Continuous chromatography using time-varying eluant flow
by Mark David Swanson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
This research was conducted to develop a method to increase the volumetric rate of flow of material that can be separated using chromatography. This was done by developing a continuous chromatography system. In this research, a square plate, 18.3 cm by 18.3 cm by 0.25 cm, was filled with Sephadex G-25, a chromatographic packing. This plate, or bed, was built so that eluent could be introduced on either of two adjacent sides, and withdrawn from the opposite sides of the bed. A dye mixture was introduced in the bed, and would flow with the eluent. By alternating the direction of eluent flow, the dyes were made to flow to characteristic points in the bed, and were extracted at these points. After the initial building of the bed, the procedure was to fix problems as they arose. Proper eluent flow proved to be very important, and specialized apparatus, "spider feeders", were developed for this. From computer simulation and observation, it was found that the fewest eluent flow direction changes possible should be used, because the flow in the bed was slow to respond to changes made at the spider feeders. The points where the dye was extracted from the bed are very interdependent, so that slight alterations in one may have large effects on the operation of the other. The system investigated did work, because a continuous separation was demonstrated, but the operation of the bed was not as consistent as desired.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

This research was conducted to develop a method to increase the volumetric rate of flow of material that can be separated using chromatography. This was done by developing a continuous chromatography system. In this research, a square plate, 18.3 cm by 18.3 cm by 0.25 cm, was filled with Sephadex G-25, a chromatographic packing. This plate, or bed, was built so that eluent could be introduced on either of two adjacent sides, and withdrawn from the opposite sides of the bed. A dye mixture was introduced in the bed, and would flow with the eluent. By alternating the direction of eluent flow, the dyes were made to flow to characteristic points in the bed, and were extracted at these points. After the initial building of the bed, the procedure was to fix problems as they arose. Proper eluent flow proved to be very important, and specialized apparatus, "spider feeders", were developed for this. From computer simulation and observation, it was found that the fewest eluent flow direction changes possible should be used, because the flow in the bed was slow to respond to changes made at the spider feeders. The points where the dye was extracted from the bed are very interdependent, so that slight alterations in one may have large effects on the operation of the other. The system investigated did work, because a continuous separation was demonstrated, but the operation of the bed was not as consistent as desired.

INTRODUCTION

Chromatography is a very useful tool in the separation of many hard to separate components. The basic theory is that a mix of the components to be separated is carried through a packed bed of some kind by a solvent or eluent. Because each component has a different affinity for the packing, each component has a different residence time in the packed bed, and hence the time for each component to travel through the bed is different. By collection of the eluent at different times, the various components can be separated. This is usually a batch process. For chromatography to be more useful to industry as a separation tool, it should be capable of separating large quantities of material. According to C. D. Scott,

> Simply scaling up a batch type column is not very desirable, because after a column has been scaled up to a 2.5 cm or more diameter, a measurable loss of resolution is noticed.[1]

Although scaling up is not very desirable, chromatography is still one of the best separation processes available. "In recent years, the purification of enzymes by chromatography on columns has become the most effective of all separation methods."[2] This use of column chromatography makes more products available, but it increases the price noticeably. S. H. Elwan says,

> We estimate the total cost of an industrial-type gel chromatography operation to be on the order of $5/gram or less. The major costs are labor (40%), gel (25%), filters (20%), chemicals (10%), and capital (5%).[3]

This chromatography expense is a factor in many of the products available today. According to J. J. Kelly,

> We surveyed the 1982 volume of the journal of Biological Chemistry and found that 60% of the published laboratory-scale protein purification schemes included at least one gel chromatography step.[4]

A continuous flow chromatography system would be capable of separating larger quantities of material, and several systems have been developed. According to P. C. Wankat,

> Although extensively studied, including industrial prototypes and pilot-plant studies, the continuous two-dimensional chromatographs have not been accepted as standard separation methods.[5]

Of the continuous chromatography systems now available, the packed annular bed seems to have the best chance of becoming accepted by industry. This system uses a packed annular space which is slowly rotated, while the feed point and extraction points are fixed.[6] This system can utilize a gradient elution technique, and has even been modified to produce three dimensional separations.[7] The problem with this technique is that it has moving parts, and needs seals where the rotating drum meets the fixed feed and withdrawal sites.

Another system is a magnetically stabilized fluidized bed, which requires the packing be a magnetic material, and

can only be used for the separation of gaseous feed products.[8] The fluidized bed is shaped like a box, with the fluidizing medium coming from the bottom. A slow gaseous flow across the bed moves the entire contents at a fixed rate. The product is introduced at the bottom, near the side where the cross flow enters the bed. The rate at which the product moves upward is determined by the affinity that the product has for the chromatographic packing, and the rate at which it moves across the bed is determined strictly by the flow across the bed. The products are then collected on the top of the fluidized bed, with those with a greater affinity for the packing, and thus a longer residence time in the bed, being further from the side where the cross flow entered.

There is a proposal for a system that uses a rectangular plate with eluent introduced on two adjacent sides, while the temperature of the system changes each time the direction of flow changes.[9] The different relative velocities of the products at the different temperatures results in the two products exiting the plate at different points.

Another system uses a slanting triangular plate, which is equilateral, with the horizontal side of the triangle at the bottom.[10] Two different eluent streams are introduced on the two upper adjacent sides, while the feed is introduced at a point on the center line of the triangle

near the top. The triangle is set at an angle so the eluents and the feed flow toward the third side of the triangle by gravity. This system has been demonstrated of being capable of continuously separating Fe+3 and Co+2.

Although there are continuous chromatography systems which have been developed, none have been accepted as a standard method. If an effective, cheap, low maintenance system were to be developed, it would give an attractive option to anyone considering using chromatography as a separation tool.

The purpose of this research is to develop an operational continuous chromatography system using time-varying eluent flow in a square plate. The purpose is not to demonstrate any particular separation, but to show that the technique is workable.

Most chromatographic columns use position and time to achieve a separation. As the components move through the column, they become separated spatially, and thus each component exits the column at a different time. By collecting the eluent when each component is leaving the column, a separation is achieved. If a continuous system is to be operated at steady state, time cannot be used as an aspect in the separation, so something must replace it. In all the continuous chromatography systems so far developed, a second spatial dimension is used to replace time.[11] This is also true for this research project.

It is desired to achieve a separation by setting up a square plate, or bed, with the capability of introducing eluent on two adjacent sides, and allowing the eluent to exit the bed on the other two sides (see Fig. 1). This eluent could be introduced entirely on one side of the bed for a fixed amount of time, then entirely on the adjacent side for the same amount of time, thus producing a square wave introduction technique. It could also be introduced entirely on one side, and then have a gradual pressure change, so that after a fixed time it would be entirely introduced on the adjacent side, creating a sinusoidal eluent introduction. Thus, time would be used as a method of operation of the bed, but not as an aspect of the actual separation.

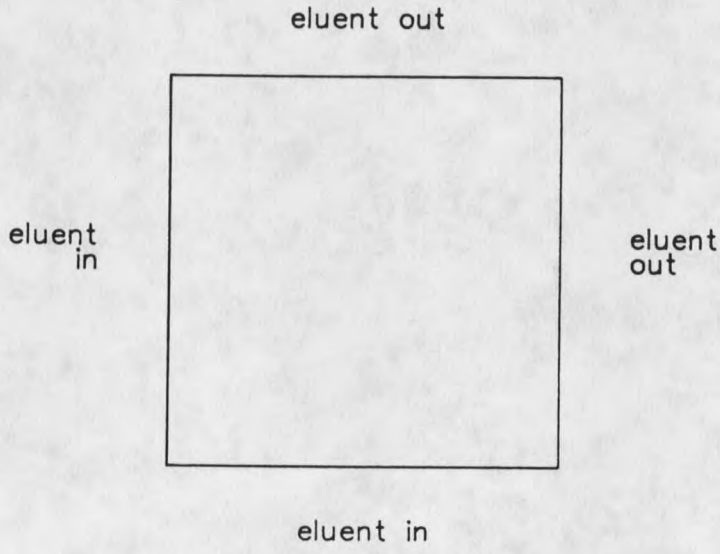

Fig. 1. Eluent flow

Because of the different distances traveled by the various components, there should be points in the bed to which each component, or dye in this case, characteristically moves. If the carrier fluid could be extracted at these points without significantly disturbing the flow pattern, a continuous separation would be achieved (see Fig. 2). As can be seen from the figure, there would be a trace of the large molecules collected with the small molecules. The large molecules collected should be free of any small molecule contamination. The optimum location for the feed would also be a consideration.

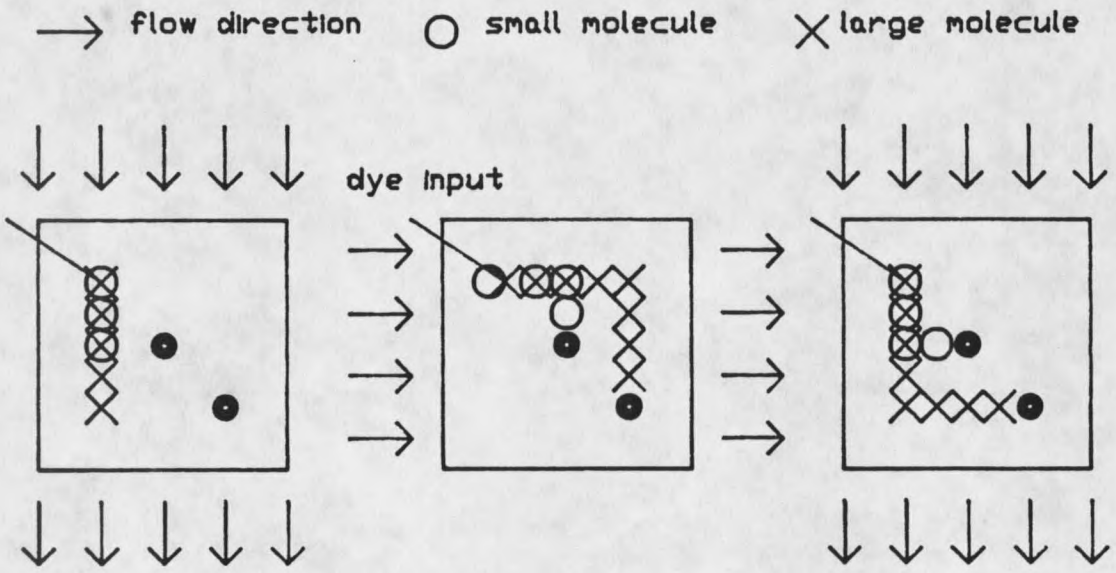

Fig. 2. Dye flow

EXPERIMENTAL

The basic experimental plan was to determine the flow characteristics in the bed, then to put in the product extraction holes, and finally to demonstrate a separation. Since this is a new technique, most of the experimental procedure was to find a problem with the system, and then fix the problem. For this reason, the experimental and the results sections of this thesis are not really separable. The results from one experiment were needed to determine the next experiment. There was an initial design, so that will be presented here in the experimental section.

