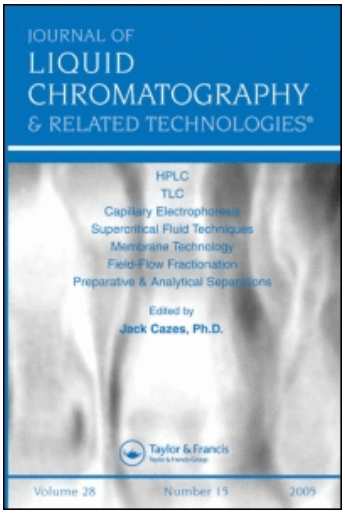


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Preparative Liquid Chromatography. I. Approaches Utilizing Highly Compressed Beds

Donald E. Nettleton Jr.[a]
[a] Dept. of Medicinal Chemistry, Bristol Laboratories, Syracuse, N.Y.

**To cite this Article** Nettleton Jr., Donald E.(1981) 'Preparative Liquid Chromatography. I. Approaches Utilizing Highly Compressed Beds', Journal of Liquid Chromatography & Related Technologies, 4: 11, 141 — 173
**To link to this Article: DOI:** 10.1080/01483918108069354
**URL:** http://dx.doi.org/10.1080/01483918108069354

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(Suppl. 1), 141-173 (1981)

# PREPARATIVE LIQUID CHROMATOGRAPHY. I. APPROACHES UTILIZING HIGHLY COMPRESSED BEDS.

Donald E. Nettleton, Jr.

Dept. of Medicinal Chemistry
Bristol Laboratories
P.O. Box 657
Syracuse, N.Y. 13201



## INTRODUCTION

In just over a decade HPLC has become an integral part of almost every phase of chemistry, leaving out only certain areas of physical and theoretical chemistry and perhaps metallurgy. In addition it has attracted considerable attention from physical and physical organic chemists with interests in surface and related phenomena.

High performance liquid chromatography (there is still some dissension as to what word the initial P does stand for) began as an analytical tool. It rapidly attracted the attention of chemists with preparative needs. The speed and efficiency of this approach have consistantly outweighed the high costs of equipment, mimicking the experience of the analytical chemists. Where funds were not available, or when commercial units did not suffice, home-made equipment has appeared.

Several excellent reviews of preparative LC have already appeared in the literature. A two-part set by DeStefano and Kirkland (1) is as timely today as when it appeared five years ago. Although its writing slightly preceded preparative LC on compressed beds, this review includes almost all the basic concepts needed to pursue that art. Today the second edition of Snyder and Kirkland's Intro-

duction to Modern Liquid Chromatography, a basic reference for anyone involved in chromatography, includes a lengthy chapter on preparative LC (2, cf. pp 615-661).

The theory involved in preparative LC is well covered in the publications cited above. In this review I shall concentrate on the application of the art, bringing in theoretical considerations only where they are pertinant. The content will be directed more to the chemist at the bench faced with a separation problem.

This review is divided into two parts. This, the first, will deal only with that portion of prepatative LC involving compressed beds, at least to the extent that this can be separated from other chromatographic modes. In the second part, to be published later, I shall cover the broad and less well delineated domain encompassing the rest of this subject.



## EQUIPMENT AND MATERIALS

### Design of Available Apparatus

Currently two firms manufacture preparative LC apparatus using compressed beds. Instruments SA, a French based firm, markets several units under the trade names of Chromatospac and Miniprep. In the U. S. A. these units are sold through the firms J.-Y. (for Jobin-Yvon) Optical Systems Division and reference will occasionally be seen to this or to J.-Y. Industries. However, officially the company is listed in directories and other publications under the first-cited name.

Waters Associates of Milford, Mass. (recently merged with the Millipore Corp.) manufactures the Prep LC/System 500A (Prep LC/System 500 before modifications). Recently it added the Radial Compression Separation System (RCSS) which, while sold as an analytical unit, the Radial Compression Module (RCM), has at least semi-preparative potentials.

In addition one home-made unit has been described (3).

The units from Instruments SA are designed to compress the column axially at its ends (4, 5). This is achieved by a piston

moving upward in a cylindrical chamber against a slurry of the packing. Excess solvent passes out at both ends, the lower frit being located in the top of the piston. In the Chromatospac units the piston sits on a telescoping shaft and is moved by compressed inert gas at pressures up to 10 bar. In the smaller Miniprep unit an oil-based hydraulic jacking system drives a straight-shaft piston.

The Chromatospac will hold either of two compression systems with one meter columns differing in diameter, one 40 mm (the Prep 10 unit) and the other 80 mm (the Prep 100 unit). The first is rated for loads up to 10 g and the second to 100 g. They are, of course, interchangeable. The Miniprep has a half meter column, 20 mm in diameter and rated for loads up to one gram. In all these units, the actual effective column height will be determined by the amount of packing used within the limits of the particular apparatus. Instruments SA will also provide, upon special order and with a one year delivery time, a larger unit rated for loads of up to 2 kg.

