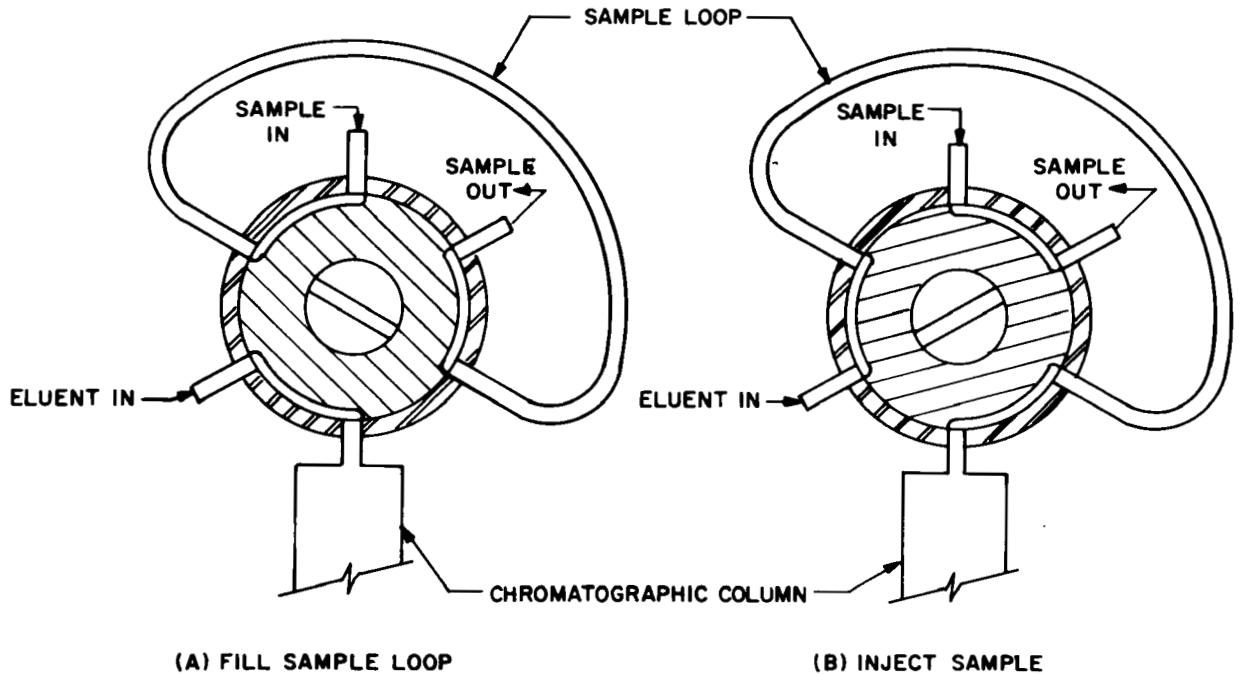


FIGURE 5

Use of a Six-Port Valve to Inject a Sample into the Eluent Stream of a Chromatograph
(From Scott in Modern Practice of Liquid Chromatography, J. J. Kirkland, ed., 1971, with permission from John Wiley and Sons, Inc.)

coupled with a controlled flow resistance, for example, narrow-bore tubing or a control valve.[21] Rotameters can be used to monitor the actual flow rate. If the reagent hydrostatic head or gas overpressure remains essentially constant during the course of a run, the reagent flow rate will remain relatively constant even at a rate as low as a few milliliters per hour.

Continuous flow photometers and colorimeters have been developed[22,23] specifically for LC, and now a variety are available commercially. Flow fluorometers[24] are also becoming available.

Typical Analyzers

Although it is possible to design an entire family of analytical systems by varying the separation system and/or eluate detector, only two of the more highly developed systems will be discussed in detail. These are an analyzer for UV-absorbing constituents (UV-Analyzer) and a carbohydrate analyzer. Other systems will be discussed in somewhat less detail in the succeeding section on Recent Advances.

UV-Analyzer

Several prototypes of the UV-Analyzer are currently being tested at various clinical and research laboratories.[25] Each uses a heated, high-pressure (up to 4000 psi) anion exchange column, concentration gradient elution with an aqueous acetate buffer for separation and transport of the constituents of the sample mixture, and a recording uv-photometer for detection and quantification of the separated constituents[2,4] (Figure 6). Recent models utilize capillary separation columns containing an anion exchange resin produced by Bio-Rad Laboratories (Aminex A-27) in the size range 10-15 μ.[3] The separation columns are fabricated from standard type 316 stainless steel tubing that is 0.22 cm ID and 150 cm long. A 1-in.-OD stainless steel heating jacket surrounds the column.