The experiments were conducted using gel permeation chromatography, which separates components on the basis of molecular size. Sephadex G-25 was used as the chromatographic packing material, and the molecules which were separated were vitamin B-12 and blue dextran. Vitamin B-12 is red, and has a molecular weight of 1355 atomic mass units, while blue dextran is blue, and has an average molecular weight of two million atomic mass units.

The bed shape was square, with internal dimensions of 18.3 cm by 18.3 cm by approx. 0.25 cm. The basic bed was constructed of closed cell foam, which allowed no eluent to flow through it. The inside surfaces were notched, and open cell foam was affixed in each of these notches (see Fig. 3). The eluent could flow through the open cell foam with little resistance, and thus bypass the bed packing.

The notches prevented this. Eluent needles introduced the eluent to the bed through the closed cell foam. The open cell foam served as a filter, preventing the chromatographic packing (Sephadex) from clogging the eluent needles. The closed cell foam frame was attached with silicone sealant to a Plexiglas plate on the bottom, and there was another Plexiglas plate that was placed on top of the foam frame. This created an empty space between the Plexiglas plates and inside the foam frame. This open space was entirely enclosed by Plexiglas and foam, and was where the Sephadex was placed.

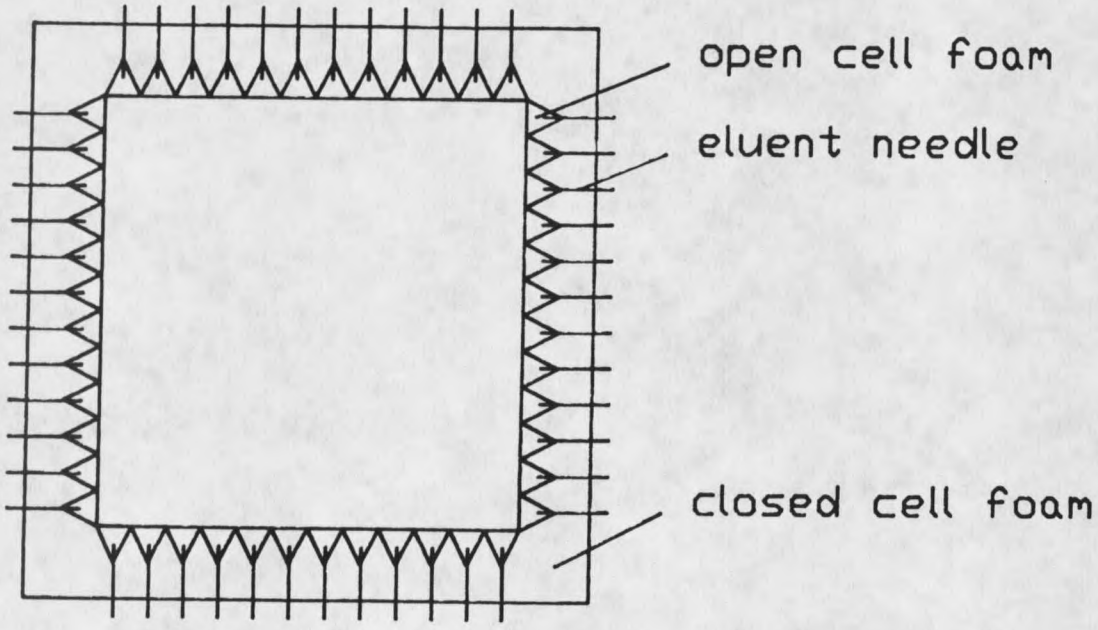

Fig. 3. Basic bed construction

There was an attempt to construct the bed out of silicone, so it would be thinner. It was found that the silicone was not compressible enough, making the bed packing process almost impossible. The wet Sephadex is a compressible mixture, and had to be compacted some in the bed packing process so it would maintain its shape while in use. The foam was compressible, hence after filling the open bed and placing the top Plexiglas plate in place, the volume of the bed would decrease in the clamping process. This bed volume change was not attainable in the silicone bed. Because the Sephadex was not compressed, it would slip and form open void spots when the eluent was introduced on a side. These open void spots disrupted the flow, and would not give reproducible results with different bed packings, so the use of the silicone bed was discontinued.

It was thought that a thinner bed would give a better separation. A thinner bed would allow the eluent less vertical motion, giving it a more constant path, and thus produce better resolution. Since the silicone did not work in making the bed thinner, a Plexiglas spacer plate was inserted into the bed to reduce the packing thickness (see Fig. 4). This Plexiglas plate had to be fixed in position, using silicone sealant, with the sealant around the outer edges of the plate, to prevent eluent from flowing under the plate, and bypassing the Sephadex. To keep the packing

a uniform thickness, it was important that this plate be very flat. It was discovered that placing a lamp close to the plate when the bed was empty and dismantled caused the plate to "lift up" and become separated from the rest of the apparatus. This proved helpful in relocating the plate if it had been positioned poorly.

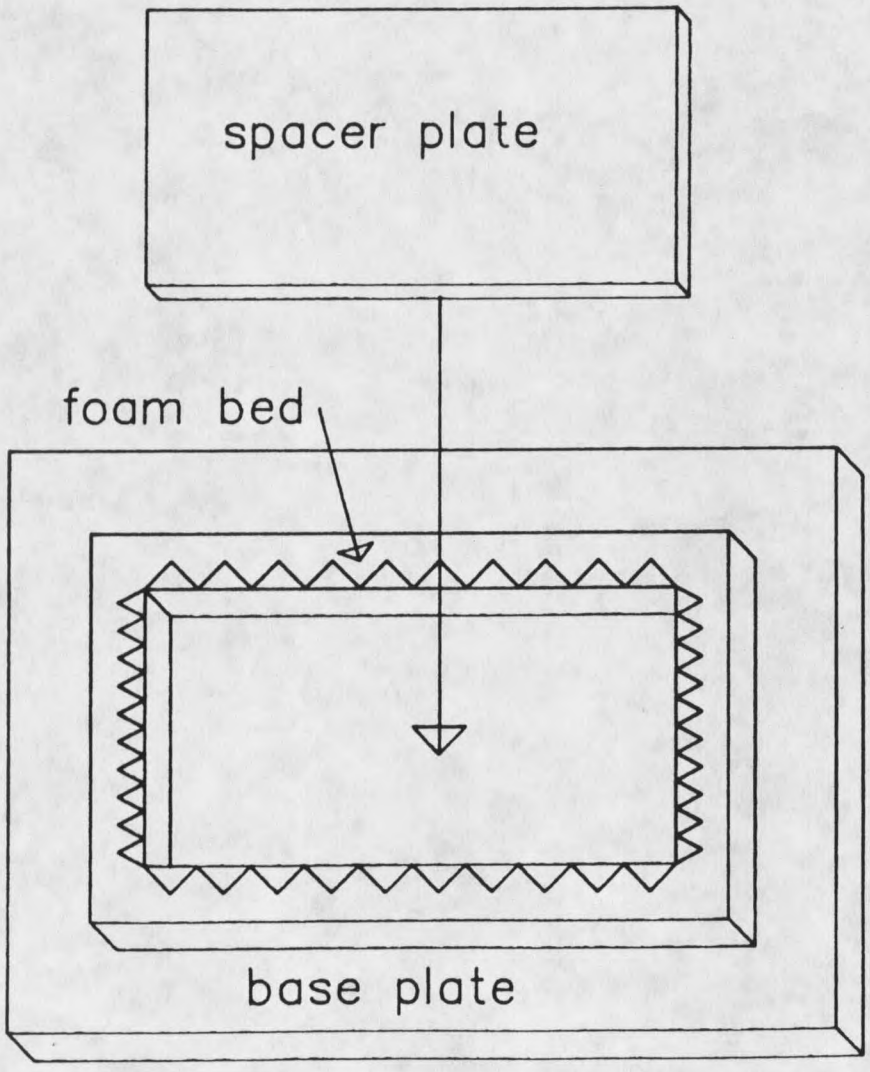

Fig. 4. Spacer plate

Because of the spacer plate, the eluent needles had to be placed high in the closed cell foam so they did not inject fluid directly into the spacer plate. To prevent leaks around these needles, they were sealed into place.

Two clamps held the upper Plexiglas plate, or cover plate, onto the base plate, which had the foam bed on it. These two clamps were flat metal bars welded into a rectangular frame, so that part of the metal bars were touching the edges of the Plexiglas plates, and the rest of the metal bars overhung the Plexiglas plates (see Fig. 5). There were six holes in the overhanging part of the metal bars, so that screws could be put in and the metal clamps could be drawn together, compressing the packing media and the bed apparatus.

An eluent feed tank with a drain hole near its base was kept on a shelf over the bed apparatus. This tank was filled with water, which was the eluent, and provided the head pressure to drive the eluent through the bed. Latex tubing connected the eluent feed tank with the bed apparatus via the "spider feeders", which will be explained later. There was also a drain tank, which was connected to the bed apparatus via latex tubing and the spider feeders.