The Chromatospac units include two solvent reservoirs, one an auxiliary, of 10 liters capacity each plus a half liter sample chamber. The auxiliary chamber flows into the main one and presumably could be used to generate some kind of gradient. However, without any stirring capability this would be quite crude. The Miniprep has two two-liter solvent reservoirs which can be used to generate a step gradient. Sample is introduced through a loop injector system. In all the units solvent flow is controlled by compressed inert gas. The multi-valve system used to direct the various stages of chromatography is easily and simply controlled on a front panel. Detectors and collection devices are supplied by the user.

Waters Associates' Prep LC/System 500 (or 500A as the newer, somewhat modified models are named) utilizes prepackaged columns under radial compression. In this somewhat more complex apparatus the pumping system, a refractive index detector with recorder, and controls are housed in one unit. Columns and an injection port are contained in a separate compression chamber assembly interlocked to

the main unit. Because of its appearance the latter is often referred to as the towers. Solvent reservoirs are external and supplied by the user (6).

The towers consist of two chambers which accept only Waters' PrepPAK 500 cartridges. These are polyethylene cylinders, 30 cm in height by 5.7 cm diameter, packed with silica or octadecyl silyl bonded silica. They hold some 325 g of the former. The wall of the cartridge extends in a modified form about 1.5 cm beyond the frits on both ends and seats tightly into polyethylene ferrules. Because of the stress induced on the ferrules by the high pressures involved, these should be replaced about every ten column changes.

Purge columns are available to fill the second chamber when it is by-passed or both chambers when the system is being flushed. The system should never be put under compression with an empty chamber due to possible damage to the fittings.



Additional sets of towers designed to be used only as slaves to the first are available at somewhat lower cost. These serve to increase total column capability. Alternatively one can buy a fully independant unit modified so that it may be used optionally as a slave. This provides the advantage that columns one wishes to reuse may be stored without removing them from the chambers. Even after decompression the seals are often difficult to break and not infrequently a column frit will be dislodged.

In the main unit a rather complex mechanical pumping system delivers solvent at flows of 50 - 500 ml/min, selected in 50 ml increments. The new System 500A modified unit has fittings allowing one inch diameter stainless steel column, one foot in length, to be attached either as a precolumn or as an alternative semi-preparative column. It can also be modified to deliver solvent at flows from 0 - 50 ml/min. A button on the control panel activates a self-priming feature in the pumping system to initiate solvent flow.

Two solvent input lines are provided to allow use of an auxiliary reservoir or changeover to another system without stopping the flow. Column effluent passes first through two stream splitters, each capable of diverting 1 - 2% of the flow. One leads to the

built-in refractometer while the other is available for an optional external detector. Next a three-way switch allows the operator the options of sending effluent to waste, to be collected, or back to the columns in a recycle mode. In the collect mode flow may be switched to either of two lines to facilitate the taking of fractions. Upon switching an air purge blows trapped liquid from the line just vacated to minimize contamination.

Interlocks between towers and the solvent delivery system provide safety features. The pump can not be started until the radial compression has been completed and the columns are completely sealed in place. During a run the system will shut off if the back pressure goes higher than 14 atm below the radial compression pressure, usually around 25 atm. The unit can be restarted by pressing a button, but it will continue to kick out unless steps are taken to prevent the back pressure rise from recurring.



Recently Waters Associates marketed their Radial Compression Separation System (RCSS)(7, 8). Although ostensibly intended for analytical work, the RCSS does have semi-preparative potentials. The Radial Compression Module (RCM) is a simple device with a single chamber holding a 10 cm by 13 mm OD polyethylene cartridge. Radial compression is provided by hydraulic pressure using glycerine as the fluid. Pressure is built up in stages by depression of three levers, these being easily manipulated by hand. The cartridges have an ID of 8 mm.

Cartridges may be exchanged in about a minute without detaching the module from the HPLC system. Originally only silica and ODS silica were provided in the cartridges, but by early 1981 Waters expects to supply all of its analytical column packings in this form. Used columns may be stored which means that they may be dedicated to a specific separation.

As noted above two French investigators have described a homemade preparative LC unit utilizing a compressed bed (3). This apparatus resembles the Chromatospac and Miniprep units in some ways. A piston, pneumatically driven by external compressed gas pressure, axially compresses the bed at 1 - 8 bar. The column, prepared from

70 cm length of stainless steel tubing with a 32 mm O.D., has an I.D. of 18 mm and a capacity for 50 g silica. The solvent reservoir has a 4 liter capcity. An auxiliary reservoir available to provide an exponential gradient is shown in a diagram but not discussed in the text. The load capacity is rated at 50 mg maximum, but presumably a larger model could be built based on its design.