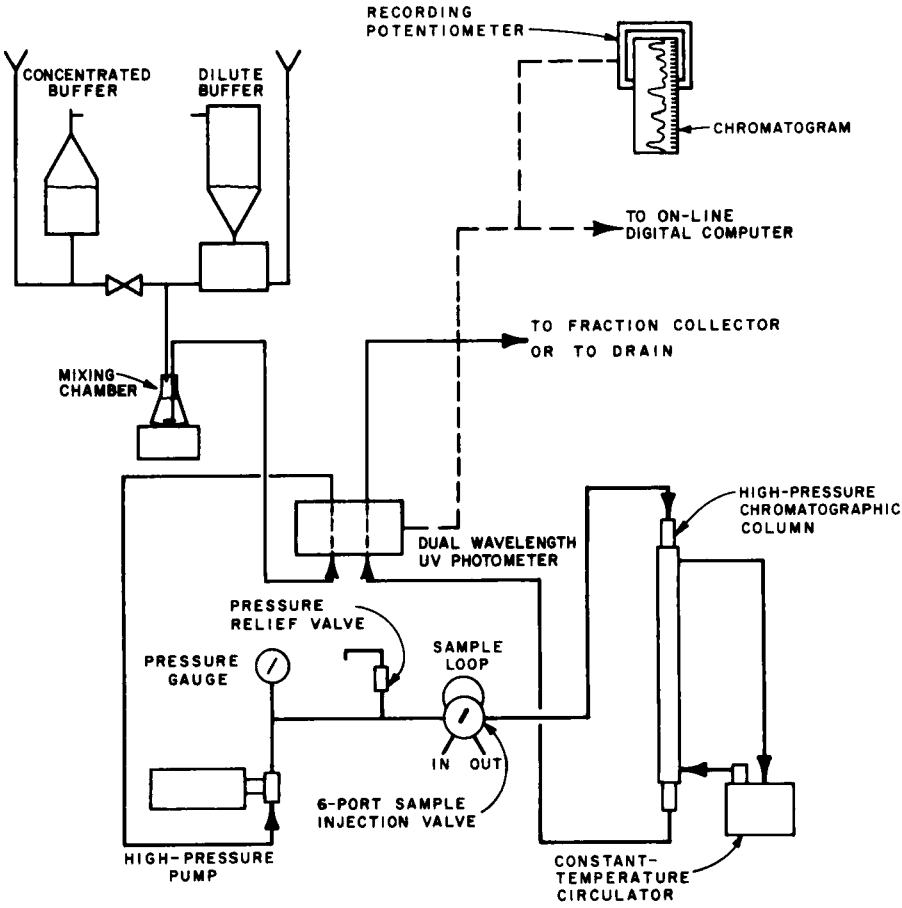


FIGURE 6

High-Pressure Anion Exchange Chromatograph for the UV-Absorbing Constituents of Body Fluids (From Scott (4) with permission from Academic Press, Inc.)

An ammonium acetate--acetic acid buffer (pH 4.4) whose concentration varies from 0.015 to 6.0 M during the course of the analysis is used as the eluent, and the separation column is maintained at 25°C for the first 30% of the run and at 60°C thereafter by a heated circulating fluid.

The detector is a miniature, recording, dual-beam flow photometer operating continuously at two different wavelengths, 254 and 280 nm. The dual-beam mode of operation provides a means of referencing the changing properties of the eluent stream by differentially comparing the eluent stream to the eluate stream.

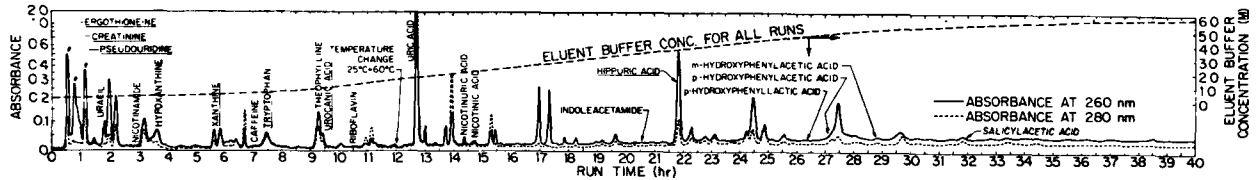
Samples are introduced by a six-port injection valve, and analytical results are presented graphically as a chromatogram showing the UV absorbance of the eluate stream vs run time, each molecular constituent being represented by a chromatographic peak (Figure 7). Typical sample sizes range from 0.1 to 0.5 ml for physiologic fluids, and the separation time varies from 25 to 40 hr for complex mixtures such as body fluids. Sensitivity is below the microgram level for many constituents.

Carbohydrate Analyzer

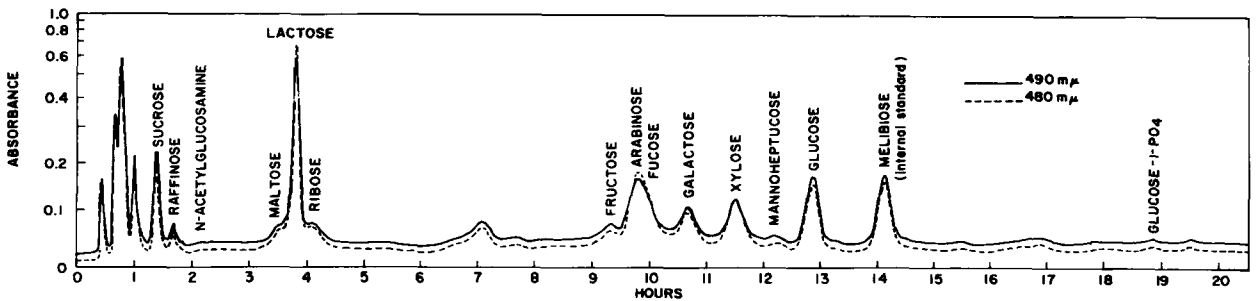
The principal component of the carbohydrate analyzer is a heated high-pressure anion exchange column having the same design and containing the same resin as that used for the UV-Analyzer.[2,8] This analyzer uses concentration gradient elution with a borate aqueous buffer, and detection and quantification are accomplished by a continuous colorimetric system (Figure 8.)[26]

The borate buffer forms complexes with the carbohydrates to give them ionic properties that subsequently allow separation by anion exchange chromatography. A sodium tetraborate--boric acid buffer (pH 8.5) whose composition varies from 0.169 to 0.845 M in the borate ion is used as the eluent. The column temperature is maintained at 55°C.

Carbohydrate detection is based on the continuous colorimetric reaction of sulfuric acid and phenol with the carbohydrates in the eluate. The reagent development system includes: (a) a reaction column into which the eluate and reagents (5% phenol solution and concentrated sulfuric acid) are continuously metered and mixed; (b) a reaction section maintained at 100°C through which



(a) UV-Chromatogram obtained from a 0.5 cc urine sample with the following operating conditions: 150-cm x 0.62-cm type 316 stainless steel column; Aminex A-27 anion exchange resin with an average particle size of 10-μ; temperature initially at 25°C increasing to 60°C at 12 hours; ammonium acetate-acetic acid buffer at pH 4.4 as the eluent varying in concentration from 0.05 to 6 M at an average flow rate of 37.5 cc/hr; and a pressure of 3000 to 3500 psi.



