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CENTRIFUGAL ELUTION CHROMATOGRAPHY WITH ELUATE MONITORING

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SUMMARY

The feasibility of using centrifugal, multicolumn, elution chromatography in conjunction with eluate monitoring is being studied. A preliminary prototype system has as many as four columns mounted in a spinning rotor that passes through a stationary photometric detection station. Cuvette windows at the terminal end of each chromatographic column allow photometric monitoring of each column eluate as it passes through the detector. The monitor is capable of associating a given output signal with the appropriate chromatographic column using suitable digital logic. The photometric output can be recorded by a conventional strip-chart recorder or, via a computer, by printed tabulations. In the present mode of operation a common sample is introduced simultaneously to each column, with a single eluent stream being used for all columns.

Preliminary tests have been directed toward establishing the best rotor design for hydrodynamic stability and overall operability. The system is being considered for the assay of several serum proteins from a single serum sample utilizing affinity chromatography.

INTRODUCTION

It is desirable to consider methods of increasing sample throughput for liquid chromatography. The greatest emphasis in this area has been on developing systems in which the time for the chromatographic separation has been significantly reduced by use of new high-efficiency sorbents such as the pellicular ion-exchange resins[1]. In this case the entire sequence of sample introduction, chromatographic separation, and eluate monitoring is carried out periodically on a single channel.

Another approach has been the use of parallel chromatographic columns in which simultaneous separations can be carried out on two or more samples using the same pumping system but separate columns and column monitors[2,3]. Sample throughput is increased by the number of columns used.

Centrifugal chromatography has also been developed to the extent that single samples can be separated on long coiled columns[4,5] or several parallel columns can

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

achieve many separations concurrently[6]. Although elution development is used in both cases, the former requires collection of the eluate from the centrifugal system with subsequent assay of eluate fractions, while the latter allows separation to occur simultaneously within each column, but the separated constituents are detected only when each column is removed from the rotor and monitored.

With the successful development of centrifugal fast analyzers[7–9] in which a stationary photometer is used to monitor a series of cuvettes in a spinning rotor, a similar technique can be considered for centrifugal elution chromatography with eluate monitoring of multiple columns. A first prototype of such a system has been developed, and preliminary results are encouraging.

OPERATING PRINCIPLES

Centrifugal elution chromatography with eluate monitoring requires a spinning rotor containing multiple chromatographic columns or channels, a means of introducing the eluent and samples, a means of monitoring each eluate stream while the rotor is spinning, and necessary controls and instrumentation to automate the operation (Fig. 1).

Eluent flow

The most obvious difference between centrifugal chromatography and the more conventional methods of column chromatography is the way in which the eluent is forced through the chromatographic column. Although a metering device will be necessary to introduce the eluent into a centrifugal chromatograph, the centrifugal field itself is used to force the eluent through the chromatographic columns.

There is a subtle difference between the forces exerted in the two systems. In the former, the differential pressure drop within the column is almost constant throughout the column and the pressure continuously decreases from the eluent entry to the eluate exit. Conversely in centrifugal chromatography, the chromatographic columns are oriented radially and the propelling force from the centrifugal field becomes greater as the eluent progresses through the column. When flowing eluent is introduced to this variable force field, the pressure drop within the column will not be constant; in fact, it can be both positive and negative within the same column. The maximum pressure will be found somewhere within the column, whereas the entrance and exit pressures should both approach ambient conditions.

During operation, the rate at which the eluent is delivered to the centrifugal chromatograph must be sufficiently high to ensure that liquid is always present in the columns. This can be achieved by maintaining a thin annulus of eluent at the column openings (Fig. 1). Since the centrifugal field tends to stabilize the fluid in such an annulus, backmixing is held to a minimum and the eluent tends to progress radially almost as a differential element.

Sample introduction

Samples can be introduced into the centrifugal chromatograph in two ways: (1) by using a conventional sample injection valve to introduce a common sample into the eluent stream just prior to its entry into the chromatograph, and (2) introduction of multiple samples by use of a removable transfer disc. The former method,

which is by far the most simple, results in a common sample being apportioned into each of the columns. The latter technique would allow several different samples to be introduced simultaneously.

Single sample introduction. Apportionation of fluid streams dynamically introduced into a spinning rotor has been studied previously[10,11]. It was demonstrated that continuous streams could be apportioned into as many as seventeen separate channels with an accuracy of better than 1 %. Thus, a single sample pulse introduced into the eluent stream could be apportioned essentially equally into each of the chromatographic channels. This would be a useful mode of operation only in cases where different separations were being carried out in each channel.