In comparing the commercial models, the equipment from Instruments SA appears to have certain advantages. It is certainly simpler in design, especially in view of the solvent delivery systems used. Flow rate is changed by altering the pumps stroke rather than its rate, for which a complex arrangement of gears and other moving parts is needed, in the Prep LC/System 500. This gives the latter a greater potential for maintenance problems.

As mentioned above the Waters towers are often difficult to open after compression, a fair amount of muscle being needed to break the seal even though the system is decompressed. Before a modification in the PrepPAK frits a few years ago, column loss was common. With the new frits, the columns are more durable. However the manufacturer still recommends keeping them under total compression if possible, otherwise in the towers with the seals intact.

Since sample injection at the port in the base of the towers requires displacement of its total volume through the entire column system, one may have to work against considerable back pressure. If the sample is viscous or just occupies a large volume, even gas-tight syringes may start leaking back through the plunger where the operator is strong enough to force the issue. An alternative is to pump the sample on to the columns through one of the two solvent input lines. This usually works but may lead to other problems. As the company's technical staff warns in its Prep LC school sessions, some solutes may precipitate under compression in the pump, especially from more concentrated solutions. This can damage the pumping system.

On the other hand the Chromatospac and Miniprep units lack recycle capability. Company representatives argue that the greater efficiencies of their units make this feature unnecessary, but the

comparisons cited are not totally convincing. One key consideration is that Waters uses a relatively poor grade of silica (as will be discussed in more detail in the next section), while users of the Instruments SA units, with the option of choosing their own packings, are likely to pick better grades. The differences may be due more to the packing quality than to design of the instrument. Regardless, there are always occasions when recycle is useful.

In working with the Instruments SA units, one may opt to stop elution when the column is partly developed and use the piston to extrude it from the chamber. In this mode fractions may be sliced out in the same way as in dry column chromatography.

In part the advantage Waters Associates have enjoyed, particularly in the U. S. A., is a measure of the presence and scope of this company's pioneering efforts and output. This diversity has also led them to place a large number of technical specialists in the field giving them additional exposure. On the other hand Instruments SA's only entries in the LC area are the units discussed above.



## Column Packings

At present only silica and octadecylsilyl (ODS) bonded silica are available in the PrepPAK cartridges. A somewhat wider range is offered in the cartridges for the RCM units, and by early 1981 Waters expects to have all the packings they market, except those which can not be used under mechanical compression, available in this mode. It may be of interest to note that a single PrepPAK cartridge holds only 325 g of silica.

One problem with the latter has been the quality of the silica used, namely W. R. Grace's chromatographic grade bulk. This is a low cost product with a number of impurities and heavy metal contaminants, especially iron. These will not cause problems in all cases, or with larger loads the problems may not be apparent. With small samples these problems can be severe. Formation of chelates which may either contaminate product or irreversibly adsorb and catalysis of degredation are the more common of these. Inciden-

tally Waters Associates does clean the silica used for their C-18 PrepPAKs before the bonding process.

For the user of units accomodating any packing, there is a large and excellent choice. Not only are normal phase packings of high quality available at reasonable prices, but a number of bulk packings are also being produced now. Under these circumstances there is little point in the individual user buying cheaper grades. Cleaning them up with hot 6 N HCl, washing and reactivation cost more in terms of labor than the price differential. And with overload, very little adsorbant is used compared to the amounts needed in classical open columns.

Considerably more will be said about packings in a later section where a number of examples will be presented.



## Solvent Considerations

There is really little to say on this subject. In an early paper describing use of the Prep 500, workers at Waters used distilled in glass solvents from Burdick and Jackson (9). Because of the volumes used, most investigators prefer to work with less expensive solvents. These do quite well. They should be of a good grade and free of particulates, but generally one does not need the quality required for analytical work.

Because of the volumes involved, certain high hazard liquids such as ether should be used with extreme caution if at all. Also, while all these units do confine the streams quite well, there still may be exposure to fumes, especially at the collection point. Appropriate precautions should be taken with toxic solvents. In particular workers should keep current regulations on substances such as chloroform or benzene in mind.

## Detectors

Only the Prep LC/System 500 (and 500A) has a built-in detector. This is a low sensitivity differential refractometer with a large attenuation range. However, the extra stream splitter allows use of an additional detector.

For small loads workers have usually been able to use the same detectors employed for analytical work. Many of these have quite wide attenuation ranges and can be kept on scale for fair amounts of material. Until recently, however, there have been few, if any, detectors capable of handling the high concentrations associated with the larger loads.

Godbille and Devaux used a Gilson Spectrochem M variable wave length detector having an 80 $\mu$l cell. With a higher upper limit in attenuation this monitor can accomodate larger loads, although with a 50 mg load limit to their homemade unit a number of detectors probably would do as well. Pharmacia, Ltd. (Pharmacia Fine Chemicals in the U. S. A.) employed a uniquely designed light source to maximize both analytical and preparative capabilities. The mercury lamp is banana shaped to enable light from it to pass through both the length and width of a quartz cell 20 mm and 1 mm respectively in these dimensions. These two path lengths are attenuated respectively for analytical and preparative use. Filters provide 254 nm or 280 nm capability, and the alternate path lengths can be used to read both simultaneously.