(b) Carbohydrate Chromatogram obtained from an 8 cc urine sample with the following operating conditions: 150-cm x 0.62-cm type 316 stainless steel column; Aminex A-27 anion exchange resin with an average particle size of 10-μ; constant temperature of 55°C; eluent borate buffer concentration varying linearally from 0.029 M $Na_2B_4O_7$-0.057 M H_3BO_3 to 0.147 M $Na_2B_4O_7$-0.283 M H_3BO_3 at an average flowrate of 68.4 cc/hr, and a pressure of about 1500 psi.

FIGURE 7

Typical Urine Chromatograms from the UV-Analyzer and the Carbohydrate Analyzer

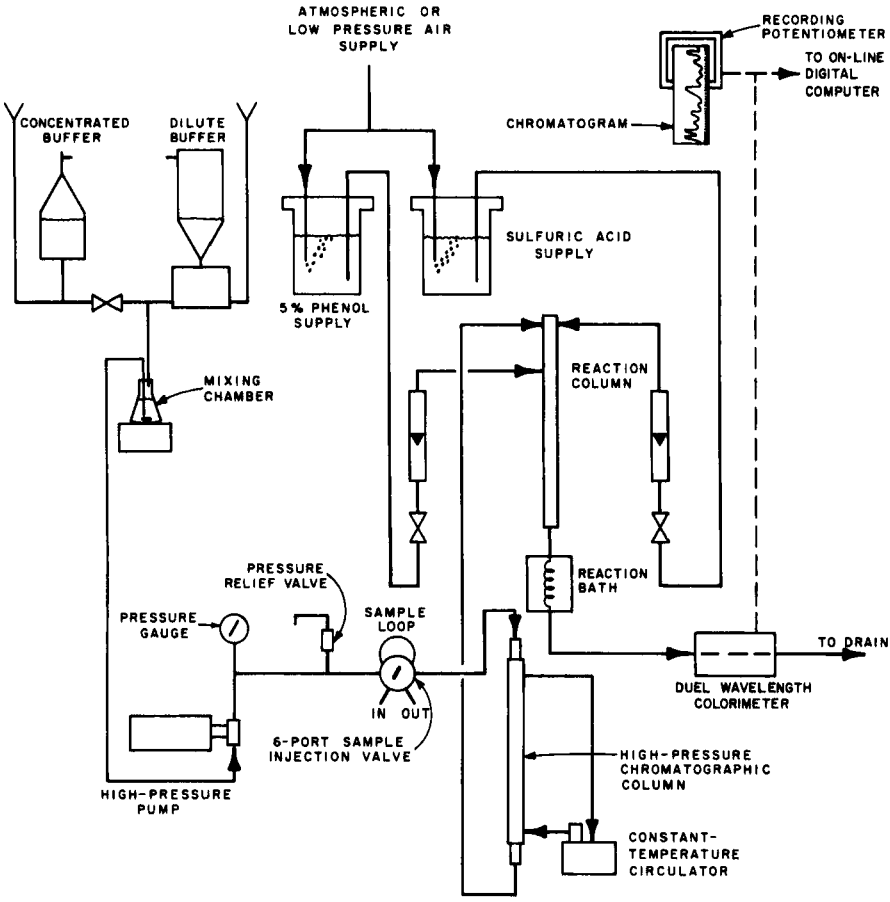


FIGURE 8

High-Pressure Anion Exchange Chromatograph for Carbohydrates in Body Fluids
(From Scott (2) with permission from J. B. Lippincott Co.)

the reaction mixture flows; and (c) a flow colorimeter that continuously measures the absorbance of the reaction mixture at wavelengths of 480 and 490 nm. The reagents are metered into the reaction column by using controlled pressure or hydrostatic head in the reagent reservoirs, a fixed pressure drop across a length of capillary tubing, and a control valve in the reagent lines.

Typically, 0.5-ml physiologic fluid samples are introduced by a six-port injection valve, and the resulting chromatogram, which is the measure of the absorbance of the eluate reaction mixture as a function of time, requires about 20 hr (Figure 7).

RECENT ADVANCES

Recent advances in the use of high-pressure ion exchange chromatography for the separation of complex biochemical mixtures have included the use of multiple columns (in parallel or in sequence) to increase the sample throughput and the development of additional reagent development systems for the column eluate monitoring.

Multiple Sorption Columns

As new ion exchange resins and reliable chromatographic components become available, additional operating options are possible. For example, multiple ion exchange columns can now be used in series to achieve higher resolution of a complex mixture or to reduce analysis time, in parallel to increase sample throughput, or in a combination of these modes to obtain both higher resolution and increased sample throughput.

Sequential Columns of Anion and Cation Resins

It is possible to take advantage of more than one sorption mechanism in separating complex mixtures. For example, an aqueous biochemical mixture containing both anions and cations can be more efficiently separated into its constituents by using both anion and cation sorption media rather than a single medium.

UV-Absorbing Constituents. — The use of sequential columns is applicable to the determination of the UV-absorbing constituents of complex mixtures such as physiological fluids, in which many of these constituents are present predominantly as anions but some

are cationic or neutral. In this type of separation, coupled anion and cation exchange columns can be used in sequence while the sample mixture is being loaded and the neutral species are being separated (by surface sorption effects). Then, after the loading phase is complete (i.e., when the remaining components are more or less segregated on each of the two columns), the columns are eluted separately but simultaneously.[27]

This type of operation allows more effective separation and, in addition, significantly decreases the total time required for separation. Operation of the columns in series during the loading phase results in increased resolution for the front end of the chromatogram. Separate elution of the components in the two columns thereafter greatly improves the resolution in the middle part of the chromatogram; in fact, it typically results in the resolution of 16 additional peaks as compared with the single anion exchange column (Figure 9). In general, the increase in resolution is due to the separate elution of anions and cations.