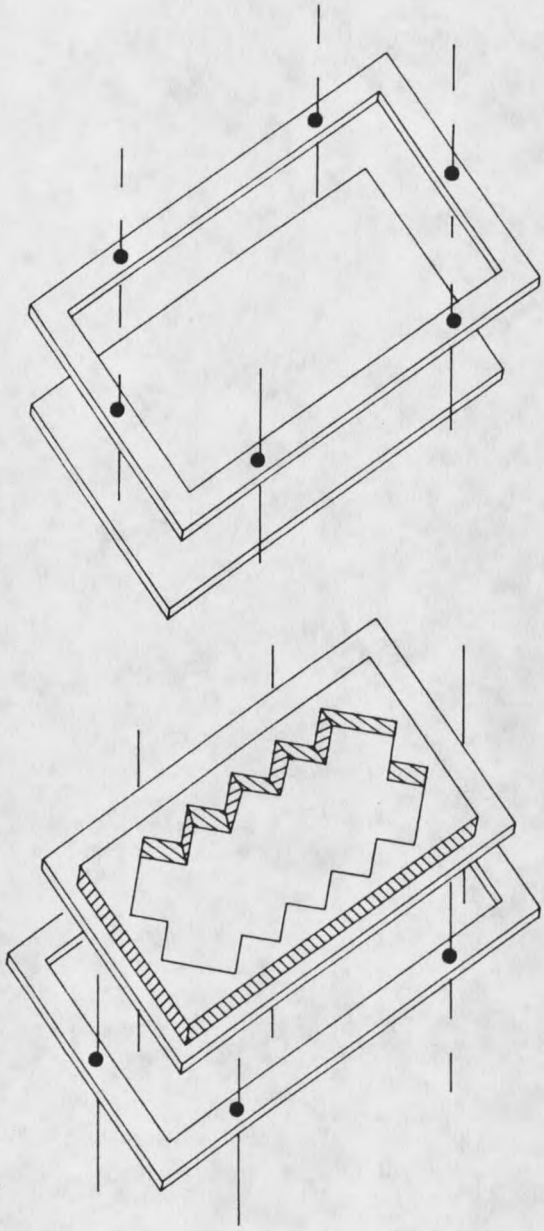

Fig. 5. Clamps

Dye was introduced by a Valcor micropump and a syringe. A small reservoir held the dye, and was connected to the pump by latex tubing. More latex tubing connected the pump to the dye introduction mount, a syringe, and the dye introduction needle (see Fig. 6). This long needle was inserted into the bed in the packing procedure, and the end of the needle was the point of dye introduction into the bed.

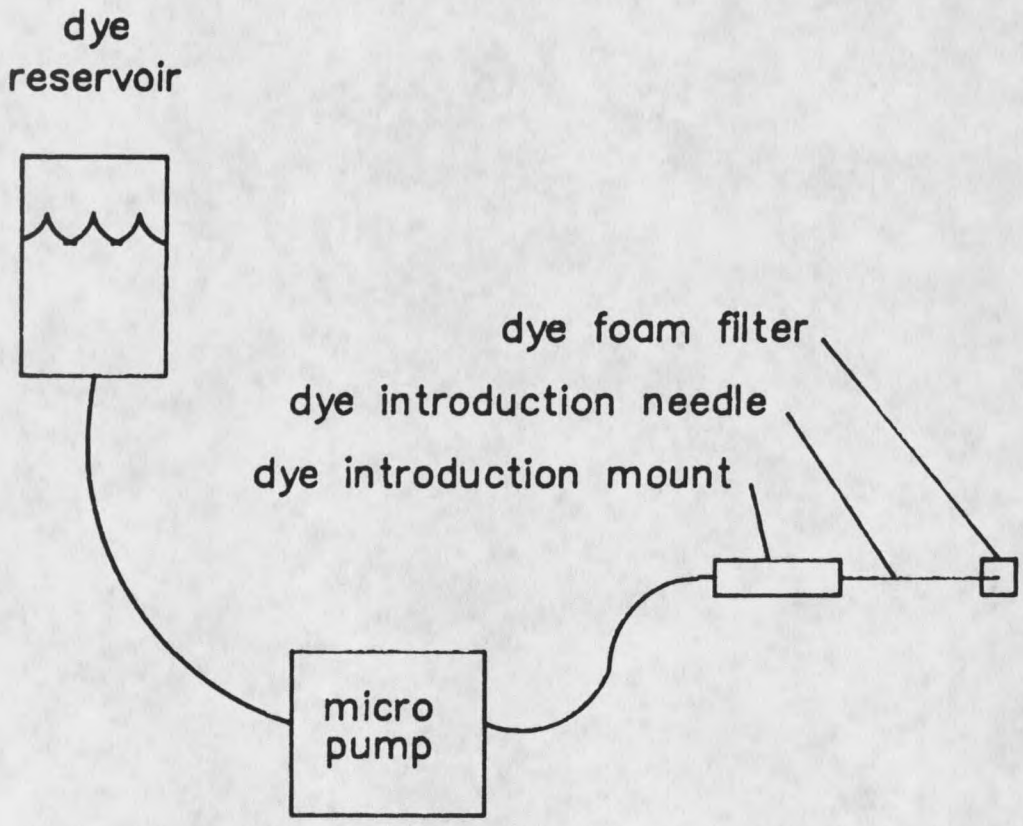

Fig. 6. Dye introduction system

The devices which controlled the flow of eluent into and out of the bed became known as spider feeders. They were constructed of Plexiglas and twelve needles each (see Fig. 7). The spider feeder needles were connected to the eluent needles by polyethylene capillary lines. The main eluent input line entered the spider feeder at the center of the circle of spider feeder needles. The spider feeder needles joined the main eluent line slightly into a trumpet bell shaped indention at the end of the main eluent line, and there was a latex diaphragm over the top of this indention. By activating a pressure source behind the latex diaphragm, the openings to the twelve needles could be closed, with no flow allowed between the different needles or between the main eluent line and any of the needles.

The pressure source behind the latex diaphragm was operated with pneumatic valves under computer control. Each spider feeder had one air line entering it behind the diaphragm. This line was branched into two lines, each of which had a valve on it. One of these lines was connected to the laboratory pressure source, and the other was open to the atmosphere. A computer would either open the pressure line and close the release line, or vice versa, for each of the spider feeders.

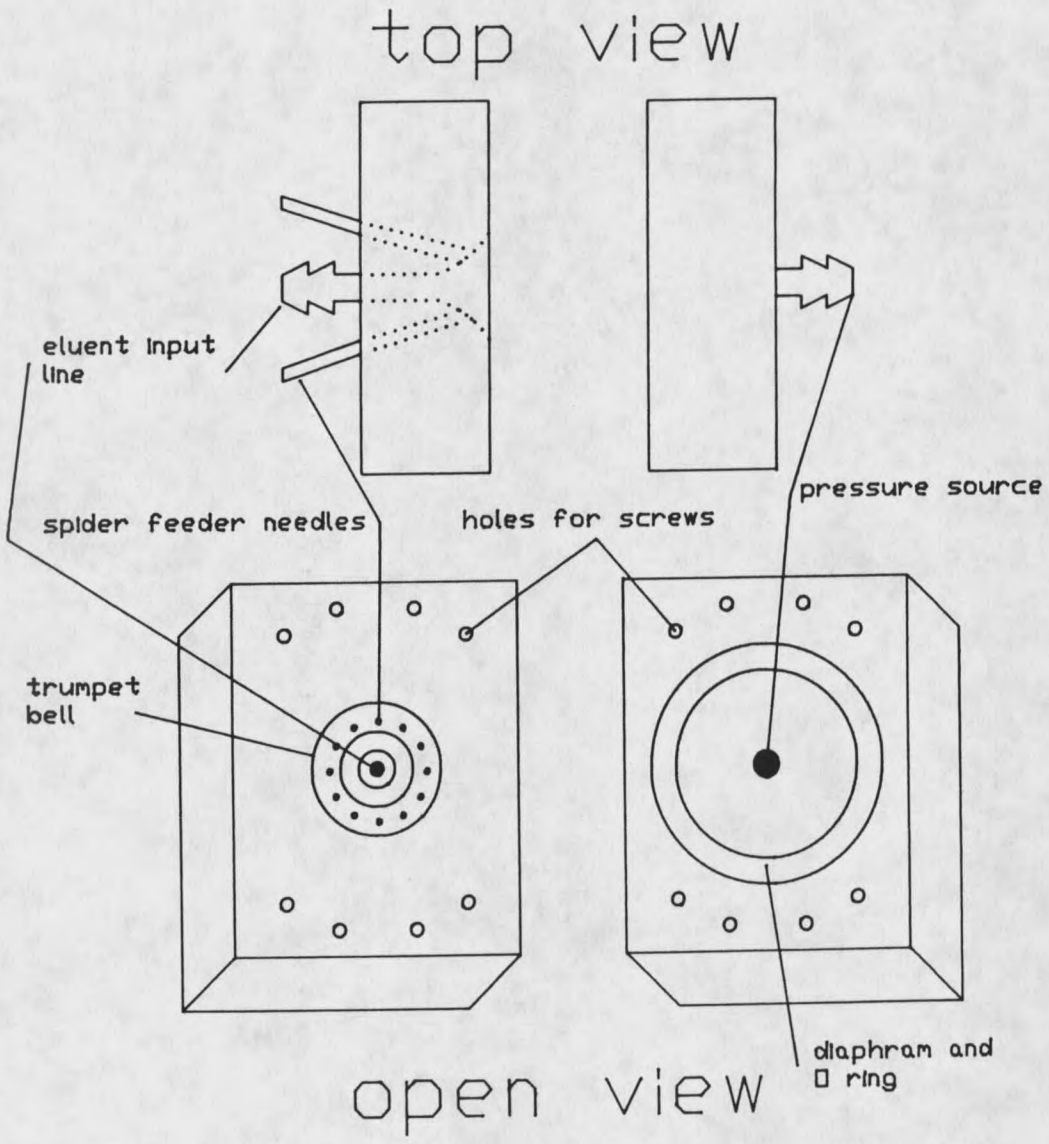

Fig. 7. Spider feeders

The product extraction holes were the last major step in the research. The relative distances travelled by the dyes were measured several times in the bed, and the results were used to determine the optimum location of the

product extraction holes (see Appendix A). For the product extraction, there were holes drilled through the spacer plate and the base plate, and matching holes drilled through the cover plate. There were eluent replacement tanks attached to the holes in the upper plate, the eluent replacement holes, which could provide pressure over the product extraction holes (see Fig. 8).