Multiple sample introduction. When separate samples are to be introduced into each chromatographic channel, a technique similar to that previously developed for the centrifugal fast analyzer can be utilized[7–10]. This technique is based on the use of a removable transfer disc containing separate samples in appropriate sample wells that are opposite to each of the channel openings when the transfer disc is in place. The disc is loaded external to the chromatograph and placed into the stationary rotor. Then, when the rotor spins, each sample is transferred radially into the chromatographic channel by the force from the centrifugal field. A slight outward slope of the sample well ensures essentially quantitative transfer of the sample. Similarly, sample wells can be provided in the rotor itself in an annular ring next to each chromatographic channel. The wells can be positioned so that a sample remains discrete when the rotor is stationary but is flushed into the column by eluent when operational speed is obtained.

Eluate monitoring

Each chromatographic channel terminates close to the outer edge of the rotor, and an eluate channel is connected to an in-line monitoring cuvette (Fig. 1). Each cuvette has transparent windows on the top and bottom.

The rotor moves through a stationary photometric station consisting of a light source and a photomultiplier vertically aligned and positioned so that the cuvettes pass through the center of the light beam as the rotor spins. This arrangement allows the photometer to periodically monitor each cuvette. With appropriate electronics, including sample-and-hold circuits, this will allow one photometer to monitor many different eluate streams almost simultaneously or at least during one revolution of the rotor.

EXPERIMENTAL SYSTEM

A first prototype of the centrifugal elution chromatograph has been built, and it is now ready for extensive testing. It is anticipated that future developments in this area will result in many changes; however, this first system will allow the feasibility of the concept to be determined.

General description

The chromatograph has a small positive-displacement pump that meters the eluent from a reservoir or gradient development system through a sample injection valve into the inner core of a rotor (Fig. 1). The rotor contains two or four chromatographic channels with eluate cuvettes that pass through a stationary photometer. (Later

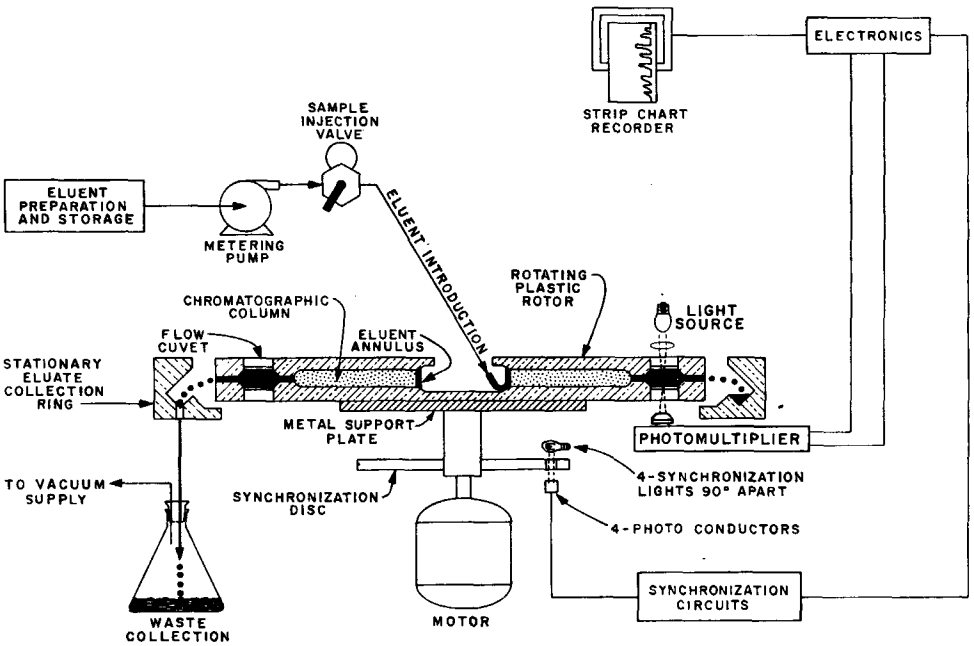

Fig. 1. Centrifugal elution chromatograph with eluate monitoring.

models will have many more channels.) The eluate streams exit the spinning rotor and pour into a stationary outer collection ring which is connected to a waste reservoir. A small negative pressure is maintained to ensure that the eluate progresses to the waste reservoir. This also retards formation of an aerosol.