Separation of Indoles. — The 5-hydroxyindoles constitute another important class of compounds found in body fluids. Although these compounds may also be detected in other ways, they can be monitored in a column eluate by fluorometric techniques with excitation at 292 nm and measurement of emission at 330 nm. This type of monitoring appears to be almost specific for indoles and related compounds.

A coupled-column configuration in which a 0.22 x 25 cm anion exchange column was connected directly before a 0.22 x 50 cm cation exchange column was developed for the separation of indoles.[11] The indoles were eluted with an ammonium acetate--acetic acid buffer that was 4 M in ammonia and 5.8 M in total acetate, and a flow fluorometer was used as the column monitor.[15] Coupling of the anion exchange column with the cation exchange column has resulted in a much more effective separation in the front end of the chromatogram (Figure 10).

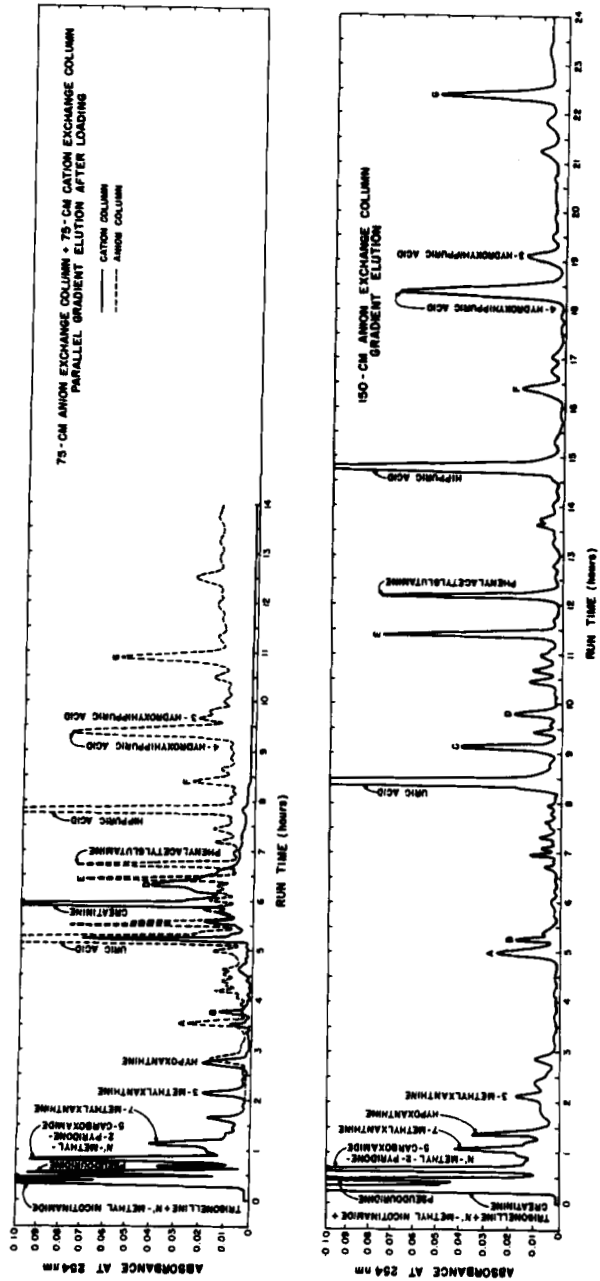


FIGURE 9

Comparison of the Separation of the UV-Absorbing Constituents of a 0.15-ml Sample of a Reference Urine by a Single Anion Exchange Column and by Coupled Anion and Cation Exchange Columns. Operating conditions are given in Reference 27