To prevent the Sephadex from draining out of the bed through the product extraction holes, filters were used. Teflon porous discs were set flush with the plates and used as the filters at first, but they were found to have too much resistance to flow, so open cell foam filters were used instead. They were also set flush with the lower and upper plates, so as not to disturb the bed pattern. There were latex tubes which connected the eluent replacement tanks to the upper holes, and the product collection tanks to the lower holes. By setting the latex tubes connected to the eluent replacement holes high over the bed, the pressure at the extraction points could be determined by measuring how high the eluent went up in the latex tube.

Fig. 9 shows all the major components in the apparatus, and has a listing of the name of each component.

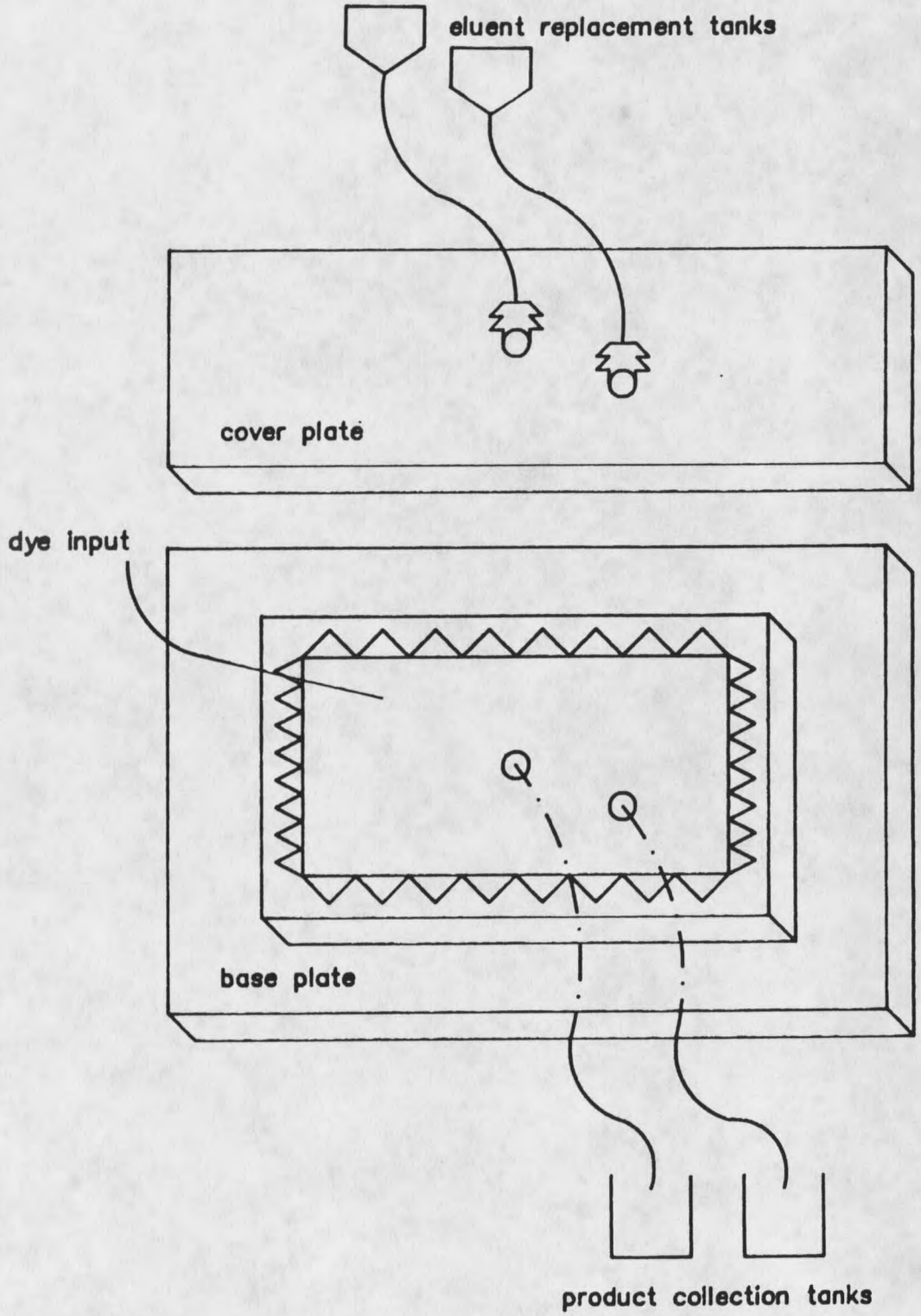

Fig. 8. Product extraction holes

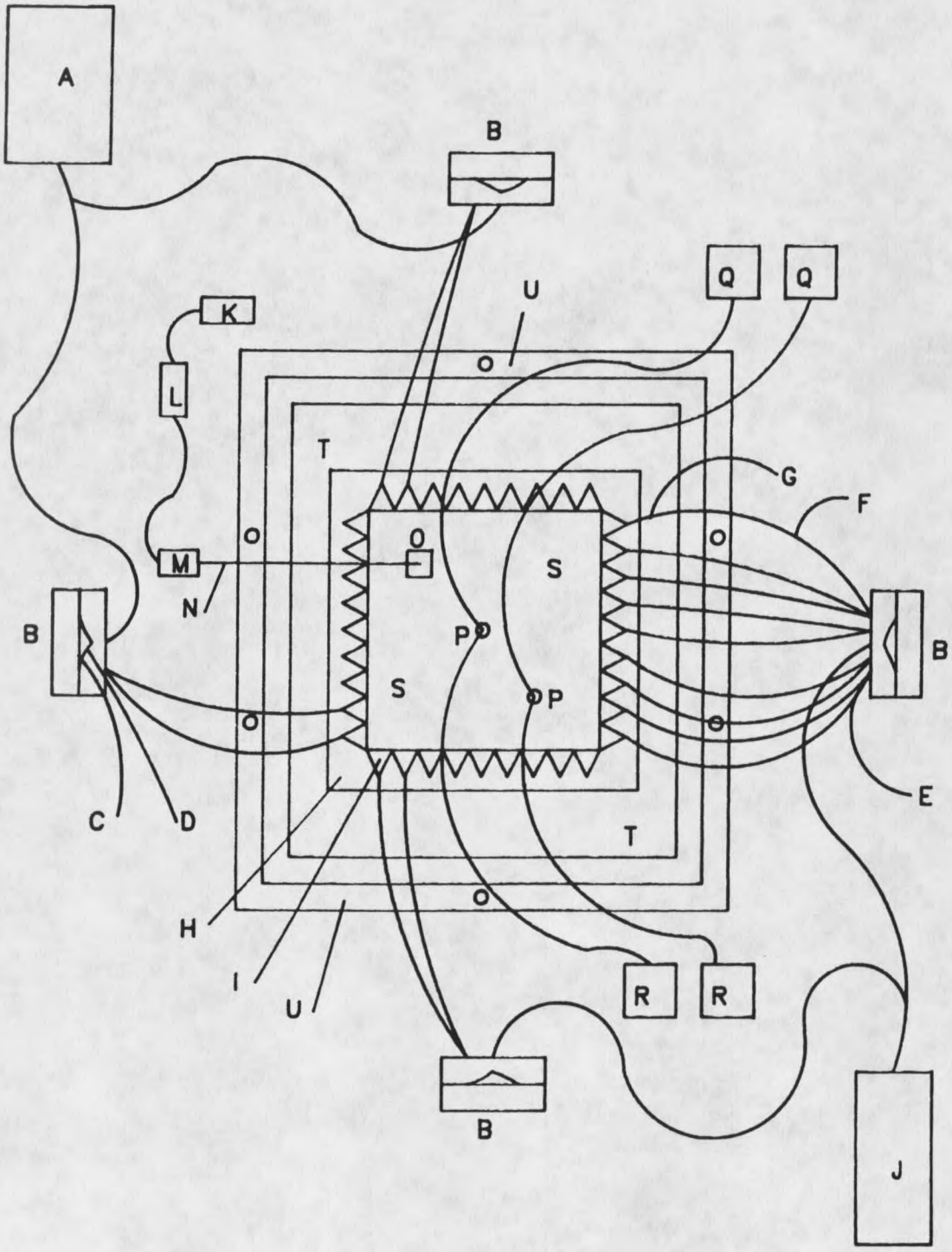

Fig. 9. Apparatus overview

A - eluent feed tank

B - spider feeder

C - diaphragm

D - trumpet bell

E - spider feeder needles

F - capillary lines

G - eluent needles

H - foam bed

I - bed foam filters

J - drain tank

K - dye reservoir

L - micropump

M - dye introduction mount

N - dye introduction needle

O - dye foam filter

P - product extraction hole (on the bottom)

P - eluent replacement hole (on the top)

Q - eluent replacement tank

R - product collection tank

S - spacer plate

T - base plate (on the bottom)

T - cover plate (on the top)

U - clamp

Fig. 9. Continued

RESULTS

In the results section, some of the difficulties which had to be overcome to make the process work will be explained. A continuous separation was demonstrated, with red dye exiting the bed from the first product extraction hole, and blue dye exiting the bed from the second product extraction hole.

To prevent leaks between the cover plate and the closed cell foam bed, some kind of gasket sealer was needed. Sno Seal, a wax-based water proofing material, was used. It did the job effectively.

Originally, a partial vacuum at the eluent extraction points, coupled with the head pressure at the introduction points, was used to move the eluent through the bed. It was discovered that the head pressure alone was adequate. This proved to be very advantageous, because the vacuum was difficult to control, and tended to draw the eluent faster than it was introduced, thus drying the Sephadex near the exit side of the bed. The head pressure alone allowed a more constant flow which was easier to adjust, and more reliable. This also made the system simpler, without the safety problems associated with a vacuum.