Today, UV and visible adsorption detectors designed specifically for preparative work are appearing on the market. Within certain limits these can be used in line. However, the larger flow rates still require a stream splitter.

So far detectors using properties other than refractive index and UV/visible adsorption have had little if any application in preparative LC. Some, no doubt, will prove useful in the future. Currently they still have problems enough in analytical HPLC.

## Additional Options

There is little that one can add presently to compressed bed preparative LC units in the market. Compared to analytical HPLC equipment the units are fairly simple and quite easy to run. They also are equally fast.

Eventually the great ongoing boom in microprocessors with language anyone can use may reach to the preparative area. On a

logistical basis, however, other features will have to be added before it will take less time to program a large scale run than to simply do it manually.

## OPERATION

### Theoretical Aspects of High Compression and Overload

In the era of open columns and classical chromatography, many researchers recognized that smaller and more uniform particles with greater surface areas would afford improved separations. This led into the present era of HPLC and modern chromatography. New types of equipment, needed to cope with the higher back pressures produced by smaller particles, followed. Methods to further increase the effective surface areas of adsorbants by increasing porosity were developed. Spherical particles, long recognized as having superior packing properties, are increasingly available. Improved classification methods are providing packings with more uniform particle sizes. All of this progress has led to columns with greatly improved efficiencies.



In analytical applications the basic concepts of classical chromatography have still prevailed, particularly those concerning load factors and column height to diameter ratios. Originally researchers doing preparative LC tried to stay within the limits set by these concepts. Fortunately they soon found this was not necessary. Certainly a balance must still be maintained between resolution, speed and capacity as graphically portrayed by DeStephano and Kirkland (1) in their now well-known triangle. This design shows each one of these qualities at an apex of the triangle, conveying the message that any one may be increased only at the expense of one or both of the others. But it was soon found that modern packings and columns had resolution to spare from a preparative point of view. One could increase loads greatly, more or less maintain the same speed, and still get quite acceptable separations.

The loss of resolution, contrary to what might have been predicted, is not directly proportional to increases in load. Rather

it seems to occur to a significantly lesser degree. This can be explained by consideration of the chemical influences arising from the immediacy of surface contact. The rates at which equilibria become established on the surface are greatly enhanced. The equilibria themselves are important features of the competition by solute components and solvent modifiers for available sites. In normal phase systems the chemistry of this process involves polar interactions, primarily through hydrogen bonding. In reverse phase systems it is mainly lypophilic in nature although some polar interactions may still be found.

Overload works in modern preparative LC, where it failed in classical chromatography, because of the rapidity with which these equilibria are established at each stage of a chromatogram. The more interactive species can compete successfully for sites before they are swept past them. This leads to what some call the cascade effect. A more reactive component at equilibrium is able to occupy a huge majority of the available sites. It literally shoves other components ahead in a cascade until its concentration drops to a level where other species can compete or until the whole mixture is diluted enough for the overload to disappear. Theoretically a column could be loaded with that amount of sample that contained the desired components and any others that are more interactive with the surface in molar amounts equivalent to the actual number of sites on the surface. Waters has calculated that one of its silica PrepPAKs (325 g of silica) contains roughly one mole of sites. Clearly this gets far beyond any practical levels.

There are many types of equilibrium interactions which may occur on columns, not all of which are beneficial, as will be discussed later with regard to examples given for silica. In addition to the differences in chemistry of various solute components, sites on the packing may differ in their chemistry as well. This is more common with normal phase packings such as silica, one of the benefits of reverse phase packings being their general chemical uniformity. Problems with the latter usually arise from poor or incomplete coverage whereby silanol sites are left in accessable positions.

The chemist controls the third parameter, namely the levels of modifiers in the solvent system. For normal phase systems these are generally more polar substances and for reverse phase less polar ones. Because these compete in the equilibrium competition for sites on the column, they are used to adjust the degree of retention and hence the speed with which the column w develop. However, as an alternative, one may choose to start with little or no modifier in order to encourage as much adsorption as possible at the start. Then by increasing the modifier content in stages, the components are encouraged to elute in increasing order of polarity. Since these will presumably already be somewhat classified on the column, care should be taken not to push this step gradient, or some may become recombined.

One of the early problems in preparative LC was in scaling up for large loads. The highly efficient small particles produced impossibly high back pressures in larger columns. Somewhat larger particles worked, but were not as efficient. Compression of the bed has not only solved this but also has allowed packings formerly usable only in classical open column to be utilized with LC efficiency. Under compression void space is enormously decreased. The ratio of surface to external solvent at each stage of the column is increased by orders of magnitude. The result is a column filled with an ordinary packing approaching the efficiency of HPLC.