The temperature of the system is essentially ambient. The rotor and support flange are driven by a $1\frac{1}{2}$ hp d.c. motor which has variable speed control up to 2000 rpm. Synchronization signals for data acquisition are provided by apertures contained in an auxiliary rotating plate which passes through stationary photometric detectors.

Optical system

The optical system consists of a focused light source (typically a miniature quartz–iodine–tungsten lamp or a low-pressure mercury lamp with interference filter) mounted above the rotor and a photomultiplier tube mounted below. Necessary power supplies are also included.

Rotor design

The rotor is fabricated from two pieces of acrylic plastic glued together. It typically contains four columns or channels, each of which is 0.3 cm diam. $\times$ 10 cm long with a 0.16-cm-diam. eluate channel connecting to a 0.32-cm-diam. $\times$ 0.32-cm-long cuvet (Fig. 2). The eluate channels are connected to the outer edge of the cuvette so that flow progresses radially inward through the cuvettes. This allows gas bubbles to properly clear the cuvette. Quartz windows cemented to the plastic body cover the cuvette on either side. An inner open annulus allows introduction of the eluent stream,

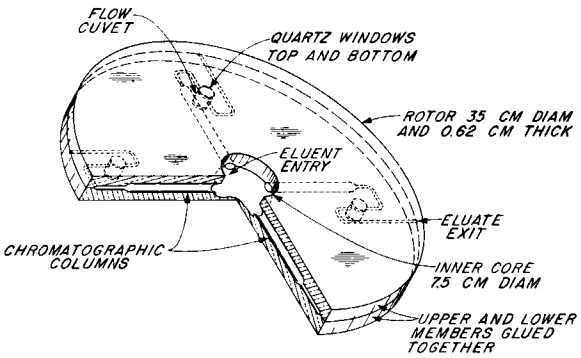

Fig. 2. Plastic rotor with four chromatographic channels fabricated from two pieces of acrylic plastic.

and an inner ring of 50-μl sample wells, one for each channel, is included in one design.

Sorption medium is loaded directly into the rotating rotor in the form of a slurry. In some cases a removable column sleeve is first introduced into the empty column. This allows the packed bed to be removed simply by removing the sleeve. A small nylon filter or a plug of glass wool is used to hold the sorption medium at the entry of the eluate channel.

Data acquisition and processing

The data from this system (the output from the photomultiplier tube) are generated in a form and at a rate suitable for direct input into a small dedicated digital computer, especially if a large number of chromatographic channels are used. However, this prototype system with only four channels uses a multipoint recording potentiometer for data display.

The monitor must be capable of associating a given signal with the appropriate chromatographic column. To achieve this, the photometer output is coupled with digital logic circuitry to keep track of the signals that are generated (Fig. 3). Synchronization signals result from four stationary lamp–photodiode combinations through which a slotted disc attached to the motor shaft passes. A synchronization signal is activated every time a cuvet passes into the photometer beam. Each of these

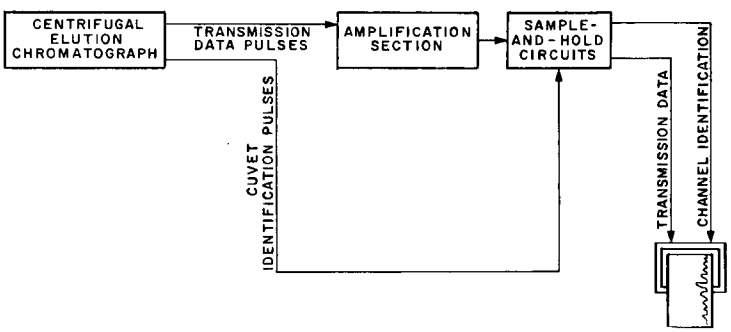

Fig. 3. Data acquisition and processing scheme.

signals is adjusted to indicate the position of the midpoint of the cuvet, at which point the output of the photometer is switched by digital logic to a sample-and-hold circuit corresponding to the cuvet. The values thus obtained are renewed each revolution of the rotor. A multipoint strip-chart recorder sequentially prints the transmission values from each cuvet.

EXPERIMENTAL RESULTS

The experimental system is undergoing feasibility tests. It was built primarily to test the feasibility of the concept. Preliminary results have been obtained for very simple separations in which a single sample is introduced to all four channels simultaneously.