There were several problems to overcome with the spider feeders. The original design had twelve needles sticking out of the side of a rectangular piece of Plexiglas. All

of these needles opened up on the inside to a hole drilled most of the way into the Plexiglas (see Fig. 10). The main fluid input was on the end of the rectangular box where the drill had entered for the main hole in the center of the Plexiglas. This spider feeder proved to be unworkable because the eluent would enter the needle(s) closest to the high pressure side of the plate, travel through the main hole in the center of the spider feeder, and exit out of the needle(s) closest to the low pressure side of the bed. Thus, the eluent would skirt the Sephadex and the bed.

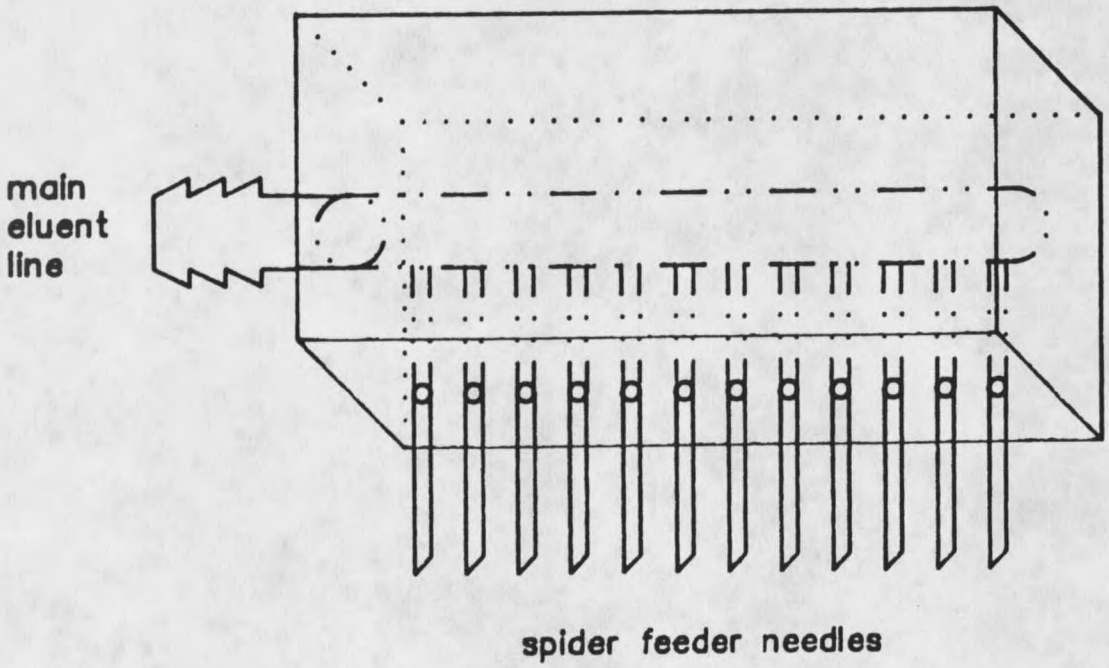

Fig. 10. Original spider feeder

Because the eluent was effectively avoiding the bed, there was no chromatographic separation. It was thought that filling the main hole in the old spider feeders with Sephadex would increase the resistance there enough so the flow would not skip the bed, but then there would be a pressure drop when that spider feeder was being used to introduce or extract eluent, and the pressure would not be uniform along the side. For this reason, it was decided that a spider feeder which was capable of closing each needle was required. This was the impetus for the design of the spider feeder explained in the experimental section.

In the operation of the system, flow would first be from left to right, then from top to bottom, then back to left to right, then to top to bottom, etc. For simplicity, the two flow directions will be referred to as the green direction, and the white direction. Shortly after the acquisition of the redesigned spider feeders, it was noticed that the flow was slower in the green direction than in the white direction. This was noticed every time for several different bed packings. So, a constant head pressure and a flow meter were used to measure the flow through each of the spider feeders. One was noticeably slower than the other three.

The first thing tried to even the flow rates was to shorten the capillary lines on the slow spider feeder. This helped, but the flow through the one spider feeder was

still significantly slower. The next step was to enlarge the trumpet bell portion of the spider feeder. This was done with sand paper, and by whittling with a razor. Enlarging the trumpet bell reduced the resistance to flow in the slow spider feeder, and resulted in the flow being much closer to that of the other spider feeders. It was used in this condition.

In the process of dismantling the bed after a run, the spider feeders and the capillary lines would become filled with air. Before repacking the bed for the next run, this air had to be cleared out of the system. This was done by hooking up the eluent feed tank to each spider feeder, running eluent through that spider feeder, and then clamping the latex tubing so the eluent could not drain out. When the Sephadex was put in place, and the cover plate clamped into position, there was always some air remaining in the capillary lines. Presumably, this was due to the open cell foam filters on the eluent needles. This open cell foam held air well, and this air would be pushed into the capillary lines from the pressure caused by clamping the cover plate onto the base plate.

It turned out that a little air in the capillary lines was not detrimental to the experiment, and proved useful because it allowed one to "see" the flow in the capillary lines. Once the air had exited the capillary lines, it was not noticed in the bed. Apparently, the open cell foam

held the air, and did not let it into the Sephadex. These bubbles proved useful for testing the operation of the computer and the valves. By pressing the eluent input line out of a spider feeder, and watching the bubbles in a capillary line, one could determine if that spider feeder was open or closed.

This testing method proved to be invaluable, because of many minor problems that happened occasionally. One of the valves would periodically become unplugged from the wall outlet, and thus one of the spider feeders would be unoperational. Also, the latex tubing connecting the valves and the spider feeders would become kinked, and prevent the operation of the spider feeders. Some of the latex tubes were kinked all the time, and did not cause any problems, so the only way to see if the kink had to be fixed was to test and see if the spider feeder was working properly. Also, the computer would sometimes be mis-keyed, and this could be seen by the flow in the bed, and double checked by testing the spider feeders with the "press test".

The capillary lines connecting the spider feeders and the bed itself sometimes became kinked, and like the latex tubing, this was only a problem some of the time. By watching the air bubbles in the kinked line, it could be determined whether or not the kink interfered with the flow. Also, the corner, or end, capillary lines often

became plugged. Presumably, this was due to the Plexiglas plates and the clamps. The Plexiglas plates which encased the bed are slightly flexible, and bend some when clamped into place. Because of this bend, the corners of the bed would get compressed the most, and this compression tended to plug or block the corner capillary lines. It was noticed that it was not essential that the corner capillary lines worked, so this was not much of a problem. This was possibly the cause of the end effects which were noticed.

There was some maintenance required for the spider feeders. One of the diaphragms broke, so they were replaced once during the course of the experiments. Also, there was a Sno Seal build up which would gradually occur. To fix that, boiling water would be run through the system. This melted the Sno Seal, and also helped to generally purge the whole system. The best results were obtained after clearing the system with boiling water.

Product Extraction Holes

The product extraction holes were the holes in the middle of the base plate where the dye would exit. These holes will be referred to as P. E. holes. There was latex tubing connecting the P. E. holes to the product collection tanks, and there were matching holes on the cover plate which were connected to eluent replacement tanks to put pressure over the P. E. holes. The eluent replacement

tanks, were connected to the eluent replacement holes with latex tubing.

To get a flow through the P. E. holes a pressure drop was needed. The pressure at these points could be measured by using the eluent replacement lines as manometers before the P. E. holes were opened. The fluid height in the eluent replacement lines could then be converted into a pressure. To get the P. E. holes to work, the product collection tanks were placed slightly lower than the measured heights of the eluent, thus giving a pressure drop. If added pressure was needed, the eluent replacement holes could be opened, and the eluent replacement tanks set at any desired height.

This pressure drop technique did not work as simply as desired. After establishing the equilibrium state at the P. E. holes, and setting the height of the product collection tanks, the P. E. holes would be opened. The opening of these holes caused the equilibrium state to change. This necessitated the establishment of a system to get both holes flowing at the same time. The first step would be getting one P. E. hole to flowing properly. The next step would be to get the other one operating. After the second hole was functioning properly, it was frequently noticed that the first hole would no longer be flowing. Because of the interdependence of the P. E. holes, their effect on the whole system, and the fact that the flows

could not be too large, it was difficult to get both holes working at the same time.

Another frequent problem was the equilibrium state would change with time. The system would be working properly, and for no apparent reason, it would quit working. The entire system was very fickle, which made operations more difficult, and less desirable to industry.

One technique which was tried to make the extraction process more efficient was to place a product extraction plate between the P. E. hole and the eluent replacement hole (see Fig. 11). This plate was supposed to help direct the eluent out of the bed, and yield a better separation. From the experiments done, it seemed as though the product extraction plate did help the operation of the P. E. holes, by making the use of the eluent replacement lines more effective, but the results were not conclusive.

Opening the eluent replacement holes had mixed results. Sometimes, this pressure would cause the flow out of the P. E. holes to increase significantly. Other times, this would cause the eluent flow to skirt the P. E. holes, because there was a higher pressure there than in the bed near the hole. This caused the dye extraction process to be greatly hindered. If the use of the eluent replacement holes could be made to work consistently, it would make the system more attractive to prospective users.