## Design of Systems from Analytical HPLC or TLC Data

Almost any chromatographic model can be used to predict a preparative system. Often a great deal of chromatographic data may have been compiled about a mixture by the time one decides to try preparative LC. Because of their speed, HPLC and TLC are favored modes for systems searching. Normally translation is a little easier from HPLC data, but TLC is used quite successfully where this is not available.

Solubility is an important consideration for any preparative LC system. Commonly concentrations of 10 mg/ml and up are desired. It often helps to explore systems in which the mixture shows good

solubility characteristics first. Another approach is to study the effect modifiers may have in improving solubility. One may even be able to dissolve the sample in the modifier and dilute out with carrier. However, the level of modifier in the final charge solution should not greatly exceed that in the running system, or the sample may be swept too far through the column and the separation destroyed. For ionizing species in reverse phase systems, another parameter is pH in the range roughly of 3 - 8.

Even with a great deal of data, some fine tuning of a system is almost always needed. In particular one will try to optimize the retentions and TLC is a fast way to explore this. Using microslides, 10 x 2.5 cm, in four ounce wide mouth bottles (sometimes called Kerr bottles), a great many variants may be explored in a short time. The best of these can then be checked on HPLC. In all cases the system chosen should show separation without tailing or streaking on the analytical model.



Two parameters, calculated from observed retentions, are used to predict behavior in a preparative system. One is the capacity factor, k', calculated by equation 1 where k is the observed retention from the point of injection and $k_0$ the void calculated from the same point. Any units, time elapsed, volume, or even squares on the chart paper may be used provided they are consistant since they cancel out in the equation. A second parameter is the selectivity factor, $\alpha_{1,2}$, calculated from the capacity factors as shown in equation 2. Although not used directly in designing a preparative system, there is a third important parameter. the resolution $R_{1,2}$, calculated as shown in equation 3. Because the first two parameters are based only on positional aspects and do not take into account such adverse effects as band spread and tailing, it is extremely important to consider resolution. If it is not good on the analytical model, it certainly is not likely to get better in the prepatative system. Note that both the selectivity factor and resolution are always calculated for two components.

EQUATION 1 $$k_1' = (k_1 - k_0)/k_0$$

EQUATION 2 $$\alpha_{1,2} = k'_2/k'_1$$

EQUATION 3 $$R_{1,2} = 2(k_2 - k_1)/(w_2 + w_1)$$

In equation 3, w is the band, or peak, width at the base. Note that k in this expression is the actual retention of the component. Also note that the retention factor is the HPLC analogue to $R_f$ values in TLC.

Retention factors are used mainly to determine the level of modifier needed in the system. If they are too low in value, material will elute too quickly and before there has been time for any resolution. If too high, components will elute much too slowly and will also show considerable tailing with poor results. There is some question as to where the ideal value of k' should be for a preparative system, or even how well it translates to an overloaded column (2). DeStephano and Kirkland (1) in 1975 reccommended that it be greater than 5 unless recycle was to be used. More recently, however, k' values in the range 1 - 5 have been preferred.

The selectivity factor is important in determining how difficult a separation is likely to be and whether recycle should be used. If its value is one, obviously there is no separation. For very low values, 1.10 or less, the separation will be very difficult and upper load limits will be small. In this range one should consider adjusting the system for a better separability. At 1.10 to 1.20 or perhaps a bit higher, recycle may be helpful. Above 1.3, the separation should be pretty smooth. On the other hand if the factor is above 2, this may mean one should decrease the retention as there is more separation potential than needed and solvent will be wasted.

The $R_f$ values derived from TLC data can be used in the same way as capacity factors. There is no thin layer equivalent to the selectivity factor, or is there any numerical determination of resolution. Possibly, by use of one of the plate scanning densitometers available in the market, one could generate data from which to make such calculations. However, it probably wouldn't cost much more to buy a good analytical HPLC system instead.

Regardless of how one approaches a preparative separation, it is best to start on a smaller scale and work up. There often is a need for some minor adjustments. Increasing loads in steps will soon give a pretty good idea of where the upper limits lie, and meantime material is being prepared. Very often actual load limits will exceed those predicted from the calculated selectivity factor. Also it may prove faster to first purify the material to a certain level, say greater than 95%, and then rerun it for a final purification. This is particularly true when one is dealing with mixtures having a lot of impurities and relatively low concentrations of the desired component or components.

## Theory and Use of Recycle

Recycle is primarily used when calculated selectivity values are low. It also may be helpful when the load levels are being pushed, provided there is not excessive tailing.

The process is simple. For example, assuming a two component mixture, one waits until the first of these begins to elute. Depending on how tight the separation is, more or less of the front is collected, or "shaved", before starting recycle. The bulk of the peak is then returned to the column. At the end of the peak, one may wish to shave another fraction. As the recycled peak comes around again, the process is repeated.