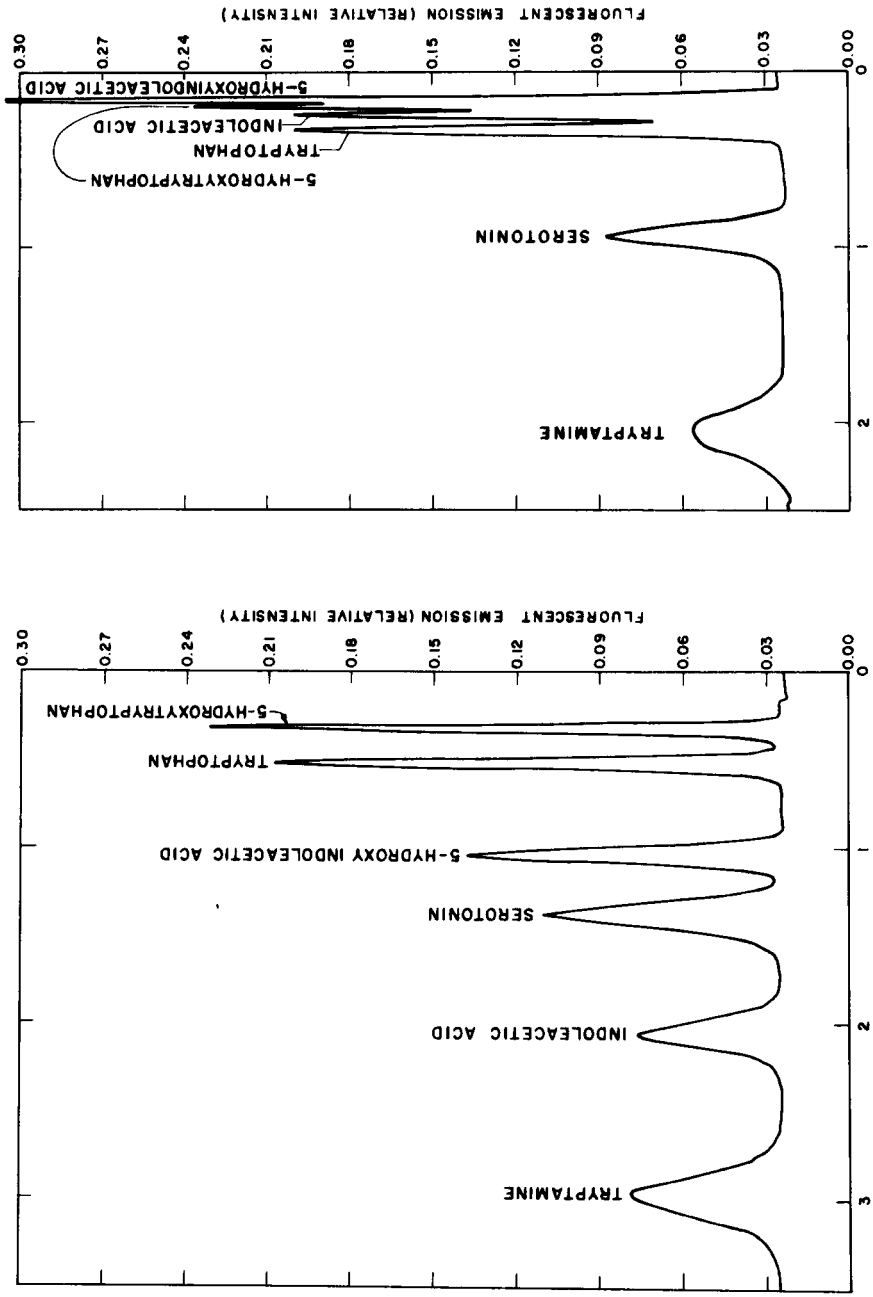


FIGURE 10. Separation of Indoles by Cation Exchange Chromatography (Left) and by Coupled Anion Exchange—Cation Exchange Chromatography (Right) with Fluorometric Monitoring

Sequential Columns of Microreticular and Pellicular Resins

The high-resolution separation of metabolites in physiologic fluids can be achieved by high-pressure ion exchange chromatographic systems that use very-small-diameter microreticular resins. Even though small particles can be used in the resin phase, diffusional resistance causes the separation process to be relatively slow. Conversely, the recently developed pellicular resins, which have only an active outer film, permit relatively rapid separation but at the expense of a severe reduction in capacity that makes high-resolution separations of complex mixtures very difficult.

An interesting concept is to combine these two types of ion exchange resins by using sequential columns of the two media. The first column, containing microreticular resin, would contribute the necessary capacity for a preliminary separation.[28] Subsequently, the second column, containing pellicular resin, would provide a rapid, final separation. Preliminary results from anion exchange chromatographic systems indicate that this technique will be useful in the separation of UV-absorbing constituents of physiologic fluids (Figure 11).

Parallel Columns

A recent development allows high-resolution ion exchange chromatographic systems for determining UV-absorbing compounds to be operated in a multi-column mode; that is, several columns can be operated simultaneously with a single pump and elution system.[29] An interesting and logical extension of this work is to consider the operation of LC systems with two parallel columns in which simultaneous analyses of two samples can be obtained, thus allowing a direct comparison of the chromatographic results either graphically or via a true differential mode of operation.[30]

Use of many parallel columns in a single system will result in a high sample throughput with only a limited increase in expense.

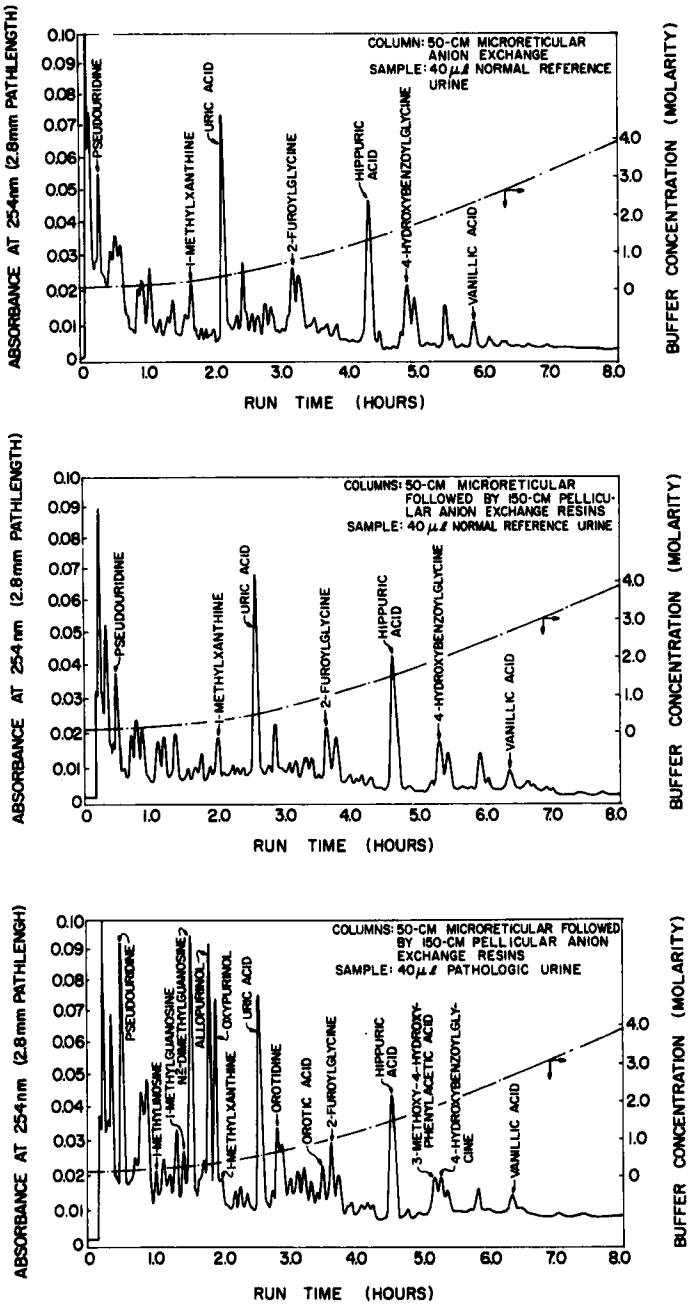


FIGURE 11

Comparison of the Separation of the UV-Absorbing Constituents of Urine on a 50-cm Column (Top Chromatogram) of Microreticular Anion Exchange Resin (Aminex A-27, 12- to 15-µm diameter) and on Sequential Columns of Microreticular (50 cm) and Pellicular (Pellionex AS) (150 cm) Resins (Two Lower Chromatograms)
(From Scott (28) with permission from Elsevier Scientific Publishing Co.)