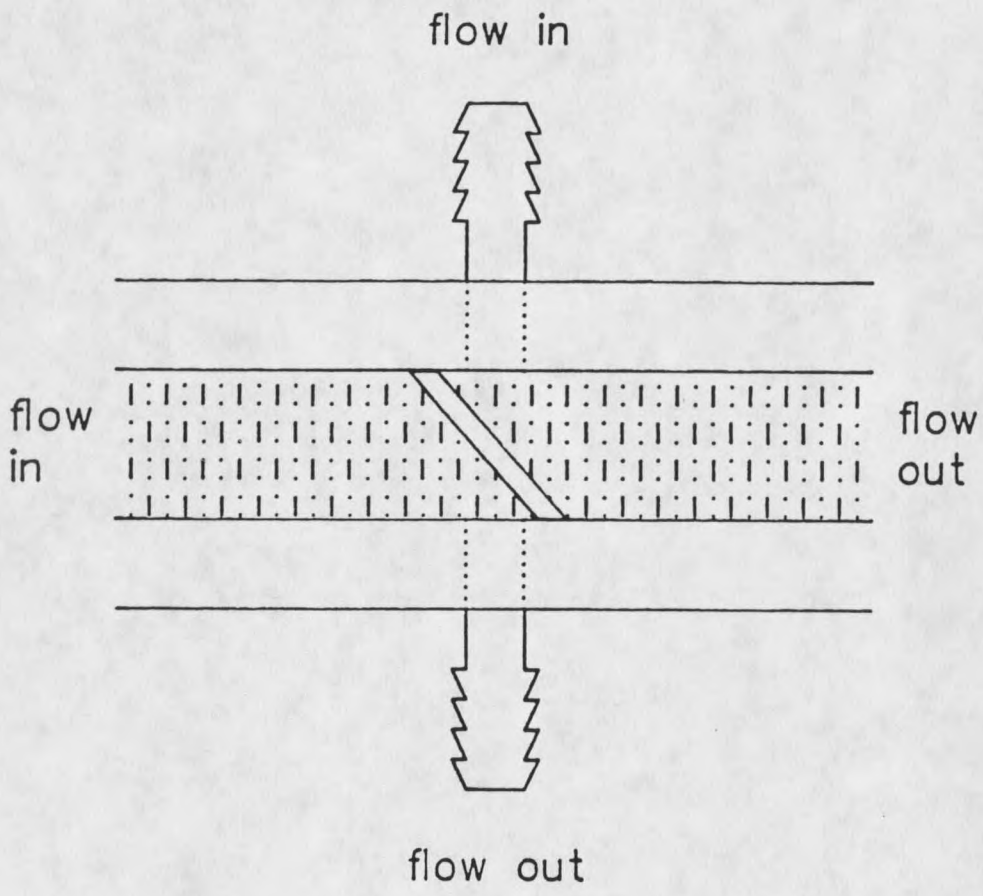

Fig. 11. Product extraction plate

The red dye moved through the Sephadex slower than the blue, and thus the red dye was extracted at a point closer to the dye introduction point. The blue dye extraction hole was set with the flow quite high, because there was no reason to worry about the bed flow pattern at the far end of the effective part of the bed. The red dye, though, had to be extracted slowly, so as not to disturb the bed flow pattern too much. Because of the slow rate of flow out of the red P. E. hole, not all of the red dye would exit, even

would exit, even if it passed directly over the hole. Part of the reason for this was that the red dye moves about half as fast as the eluent through the Sephadex. In the time it takes eluent to travel from a point close to the cover plate down through the Sephadex to the P. E. hole, a red dye molecule would have only gone half way through the Sephadex, and would not be ready to exit the bed. Hence there has to be about twice the flow going down the first (red) hole as down the second (blue) hole to achieve the same amount of dye being carried down the hole. This was a problem, because the first hole was the one which should have had the slower flow rate, so as not to disturb the bed flow pattern.

A second problem caused by the red dye not all going down the first hole was the extra dye would be carried to the second hole. Because the red dye moves at about half the rate of the blue dye, all the red dye that did not drain in the first hole was then in position to drain down the second hole. Also, because of the high flow rate down the blue hole, even if the red dye did not flow directly to the blue hole, it would probably be drawn down it anyhow. Furthermore the red dye left from the first hole would be in the lower part of the Sephadex, and thus more likely to go down the second hole. It would be in the lower part of the Sephadex because the flow down the first hole would carry everything that passed over it downward.

Experimental Protocol

In the process of making a run, the first step was to pack the bed. This involved (1), clearing the spider feeders of air, (2), putting the Sephadex in the bed, (3), placing the Sno Seal on the closed cell foam, (4), placing the dye introduction needle in the proper place, (5), placing the cover plate in position, (6), placing the clamp, and (7), tightening the clamp. Better results were obtained by putting the Sephadex in after it had been soaked in water for only a short period. It would then swell slightly after it was packed in the bed, producing a tighter fit. The wet Sephadex would be poured into the bed, and then moved around with a glass rod until it was of fairly uniform depth, and just a little bit higher than the closed cell foam.

The location of the proper dye introduction point could be found using a ruler. The distance from the first P. E. hole to the dye introduction point was 5.1 cm. The point was also in line with the two P. E. holes. A mark was made on the cover plate at the proper dye introduction position with a water soluble pen. When the ink washed off, the point would have to be found again using this technique. Since the eluent replacement holes were directly over the P. E. holes, this mark would always correspond to the same point in the foam bed. By holding the cover plate over the

foam bed, and looking at the mark, the dye introduction needle could be placed quickly.

When the cover plate was placed, it was done so as not to get any air in the bed. This was done by first setting one edge where it would go, and then slowly laying the plate on top of the Sephadex. The part of the plate which would be coming into contact with the Sephadex would be pushed down, and the far end held up, so that the air would be pushed out as the plate settled down onto the Sephadex and the foam bed. The slight flexibility of the Plexiglas helped significantly. The main problem when packing the bed was not to get any air trapped inside, and to prevent any cracks from forming when eluent was introduced. The packing of the bed was a difficult step, and some practice was definitely needed. Experience seems to be the only way to get reliable results.

After the cover plate was in place, the clamp would be put in position and tightened down. It was found that if the clamp was tightened too much, the flow through the bed would be restricted. However, if the clamp was not tight enough, the bed would leak. Experience seems to be the only way to determine how tight the clamps have to be.

If the product extraction plates at the P. E. holes were to be used, they had to be placed in the Sephadex before the cover plate was put in position. They would be slid into the Sephadex at the desired locations so that

only the tips of the top edges were above the Sephadex. If too much of the top edges were sticking up, the placement of the cover plate would cause a shifting of position of the product extraction plates.

After the bed was packed, and it was ready for use, the eluent from the eluent feed tank would be released. This caused a pressure which would enter one side of the bed all at once, and if the conditions inside the bed were not proper, the Sephadex would develop cracks. These cracks were either void, or full of air, and would not allow eluent to pass through them. This disrupted the flow pattern in the bed, which would then have to be dismantled and repacked. After putting in the P. E. holes, it was found that the bed could not be packed so that cracks did not develop. This was because the open cell foam used as filters for the P. E. holes allowed too much compression under the higher pressures of the bed in operation. When the foam compressed, it gave room for the Sephadex to move in the bed, and cracks developed. To fix this, the foam filters were made as small as possible.

From the repeated use of the bed, the closed cell foam became compressed, and the height of the bed decreased during periods of heavy use. One time the foam had compressed so much that the spacer plate under the Sephadex was actually higher than the foam bed. A period of rest for the foam would bring it back to a nearly non-compressed

state. The corners would get the most compression because of the way the Plexiglas plates would bend, and they would not recover their shape after a rest. Because of this, foam inserts were needed at the corners of the bed to keep the foam higher than the spacer plate, and thus keep the bed operational.

It was found that waiting a day after unpacking the bed allowed the foam enough time to swell again to a usable degree. Occasionally, longer waits were needed for the sake of the foam. Also, after packing the bed with Sephadex, it was found that giving it a day to settle and swell caused the bed to crack much less frequently. This was perhaps due to the swelling effects of the Sephadex. Sephadex swells to almost its full amount quite quickly, but then slowly swells until it has reached its maximum volume. So, every time the bed had to be packed caused a two-day delay in experiments, because of the day for the foam to swell, and the day for the Sephadex to swell.

The dye input was achieved by a micropump, and an hypodermic needle. The dye was stored in a small reservoir made out of a syringe, and another syringe made a mount for the hypodermic needle. Latex tubing was used to hook the various parts together. The dye introduction and the P. E. hole locations were determined by the tests summarized in Appendix A, so the dye introduction location was fixed after the P. E. holes were drilled. To avoid end effects,

the P. E. holes and the dye introduction point were put in the middle of the bed. It was found that using different sized hypodermic needles to prevent plugging with Sephadex did not work, so a small open cell foam filter was used. The disadvantage of the filter was it allowed the dye to pass through it very fast. Because of this, the location of dye introduction was not a point, but an area. Making the foam filter small, though, minimized this problem. The hypodermic needle caused indentations in the closed cell foam which would not expand out, so these had to be filled with Sno Seal to prevent leakage.

The flow rates in the green and white directions were not always the same. There was work done on one of the spider feeders, as was previously mentioned, but the problem was still there. Sometimes the flow in the white direction would be faster, and sometimes the flow in the green direction would be faster. Also, the equilibrium pressure in the P. E. holes would be different for the green and white directions. The different flow rates could be handled by having the flow go longer in one direction than the other, but the pressures in the P. E. holes had to be the same in both directions. To even these pressures, a clamp would be placed on the main flow line from one of the spider feeders, thus increasing the resistance to flow in that spider feeder. By using this clamp, the pressures in

the P. E. holes would be made the same. As a result, the flow rates usually came out pretty close to the same.

Because the pressure at the P. E. holes can be measured, and the pressures at the input and the output are also measurable, the pressure drop in the spider feeders and in the Sephadex can be calculated. A linear pressure drop is assumed because the packing is homogenous, and of a constant thickness. The red hole was 8.3 cm. from the high pressure side of the bed, which was 18.3 cm. square. Thus, the pressure at the red hole should be (18.3 - 8.3)/18.3 = 0.55, or 55 percent of the total bed pressure drop more than the pressure at the eluent extraction side. Similarly, the pressure at the blue hole should be 37 percent of the total bed pressure drop more than the pressure at the extraction side. Thus, the following equations hold.

0.55*X = red pressure - Y

0.37*X = blue pressure - Y

where

X = pressure drop in the bed.

Y = pressure at the extraction side.

The pressure at the eluent input side is X + Y. By comparing the measured exit pressure with Y, and the measured input pressure with X + Y, the pressure drop in the spider feeders can be obtained.

Table 1. Pressure drop in the spider feeders.

run number	pressure drop at input spider (cm. water)	pressure drop at output spider (cm. water)
1	8.1	24.4
2	7.0	17.6
3	14.1	27.4
average	9.7	23.1

The pressure at the product extraction holes was averaged for the two flow directions. The pressure drops were not very consistent, due to the clamp used to even the pressures in the P. E. holes, Sno Seal build up, and other unknown factors.