The tricky part of recycle is knowing just where to shave and how many recycles are needed. Usually there is some leeway and it is better to take the conservative route. Also the more runs made with a given mixture, the more exact these decisions become.

The amount which can be shaved on both sides depends on how close the components are eluting. Sometimes several recycles must be run before any shaving can be done. On the other hand if one is too conservative in beginning to shave, band spread may cause the front to come about before the end is off and remix them. Each shave reduces band width, thus counteracting spread. Also each shave removes material thus reducing the overload and increasing efficiency with regard to the remaining material.

In overload, normal phase columns tail to the rear as a rule, while reverse phase columns tail forward. Thus the degree of shaving should always be less on the side of the tail. This is modified somewhat by the relative amounts of the components involved.

For more complex mixtures any number of things may elute ahead of the area of interest. Late eluting species prohibit recycle for obvious reasons, but usually these can be removed by a preliminary preparative run made at as high a load as the traffic will bear. If one is after the first or last of a multi-component mixture, recycle can often be used as well by treating the desired component as one species and the mixture of the others as a second species. This works as long as the whole complex is not spread out over two great an area, and even in the latter case when the last component is being sought.

Much of the success in using recycle comes from experience. After using it several times one comes to know what to expect from it and how to apply auxilliary data.

If one is using HPLC or TLC to analyze fraction, there is an additional trick that can be used with the Prep LC/System 500. This is to collect samples of the outflow from the refractive index detector while in the recycle mode. Analysis of these can not be made before the current run is finished, but it certainly will provide good direction for the next one.



## Scope and Limitations

Much of this has been covered in the preceding discussion. Preparative LC has benefitted many areas of chemistry and allied fields. Its impact on natural products research is obvious. Synthetic organic chemists have benefitted too. Now many of these isolate products from mixtures they would have thrown away only a few years ago and have them evaluated. In this way they can concentrate their efforts to improve yields on those products with real potential.

The process is not limited to isolating pure compounds. Rapid class separations can be effected to concentrate desired components

for later refinement. Step gradients can be applied on preparative units in the same manner as flash chromatography is practised in open columns. Speed is the big advantage and the chemist's main limitation is his ability to keep up with it all.

Poor solubility is probably the main limitation. There are caes when one must work with more dilute solutions and make more runs, although there are a few tricks one can try. One old chromatographer's trick is to evaporate a solution of the material down with some of the packing so that the sample becomes coated on the surface of the latter. Presumably a solvent system unsuited for chromatography can be found to accomplish this purpose. The coated packing is then layered on top of the column, and developement is begun.

Obviously some modifications will be needed for the instruments available. With the slurry packing devices such as the Chromatospac and Miniprep units it should be possible to layer on a thick slurry of the coated material using a less modified system. The degree of success will depend on how much or how little mixing occurs during bed compression. With units requiring prepackaged cartridges, the coated packing can be dry packed into some kind of a pre-column. The one foot by one inch diameter stainless steel column provided with the Prep LC/System 500A unit is ideal for the purpose.

This approach is successful only to the extent that the sample will rapidly come off in the running system and go onto the bed itself. While generally in this finely divided state considerably more sample can be pursuaded to do just this, there are limits. If these are exceeded, sample will bleed onto the bed and the separation will be wrecked.

In reverse phase systems PIC reagents have been very successful for analytical work. Unfortunately these do not appear to be applicable to preparative work. Aside from cost, which is a real consideration, they tend to contaminate both the bonded phase, giving problems in subsequent runs, and product, where they may be difficult to remove.

A lesser limitation of compressed bed systems is that they require packings with rigid matrices. This precludes against such materials as the polystyrene packings used for size separations. Even though these may seem fairly firm, they will compress and virtually seal off the flow. Currently people are discovering that microporous silicas where pore sizes are controlled seem to be displaying molecular sieving properties. Actually over ten years ago Corning produced glass beads with controlled pore sizes to be used for just this type of chromatography, but the product was allowed to die. Perhaps now this technology will be rejuvenated. With proper bonding an inert silica presumably could be developed for the purpose.

It is often said that the insatiable solvent demands of the compressed bed preparative units place a severe cost limitation on their use. Actually, where direct comparisons can be made to open column work, the former use less solvent per gram of sample and accomplish the job in minutes as opposed to hours or days. It's just that with the modern units one is psychologically more aware of the volume involved.



Other Considerations

A number of theoretical studies have been made on preparative LC systems, some utilizing compressed beds. Although some of the literature from Waters Associates has claimed reduced wall effects in the case of the latter, none of these studies seem to make any distinction on this point.

Of particular interest is research on the effects of volume overload. Since distribution isotherms are the parameters used for this work, sample amounts are kept below overload levels where the isotherms would cease to be linear. The latter are determined by plotting solute concentrations (for a model compound) on the stationary phase vs those in the mobile phase for a series of total solute levels. There are several ways to do this, the simplest being a batch process. Linearity of the isotherm breaks down at sample overload because the stationary phase can no longer accomodate its share of the solute.