Operation

The system is operated by first establishing a steady-state eluent flow-rate into the spinning rotor. This is determined when the inner annulus of eluent has a constant thickness. Shortly thereafter, a sample is injected into the eluent stream by an in-line sample injection valve. This constitutes time zero for the separation. The eluate monitoring system is operated throughout the run, and the multipoint strip-chart recorder is operated with four different colors of ink, one for each chromatographic channel.

Experimental results

Preliminary tests have been made with a mixture of riboflavin and Blue Dextran 2000 (Pharmacia, Uppsala, Sweden). This mixture was separated on columns of Sephadex G-10 gel in the size range of 40 to 100 μm. Water was used as the eluent.

Dextran blue has a molecular weight much too large to penetrate the G-10; thus it was eluted at breakthrough. Riboflavin, which penetrates the gel matrix, was retained longer in the column. Monitoring was at a wavelength of 254 nm.

As expected, the steady-state eluent flow-rate increased with the square of the rotor speed (Fig. 4) since the centrifugal force has a similar dependence on angular velocity. The flow resistance was due to the small eluate channels as well as the bed

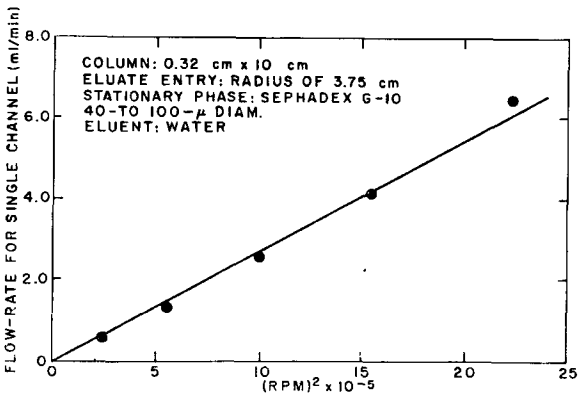

Fig. 4. Steady-state eluent flow-rate as a function of rotor speed for a typical rotor system.

of sorbent. Within the rotor speeds tested, the photometer and associated electronics were adequate to essentially give a continuous chromatographic trace (insofar as a multipoint recorder is continuous) for each channel.

The reference separation for a typical run at 500 rpm required approximately 6 min. There was some tailing in the last peak perhaps indicating poor design for the fluid dynamics of the system (Fig. 5). At this stage of development no attempt has been made to optimize the system for high efficiency. The apparent efficiency of the entire system for this simple separation can be represented by about thirty theoretical plates. This will probably be adequate for affinity chromatography. The comparable chromatographic peaks from each channel had very close elution times indicating that eluent flow-rates for the various channels were very close to the same.

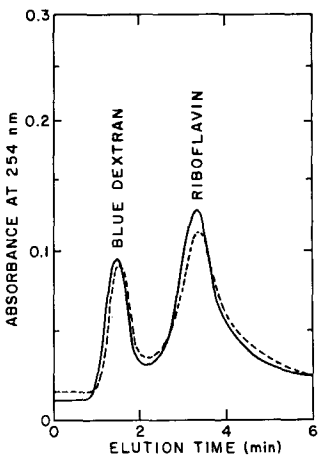

Fig. 5. Typical chromatograms from two channels for the simultaneous separation of a mixture of blue dextran (1 mg/ml) and riboflavin (0.03 mg/ml) on a centrifugal elution chromatograph. The rotational speed was 500 rpm and the sample size was 50 μl.

FUTURE APPLICATIONS

Although this system has the capability for several important separations, one of particular interest is the use of affinity chromatography for serum protein determination. In this type of analysis, an antibody to a specific protein is bound to the stationary phase and, as a serum sample passes through the column, only the specific protein will be held up in the column. This protein can be subsequently eluted from the column with a change in eluent. The chromatographic pattern (*e.g.*, with photometric monitoring at 280 nm) from such an affinity column would first give a large peak for all of the initially non-sorbed species, followed by a much smaller chromatographic peak for the specific protein. The smaller, more specific peak could be used to quantitate the protein.

In the centrifugal chromatograph, a whole series of different bound antibodies could be used —one for each chromatographic channel. A single serum sample introduced into the chromatograph would then be analyzed for a different protein in each

channel. Initially, Sepharose-bound antibody will be used as the stationary phase and several of the immunoglobulins will be analyzed from a single serum sample.

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