The flow rates and the pressures at the P. E. holes were variable, and would often change in the middle of a run for no apparent reason. This requires the system to be supervised when in operation, which is a disadvantage. The average flow rate through the whole bed was about 2 ml per minute, and a list of the measured flow rates is in Appendix A.

The reproducibility of the bed, as was mentioned, was not good. However, the path taken by the dye was consistent. After one bed sat for several days, the dye would follow the same route as before. If a new bed was packed, the dye would follow a route close to the one of the previous bed. This makes the system usable, if only the reproducability of the flow rates and pressures can be

improved. It was also noticed that the dye near the edge of the bed moved slower than that in the middle. These end effects were not a problem, though, because the separation was done in the central part of the bed.

It was noticed that after a flow direction change, the bed was not in equilibrium immediately. Because of the unsteady state following flow direction changes, it was decided that the best technique would use the fewest flow direction changes possible. This produced a square wave eluent introduction technique. A mathematical model of the system after a flow direction change is described below.

The equation of continuity is

$$\partial\rho/\partial t + \partial/\partial x\ (\rho V_x) + \partial/\partial y\ (\rho V_y) + \partial/\partial z\ (\rho V_z) = 0$$

where

ρ = density t = time

V_x = velocity in the x direction

V_y = velocity in the y direction

x = length in the x direction

y = length in the y direction

In this work, V_z is assumed to be zero at all times, so the $\partial/\partial z$ term drops out of the equation. Rearrangement produces

$$\partial\rho/\partial t + \rho*(\partial V_x/\partial x + \partial V_y/\partial y) + V_x*\partial\rho/\partial x + V_y*\partial\rho/\partial y = 0 \quad (1)$$

Density is a function of T(temperature) and P(pressure). The system is assumed to be isothermal, so ρ is a function

of P only. Thus $\rho = f(P)$. A Taylor expansion on the pressure function gives

$$\rho = f(P) = f(Po) + (P-Po)*f'(Po) + \ldots$$

where

Po - some set initial pressure

Truncation after the first term gives

$$\rho = k + C*P$$

$$k = f(Po) - Po*f'(Po) \qquad C = f'(Po)$$

Differentiation of both sides of the last equation with respect to time gives

$$\partial\rho/\partial t = C*\partial P/\partial t \quad (2)$$

This assumes that the density is not constant. This is true, because Sephadex G-25 is a compressible gel. The density does not change very much, since the gel remains fixed in the bed, but the gel mixture is compressible.

If Eq. (2) is plugged into Eq. (1), the result is

$$\rho*(\partial V_x/\partial x + \partial V_y/\partial y) + V_x*\partial\rho/\partial x + V_y*\partial\rho/\partial y = -C*\partial P/\partial t \quad (3)$$

The $V_x*\partial\rho/\partial x$ and $V_y*\partial\rho/\partial y$ terms are neglected, because they are very small when compared to the $\rho*\partial V_x/\partial x$ and $\rho*\partial V_y/\partial y$ terms. Darcy's law says that

$$V_x = c*\partial P/\partial x \text{ and } V_y = c*\partial P/\partial y \quad (4)$$

c = a constant

If Eq. (4) is substituted into Eq. (3), the result is

$$\rho*c*(\partial^2 P/\partial x^2 + \partial^2 P/\partial y^2) = -C*\partial P/\partial t$$

This can be rearranged to

$$\partial P/\partial t = KK*(\partial^2 P/\partial x^2 + \partial^2 P/\partial y^2) \quad (5)$$

$$KK = -(\rho*c)/C$$

This gives an equation which predicts the pressure anywhere in the bed. The fluid velocity is directly proportional to the pressure drop in the bed by Darcy's law, so the velocity could be calculated anywhere in the bed if the constants c and KK were known.

These constants are not known, and would have to be measured. There was a tube connected to the eluent replacement holes, which could serve as a manometer to measure the pressure at those points. But, because the tubes were so large, they took a lot of water to fill, and this upset the normal operation of the bed. These manometers caused a new pressure source or sink. To find accurate values for the constants, a pressure measurement device which did not disturb the bed would be needed.

The exact values of the constants are not needed, though. It is known that there is a transition stage between equilibrium flow in the two directions, and this suggests that the fewest direction changes possible for the fluid is the best way to get a good separation.

Darcy's law holds for flow in a porous medium if the Reynolds number is less than one[12].

$N_{re} = D_p * G_o / \mu$

where

N_{re} = Reynolds number D_p = average particle diameter

μ - viscosity G_o = mass velocity of fluid

For Sephadex G-25, the average particle diameter was 100 microns, or 0.01 cm. The mass velocity of the fluid can be calculated from the cross sectional area of the bed, and the fluid flow rate. The cross sectional area was the product of 18 cm by 0.25 cm, or 4.5 cm^2. The average flow rate was 2 ml per minute, or 2 grams per minute, since the fluid was water. This gives a mass velocity of the fluid of 0.0074 grams/cm^2 sec. The fluid is water, so the viscosity is 0.01 grams/cm sec, or 0.01 poise. This gives a Reynolds number of 0.0074, which is less than one, so the use of Darcy's law is valid.

A computer program which predicts pressures at various points and times can be found in Appendix B. Some results from this program can also be found in Appendix B.

The bed was watched, and when the dyes were in line with the P. E. holes, a flow direction change would be made manually. Because of the variability of the flow rates, the computer was not set on a timer for the flow direction changes. The dyes formed broad bands, especially the red dye, and would be aligned with the P. E. hole for thirty seconds or more. If the timing was off some, it did not

cause much of a problem. This would not be so if the dyes were to stay more concentrated in the Sephadex.

The blue dye concentrations were measured before the dye entered the bed, and after it exited the bed. By making up known concentrations of blue dextran dye in water and measuring the absorbance of light by these mixtures, a calibration curve was made (see Fig. 12). Next, the dye entering the bed and the blue dye product were tested for their light absorbancies. The concentrations of these unknowns were then found by interpolating between the known points in the calibration curve.

Filters were used so that the light for these tests would be absorbed by the blue dye, but not by the red dye. A wavelength of 710 microns was found to be effective. The concentrations and their corresponding absorbancies are in Table 2.

This shows that the concentration of the dye was diluted by a factor of about 14 in the chromatographic process. There was a dilution of the blue dye, but no attempt was made to maximize the dye introduction rate, or to minimize the dye dilution. It is possible that the dyes would not be diluted as much under different operating conditions.

Table 2. Dilution of the product.

Absorbance	Concentration (grams/liter)
0.789	0.029
0.498	0.0145
0.348	0.010
0.192	0.005
0.097	0.0025
0.049	0.00125
0.635*	0.0213
0.057**	0.0015

* - The sample which was injected into the bed.
** - The sample which was recovered from the bed.

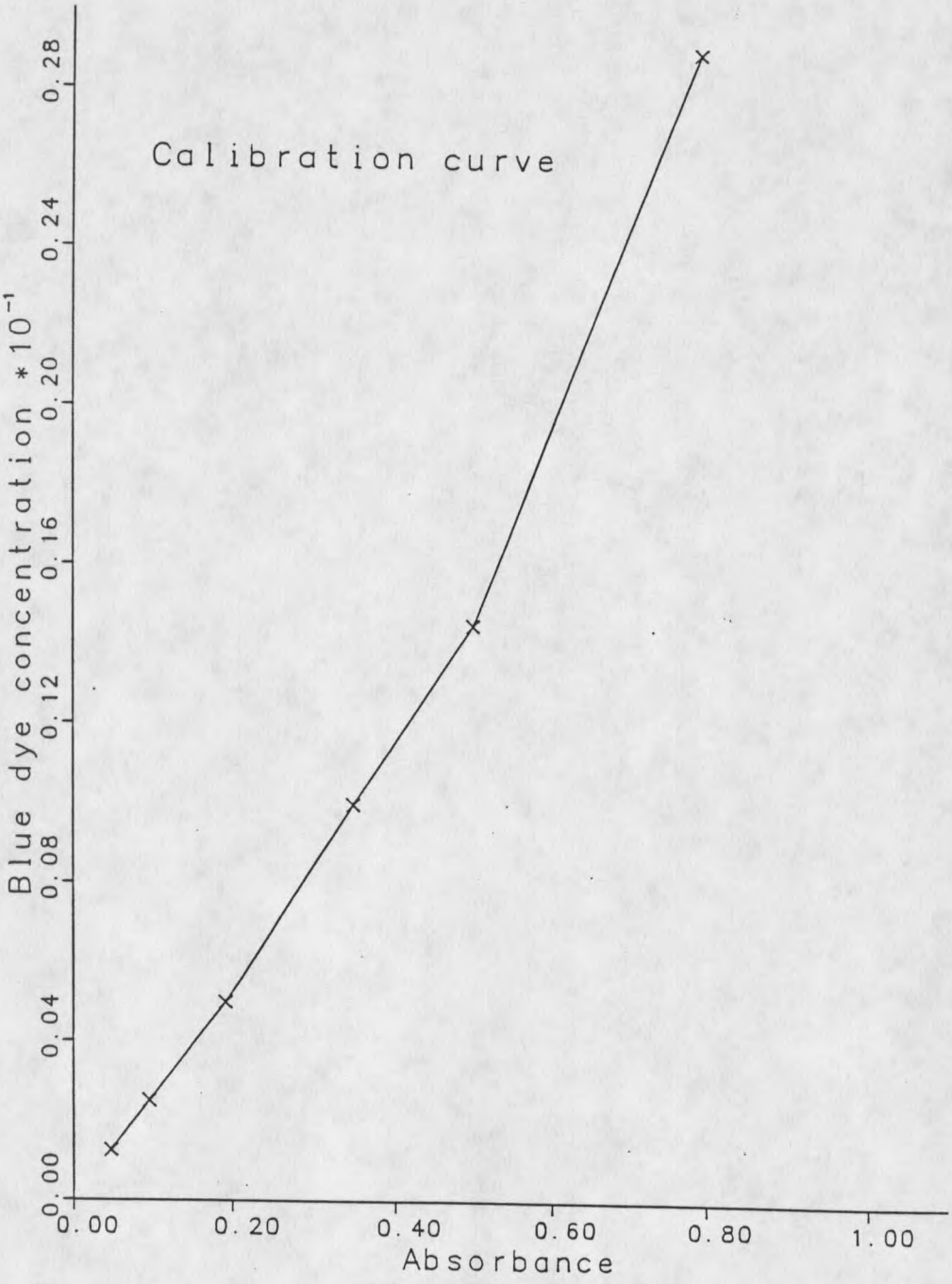

Fig. 12. Concentration calibration curve

SUMMARY

This experiment shows that a continuous separation using the apparatus discussed is possible. A continuous separation was achieved with red dye exiting the bed from the first P. E. hole, and blue dye exiting from the second P. E. hole. The solutions from the P. E. holes were either red or blue, depending on which hole they came from, and were easily distinguished from the dye which was injected into the bed. This adds to the available tools for the chemist or the chemical engineer to use. Perhaps with further development of this technique, it will enter the industrial market.