Use of dual columns for differential chromatography will allow, for example, quantitative comparisons to be made between the urinary constituents of subjects with a pathology versus normal subjects, and the urinary constituents of subjects prior to and following drug intake (Figure 12). The effects of processing steps can also be investigated by simultaneous chromatography of samples from before and after the processing. The differential chromatograph yields almost superimposable chromatograms when identical samples are injected into both columns, but striking dissimilarities are noted when different samples are compared.

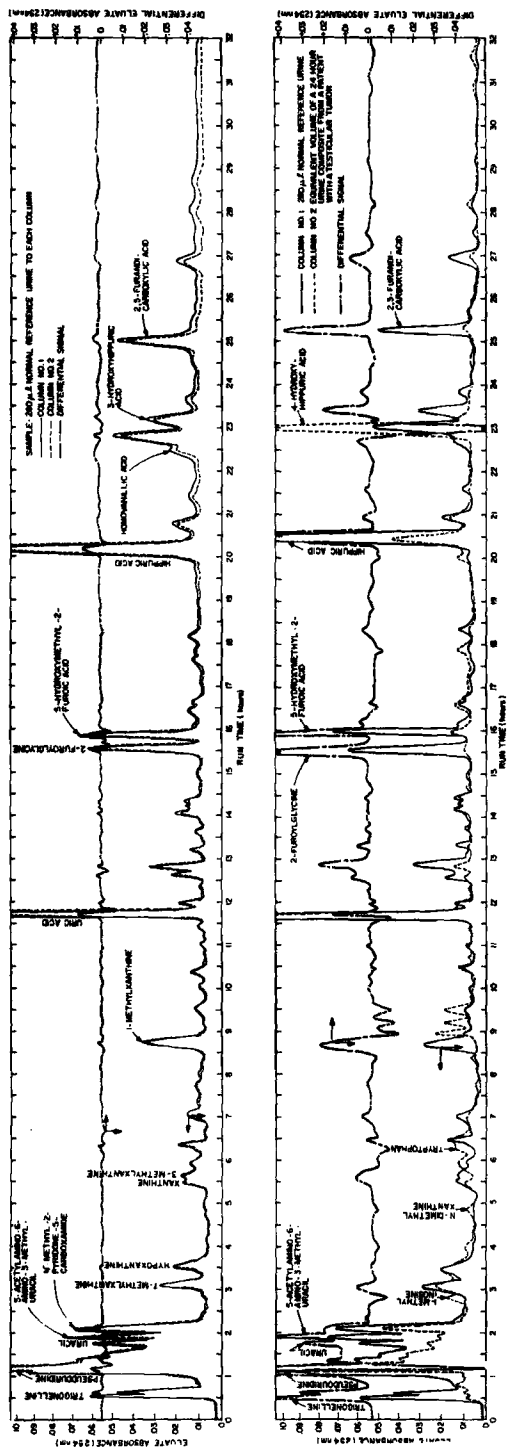
Reagent Development Systems

Although there are some strong arguments for a universal column monitor for LC, specific detectors are also extremely useful, particularly when complex biochemical mixtures are to be separated. In this case, a deficiency in the separation system can be overcome by monitoring or detecting only a very small fraction of the total compounds present.

The most important recent advances in detection and monitoring systems involve the adaptation of new reagent development schemes. In each case, a reagent stream is continuously mixed with the column eluate stream, and the mixture is allowed to react as it progresses to a flow monitor such as a fluorometer or colorimeter.

Cerate Oxidimetry

A recent interesting development in this area concerns the use of cerate oxidimetry in conjunction with fluorometric monitoring of the resulting reduced cerium. In this concept, the reduction of cerium(IV) to fluorescent cerium(III) by separated compounds in the column eluate is used to analyze for organic acids and other oxidizable compounds.[9] This method of monitoring is highly sensitive (capable of detecting a few nanograms of some biochemicals), and the measurement is made on a developed, rather than a consumed, species.