As a simpler alternative to isotherms, column efficiency has also been used as a parameter. Godbille and Devaux (3) followed this property in using their home-made apparatus to study the effects of flow rates and sample volumes. They also designed two types of injectors. One introduced the sample over the entire cross-sectional area of the column in the more traditional manner. The other introduced it in the center to produce the effect of an infinite diameter column. In the latter case they could calculate that less than 5% of the sample ever reached the wall.

The authors studied both normal and reverse phase systems at both analytical and preparative levels, using a steroid, nordienolone, as a model. Flow rate and injector effects were studied on both silica (10 $\mu$) and octylsilyl bonded silica using respectively 5% methanol in methylene chloride and 65:35 water:methanol systems. Optimal flow rates were found which seemed independant of injection mode on normal phase but varied somewhat on reverse phase. Center injection was slightly superior in terms of efficiency in the normal phase system but not on the other. Overall they found the bonded phase system to be less efficient, but this is not uncommon.

At the preparative level the effects of volume overload were studied on the normal phase system alone. For smaller volumes the center injection mode was somewhat more efficient, but it became dramatically less efficient as volumes were increased.

Another group from France has also studied volume overload on compressed beds (10). These workers, using some rather complex mathematics, determined maximal injection volumes. They compared four types of on-column injectors including a rather interesting sprinkler type.

Dutch workers began by studying the dispersion effect in preparative LC, using in this work packed metal columns rather than mechanically compressed beds (11). This led to studies using the distribution isotherms discussed above as a means to determine optimal conditions for both analytical and preparative LC (12). Several ways of determining the isotherms are described. Of interest was the discovery that for hydrocarbon bonded silicas,

increasing chain length has an adverse effect on isotherm linearity. The standard chain lengths of 18, 8 and 2 carbon atoms were compared. The authors had some reservations about the study and cautioned that this does not mean that $C_2$-bonded phases are superior.

Unfortunately none of this has application as yet to large scale separations. With large weight overload, volume overload effects may be buried. Their actual contribution is still too difficult to measure. One interpretation of the data on the differences due to carbon chain length may be that capacity is reduced with increasing length. Otherwise in preparative LC one must still rely on empirical data and use imagination. This area of science remains largely an art.

## EXAMPLES

### Silica Chromatography

Most of the preparative LC on compressed beds reported to date have used silica. That many hold this packing to be the most versatile adsorbant available today may be a factor. Certainly it is the most available and this, too, has its influence.

Things can happen on this medium which the chromatographer is well advised to take into consideration. Silicas from different sources, and even in different batches from the same source, can vary considerably. Much of this is due to differences in adsorptive sites on the surface. Silanol groups, which may act both as hydrogen bonding donors and acceptors, vary in reactivity and acidity. Non-protonated silyl oxygens, mostly ether type functions, act as hydrogen bonding acceptors and vary in reactivity as well. The influences may be chemical or physical (eg. steric influences) or both. There are also $\pi$-electron bonding effects which are at best little understood. In addition varying amounts of water, polar modifiers, and other solvent constituents are spread about on the surface contributing their effects. In overload, solute constituents may even contribute to some extent.

In isocratic systems most of these features average out to a sort of norm for the particular environment created. Even with a

gradient, as long as equilibrium is maintained, surface features gradually change in a continuum. However, if there is a wide divergency in the reactivity of different sites towards solute components, this breaks down. An extreme example is the well-known case of amines interacting with acid silanol functions. In any given region of a column there are not enough of the latter to accomodate all the basic groups, even at analytical loads. In a sense the column is overloaded with respect to these acidic sites. The result is tailing and streaking.

In preparative LC, even with a general overload situation, the hyperactive sites can cause problems, especially when interactions with a particular functional group do not distinguish between the different molecular species bearing it. In addition the different capacities of subsets of sites with widely differing reactivity levels may provoke additional tailing effects. In working with overload it is often better to have a more uniformly reactive column than a highly active one.

The degree to which one needs to deactivate silica depends upon how solute molecules are likely to perceive the various active sites. Neutral, fairly non-polar substances find the whole spectrum of these sites pretty much equivalent. As more polar functions are added to the solute molecules, a complex range of interactions develops and the more reactive sites must be suppressed. Quite often this occurs as a natural consequence from the addition of polar solvent modifiers to reduce retention to a usable level. In extreme cases, as evidenced by severe tailing in the trial analytical systems, additional modifiers will be needed.

Antitumor agents from fermentations (particularly fermentations of members of the order Actinomycetales) seem to display all of the undesirable properties possible from a chromatographer's point of view. They are usually very strong binding agents in their own right, probably a natural consequence of their antitumor properties. Some are bases and not a few have the solubility characteristics of gravel.