> While there are examples of industrial use of gel chromatography for protein purification and even though it continues to enjoy great popularity among biochemists for small-scale separations, we perceive a hesitancy to apply the method within the Biotechnology industry, due to some inherent problems.
> These problems are:
> 1.) Low productivities due to limited feed and flow rate capabilities;
> 2.) Low column efficiencies;
> 3.) solute dilution;
> 4.) Lack of information about costs involved in large-scale operations.[13]

The utilization of this technique could help solve the first inherent problem listed above, by separating large quantities at a time. Although the technique is not yet perfected, it does work, and deserves further study.

This research has shown several things necessary for this system to work. (1) The eluent must be forced to flow

through the packing, and not be allowed to avoid it by going through the spider feeders or any sort of eluent introduction system. (2) The bed must be packed so that no cracks or air pockets are present. (3) Gravity flow systems seem to work the best, but if a larger pressure drop is needed, it should be done by increasing pressure on the high pressure side, not by lowering pressure on the low pressure side. (4) If a compressible packing is being used, the fewest flow direction changes possible should be used. (5) Filters of some kind are needed at all points where needles enter into the bed packing material. (6) The product extraction holes are very interdependent, and must be considered together.

For this system to be effective, there are some points which must be dealt with. (1) The eluent introduction system has to be more consistent, producing constant and reliable flows. Some sort of constant flow pump for each eluent needle, at least on the eluent introduction side of the bed, might work. (2) The product extraction system has to be made more consistent. Perhaps a constant flow pump at the P. E. sites would be effective. It is possible that the open cell foam used as the filters would become blocked, or partially blocked, by the Sephadex. This would not necessarily happen immediately, and could be responsible for a significant portion of the difficulties encountered. Suggestions for further work are below.

1) The results should be compared to the results of a column to find out how effective the new technique is.

2) Different plate arrangements should be tried. It seems quite possible that problems like the red dye going down the blue hole could be avoided. For example, consider the dye introduction point and the P. E. holes were in one line, a base line, parallel with one side of the bed. First, introduce eluent perpendicular to this line, then parallel to the line. The dye introduction would have to be shut off while the flow was parallel to the base line. The parallel flow would leave lines of dye, or product, perpendicular to the base line at the P. E. hole locations. At this point, the dye introduction would be turned on again, and the flow set perpendicular to the base line, in the opposite direction as before. The dye would drain down the P. E. holes, and the system would be ready for parallel flow again.

3) Different packings should be tried. There are many chromatographic packings for different uses, and they would make this tool much more powerful if it were demonstrated that they can be used. HPLC packings and pressures result in much better resolutions, and make possible separations which are not possible with more conventional systems. For these reasons, they should be tested also.

4) A Bio-Rad packing was tested, and found to be too difficult for the packing process because it was not

compressible. Some sort of solid packing, or a matrix of some kind to attach the packing to, would help.

5) The system should be tested to find what the maximum dye input capabilities are, and compared to the results from column chromatography work.

6) Constant flow spider feeders should be developed to give the system more reliability. These should be set up so the pressure at the P. E. holes is the same for flow in both directions, and the pressure stays the same over extended periods of time.

7) The product extraction plate should be experimented with more.

8) A different filter, other than the open cell foam used, should be tried at all points were the open cell foam filters were used.

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APPENDICES

APPENDIX A

PRODUCT EXTRACTION DATA

Table 3. Relative distances traveled by the dyes.

red dye	blue dye	ratio (red/blue)
5.3	12.6	0.42
6.1	11.2	0.54
8.5	15.0	0.57
4.4	7.9	0.56
4.4	7.2	0.61
6.2	10.6	0.58
6.5	14.0	0.46
6.0	11.1	0.54
8.7	16.0	0.54
5.5	11.6	0.47
5.6	12.4	0.45
4.3	8.5	0.51
5.4	11.3	0.48
4.1	8.6	0.48
3.2	7.3	0.44
4.0	9.3	0.43
4.9	9.7	0.51
5.5	11.6	0.47
6.0	11.9	0.50
5.1	10.6	0.48
5.2	10.0	0.52
3.8	6.8	0.56
4.5	8.8	0.51
5.1	10.1	0.50
4.8	10.1	0.48
5.3	10.4	0.51
4.9	9.7	0.51
4.5	9.0	0.50
4.8	8.9	0.54
3.7	7.4	0.50
4.5	9.8	0.46
3.9	6.8	0.57
5.5	10.8	0.51
6.3	12.0	0.53
5.1	9.2	0.55
3.6	6.7	0.54

Before drilling the product extraction holes, the relative distances traveled by the red and blue dyes were measured several times. The ratios of these distances were calculated, and averaged. The averaged ratio was then used

to determine the location of the product extraction holes. These dye distances and ratios are listed above in Table 3.

The average of these ratios is 0.51. The standard deviation is 0.044. The first hole was drilled 5.1 cm. from the dye input point, and the second hole was 10.0 cm. from the dye input. A list of the recorded flow rates through the entire bed is below. The numbers are in ml./min.

1.25 1.20 1.45 2.29 1.00 0.67 3.70 4.00 2.20 2.50

The average flow is 2.03 ml./min. with a standard deviation of 1.07.

APPENDIX B

COMPUTERIZED MATH MODEL

```
      SUBROUTINE INITAL
      COMMON/T/TH,NFIN,NRUN/Y/P(7,7)/F/DP(7,7)
C     THIS IS USING THE RESULTS OF THE FIRST RUN AFTER
IT C       REACHED STEADY STATE
      DO 100,I=1,7
      P(I,1) = 0.0
      P(I,2) = 16.667
      P(I,3) = 33.333
      P(I,4) = 50.0
      P(I,5) = 66.667
      P(I,6) = 83.333
      P(I,7) = 100.0
100   CONTINUE
      RETURN
      END
C
      SUBROUTINE DERV
      COMMON/T/TH,NFIN,NRUN/Y/P(7,7)/F/DP(7,7)
      REAL DPX(7,7), DPY(7,7), DDPX(7,7),  DDPY(7,7)
      DO 200, I=1,7
      P(1,I) = 100.0
      P(7,I) = 0.0
200   CONTINUE
      CALL DSS022(0.0,18.0,7,7,1,P,DPX)
      CALL DSS022(0.0,18.0,7,7,2,P,DPY)
      DO 300, I=1,7
      DPY(I,1) = 0.0
      DPY(I,7) = 0.0
300   CONTINUE
      CALL DSS022(0.0,18.0,7,7,1,DPX,DDPX)
      CALL DSS022(0.0,18.0,7,7,2,DPY,DDPY)
      DO 400, I=1,7
      DO 400, J=1,7
      DP(I,J) = DDPX(I,J) + DDPY(I,J)
400   CONTINUE
      DO 350, I=1,7
      DP(1,I) = 0.0
      DP(7,I) = 0.0
350   CONTINUE
      RETURN
      END
C
      SUBROUTINE PRINT
      COMMON/T/TH,NFIN,NRUN/Y/P(7,7)/F/DP(7,7)
      WRITE(2,550),TH
      WRITE(6,550),TH
550   FORMAT(1X,2(/),1X,'TIME',2X,F8.3)
      DO FOO, I=1,7
      WRITE(2,600),P(I,1),P(I,2),P(I,3),P(I,4),P(I,5),
     @          P(I,6),P(I,7)
      WRITE(6,600),P(I,1),P(I,2),P(I,3),P(I,4),P(I,5),
```

```
     @          P(I,6),P(I,7)
500       CONTINUE
600       FORMAT(1X,7(F8.2,3X)
          RETURN
          END
```

Fig. 13. Computer program to simulate flow in the bed

This program predicts the pressure at various points in the bed at different times. The pressures were predicted in the bed in a seven by seven matrix, which was evenly spaced. The flow should go from the high pressures toward the low pressures, and be proportional to the pressure drop, by Darcy's law. The constants were not known for the equations, so the time units are unknown, and the exact pressures are also unknown. This equation, therefore, only gives the direction of flow in the bed, but it is adequate to demonstrate that the flow direction in the bed does not change quickly. Some results from the computer program can be found in Fig. 14.

TIME	20.000					
100.00	100.00	100.00	100.00	100.00	100.00	100.00
72.63	73.75	76.42	80.20	83.98	86.65	87.78
50.64	52.55	57.10	63.52	69.94	74.49	76.39
34.93	37.15	42.48	50.00	57.52	62.85	65.07
23.61	25.51	30.06	36.48	42.91	47.46	49.36
12.23	13.35	16.02	19.80	23.58	26.25	27.37
0.00	0.00	0.00	0.00	0.00	0.00	0.00
TIME	60.000					
100.00	100.00	100.00	100.00	100.00	100.00	100.00
82.58	82.64	82.92	83.27	83.63	83.91	83.96
65.26	65.37	65.89	66.55	67.22	67.74	67.85
48.55	48.67	49.26	50.00	50.74	51.33	51.45
32.15	32.26	32.78	33.45	34.11	34.64	34.74
16.04	16.10	16.38	16.73	17.08	17.36	17.42
0.00	0.00	0.00	0.00	0.00	0.00	0.00

Fig. 14. Results from computer program in Fig. 13