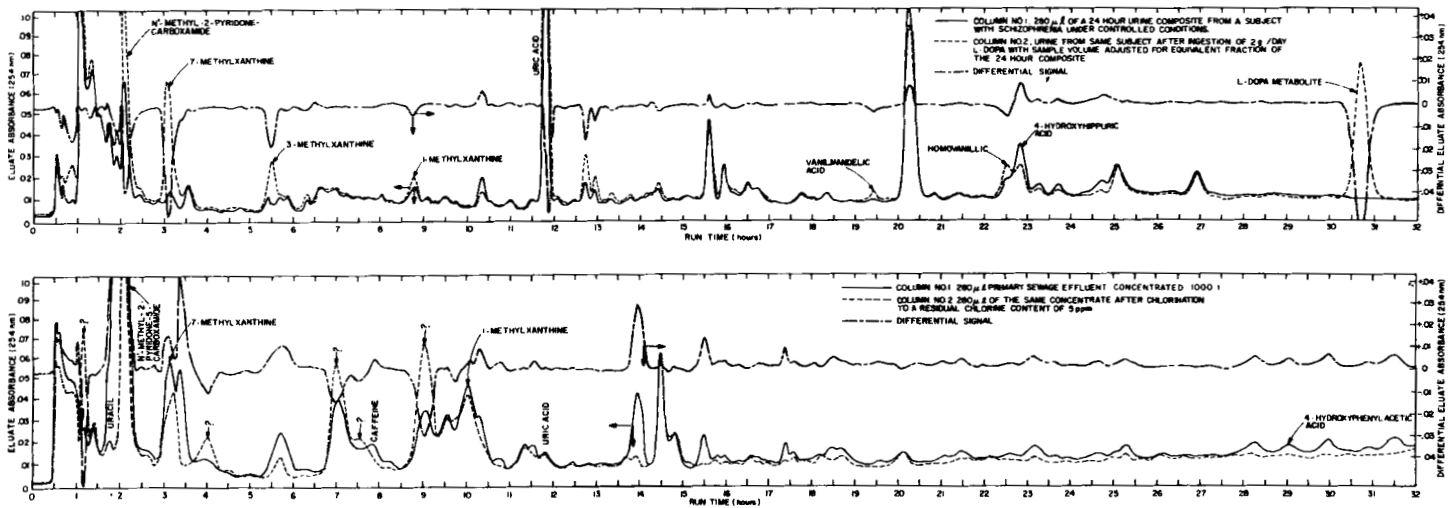


FIGURE 12

Differential Chromatograms of the UV-Absorbing Molecular Constituents in Complex Biochemical Mixtures (From Scott and Pitt (29) with permission from Journal of Chromatographic Science)

Cerium(IV) in 2 N H_2SO_4 is metered and mixed continuously at an approximate equal volume with the eluate from a high-resolution LC system, typically one of the anion exchange columns designed for measuring the UV-absorbing constituents in body fluids (Figure 13). An accurate reagent flow to match the flow of the column eluate (i.e., 7 to 12 ml/hr) is provided by flow resistance through a capillary with gas pressure over the reagent.

This type of detection system, when connected in series with a UV-photometer, will increase the amount of information obtained from a single separation system (Figure 14). Obviously, as other detection and monitoring systems are developed, they may also be added to existing separation systems to give more definitive data.

Detection of Polyamines with Fluorescamine

Another useful reagent development scheme takes advantage of the interaction of primary amines with a fluorescent reagent to detect polyamines that have been separated by a small cation exchange system. Of primary interest were the urinary polyamines: 1,3-diaminopropane, putrescine, spermidine, cadaverine, and spermine. The determination of polyamines in physiological fluids may be important in clinical diagnosis since recent studies have suggested that the urine of cancer patients contains elevated levels of these compounds.

Several of these polyamines were separated on a 15 x 0.45 cm cation exchange column (70°C) employing a combined pH-NaCl gradient. Detection was accomplished fluorometrically by reagent development, utilizing the reaction between polyamines and the recently developed reagent Fluorescamine[12] which, although non-fluorescent itself, forms a fluorophor with most primary amines. A typical analysis for these compounds requires approximately 90 min (Figure 15).

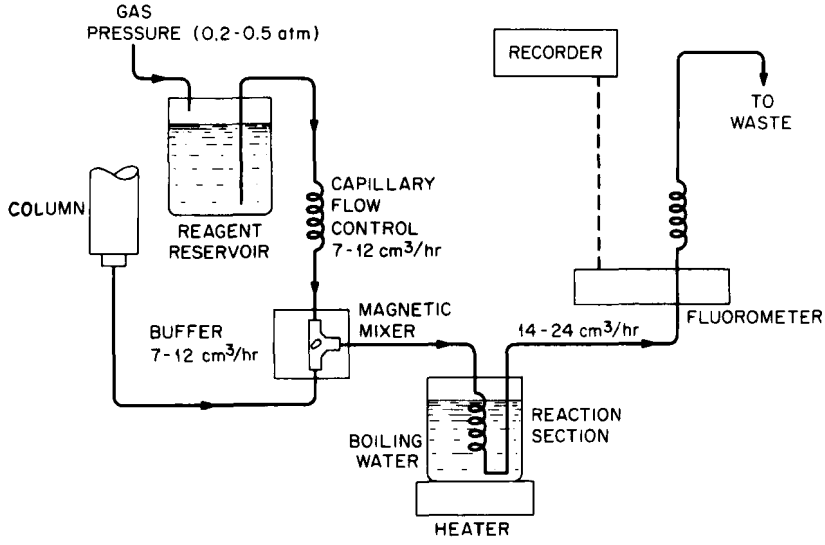
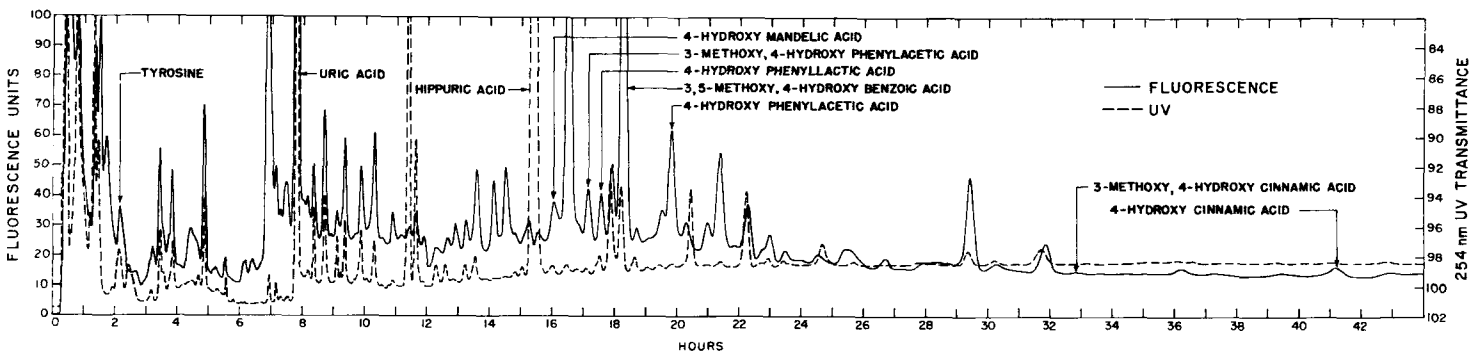


FIGURE 13

Cerate Oxidative Column Monitor for Organic Acids and Other Oxidizable Compounds
(From Katz and Pitt (10) with permission from Analytical Letters)

FUTURE TRENDS

New separation systems, especially those that are designed to resolve complex biochemical mixtures such as body fluids, will continue to be explored. As other sorption media become available, we can anticipate additional separation systems that utilize more than one sorbent in sequential columns. Work on detection systems will be primarily centered around additional reagent development methods. Such approaches will undoubtedly include new devices for colorimetric and fluorometric monitoring, and perhaps even luminescence development systems will become available.



CHROMATOGRAM OF A NORMAL URINE
(0.27 cc SAMPLE)

FIGURE 14

Detection of Organic Acids and Other Oxidizable Organic Compounds
by Cerate Oxidimetry After Separation by
Anion Exchange Chromatography

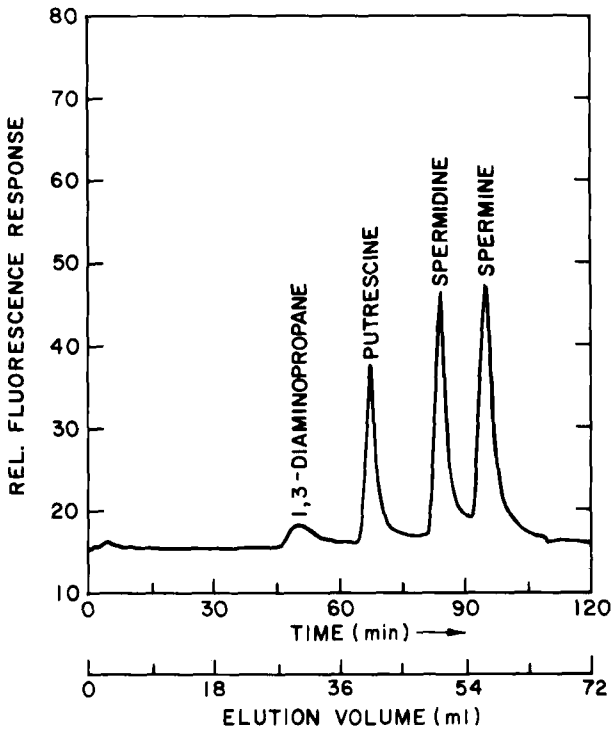


FIGURE 15

Separation of Several Polyamines by Cation Exchange Chromatography with Detection by Fluorimetric Development Using Fluorescamine. The separation was achieved on a 15 x 0.45 column of Aminex A-7 resin at 70°C using a combined pH-NaCl gradient

REFERENCES

1. Operated for the U.S. Atomic Energy Commission by the Union Carbide Corporation.

2. C. D. Scott, R. L. Jolley, and W. F. Johnson, Am. J. Clin. Pathol. 53, 701 (1970).

3. W. W. Pitt, C. D. Scott, W. F. Johnson, and G. Jones, Clin. Chem. 16, 657 (1970).

4. C. D. Scott, in "Advances in Clinical Chemistry," Vol. 15, O. Bodansky and A. L. Latner, eds., Academic Press, New York, 1972, p. 1.

5. S. Katz, W. W. Pitt, C. D. Scott, and A. A. Rosen, Water Research 6, 1029 (1973).

6. P. B. Hamilton, Anal. Chem. 35, 2055 (1963).

7. P. B. Hamilton, in "Handbook of Biochemistry. Selected Data for Molecular Biology," H. A. Sober, ed., Chemical Rubber Publishing Co., Cleveland, 1968, p. B43.

8. S. Katz, S. R. Dinsmore, and W. W. Pitt, Clin. Chem. 17, 731 (1971).

9. R. W. Rosevear, K. J. Pfaff, and E. A. Moffitt, Clin. Chem. 17, 721 (1971).

10. S. Katz and W. W. Pitt, Anal. Lett. 5, 177 (1972).

11. D. D. Chilcote and J. E. Mrochek, Clin. Chem. 18, 778 (1972).

12. H. Veening, W. W. Pitt, and G. Jones, J. Chromatogr. 90, 129 (1974).

13. N. G. Anderson, J. G. Green, M. L. Barber, and F. C. Ladd, Anal. Biochem. 6, 153 (1963).

14. M. Uziel, C. K. Koh, and W. E. Cohn, Anal. Biochem. 25, 77 (1968).

15. C. D. Scott, Anal. Biochem. 24, 292 (1968).

16. P. B. Hamilton, Anal. Chem. 32, 1779 (1960).

17. S. Ergun, Chem. Eng. Progr. 48(No. 2), 89 (1952).

18. C. D. Scott and N. E. Lee, J. Chromatog. 42, 263 (1969).

19. D. D. Chilcote, C. D. Scott, and W. W. Pitt, J. Chromatog. 75, 175 (1973).

20. C. D. Scott, W. F. Johnson, and V. E. Walker, Anal. Biochem. 32, 182 (1969).

21. R. L. Jolley, W. W. Pitt, and C. D. Scott, Anal. Biochem. 28, 300 (1969).

22. L. H. Thacker, W. W. Pitt, S. Katz, and C. D. Scott, Clin. Chem. 16, 626 (1970).

23. L. H. Thacker, C. D. Scott, and W. W. Pitt, J. Chromatog. 51, 175 (1970).

24. S. Katz and L. H. Thacker, J. Chromatog. 64, 247 (1972).

25. Construction prints of the earlier models are available as CAPE-1753 from the National Technical Information Service, U.S. Department of Commerce, 5285 Port Royal Road, Springfield, Virginia 22151.

26. Construction prints of the earlier models are available as CAPE-1779 from the National Technical Information Service, U.S. Department of Commerce, 5285 Port Royal Road, Springfield, Virginia 22151.

27. C. D. Scott, D. D. Chilcote, and N. E. Lee, Anal. Chem. 44, 84 (1972).

28. C. D. Scott and N. E. Lee, J. Chromatog. 83, 383 (1973).

29. W. W. Pitt, C. D. Scott, and G. Jones, Clin. Chem. 18, 767 (1972).

30. C. D. Scott and W. W. Pitt, J. Chromatog. Sci. 10, 740 (1972).