The anthracycline mixture, Bohemic Acid Complex, which was resolved at Bristol Laboratories (13, 14, 15), provides a good example of most of these problems except solubility. The general structure of its components (cf. Fig. 1) shows a variety of polar functions including an amine. In the early stages it was difficult to find a thin-layer chromatographic system that would afford zones which didn't streak.

On analytical HPLC the chromatography profiles were no better, but this approach allowed experimentation with modifiers difficult at best to use in TLC systems. Earlier experience with open columns had taught us that pretreatment of silica with aqueous ammonium hydroxide neutralized acidic silanols and reduced tailing. Concerned at the time that water would excessively deactivate the HPLC analytical column used for a systems search, we studied the modifying effects of lower alkyl amines and even anydrous ammonia in methanol. With a basic system of 4 - 5% methanol in methylene chloride, some improvement was noted, but tailing was still severe. Only when water (in the form of aq. $NH_4OH$ stirred with the system to saturate it) was added was complete resolution achieved. Where



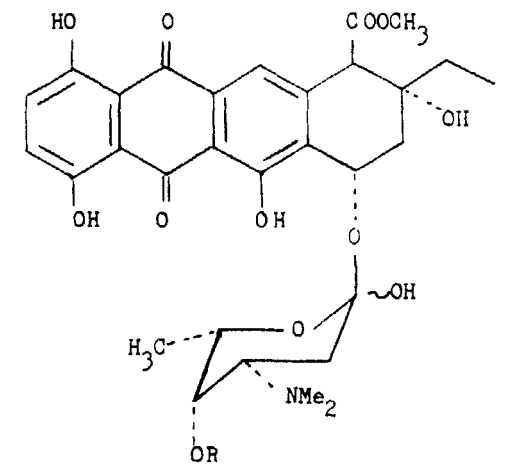


FIGURE 1. General structure of the anthracyclines of Bohemic Acid Complex; R = H or various mono- and disaccharides.

at best some two or three components had been seen before, seven or eight, including the six anthracyclines eventually isolated, appeared in sharply resolved peaks.

In the early days of HPLC such stress was laid on maintaining anhydrous conditions that most researchers avoided water like the plague. In recent years reports of the beneficial effects of this agent have appeared with increasing regularity. Highly active columns with huge plate counts are impressive but not particularly useful if they do not translate to the separation at hand. It is important to recognize that column efficiency is a property of solute-adsorbant interactions, not of the adsorbant alone.

While bases will specifically neutralize acid functions, water will modify other highly active sites where lower alcohols fail. For solutes with high binding potentials such as antitumor agents, this will actually lead to improved column efficiency even though its plate count will drop markedly in the systems usually employed to measure this property.



The translation of the separation of Bohemic Acid Complex to preparative LC was excellent. Because of the complexity of the mixture, refractionation was necessary, and recycle proved very profitable in isolating certain of the components. The process developed has proven of commercial value, one of the components (marcellomycin) now being produced for clinical testing.

In using ammoniacal systems it is important to remember that silica does dissolve in base, particularly with ammonia in methanol. In compressed bed systems a certain amount of loss can be tolerated since voids are squeezed out. However, eventually even compression can not keep up with the losses. Because of the iron contamination in the PrepPAKs used for our anthracycline work, there were always losses on new columns and contamination of the latter with iron chelates of the compounds. Since these could contaminate later runs, they had to be removed, a methanol wash being best for this purpose. Fortunately one wash after the first run removed not only the chelates but with them all the iron, making washes after subsequent runs unnecessary. Furthermore, in these subsequent runs

the resolution and column behavior was vastly improved. Now we use unwanted anthracycline by-products to precondition columns.

In working with modifiers such as ammonia, it is important to be mindful of any other interactions that may take place. Concentrated ammonium hydroxide is not completely miscible with 4% methanol in methylene chloride, hence a quantity of the reagent (usually 0.5% v/v) was stirred into the solvent system and allowed to settle out on top. However, when the methanol content was increased to 5%, this quantity of the aqueous ammonia completely dissolved. In the 4% methanolic system most of the ammonia distributed to the aqueous phase, hence its concentration in the running solvent was quite low. With all the ammonia dissolving, the relative increase in ammonia concentration in the latter was huge. In the following preparative run a peculiar array of peaks was seen and every fraction, by HPLC analysis, looked exactly like the starting material. Apparently all that had separated were various ammonia complexes of the polyhydroxy anthraquinone system (where the hydroxyl functions have pK's close to those of carboxylic acids) without regard to other molecular differences.



Probably no single investigator has demonstrated the values of preparative LC in more diverse ways than Prof. Koji Nakanishi of Columbia University. An excellent review in 1979 (16) covers much of his and others' work on a variety of chemotypes.

In conjunction with his work on vision research, Prof. Nakanishi has done extensive experimentation with the retinals and retinoids. Many of these compounds are not only light sensitive but labile in other ways as well. Needing small quantities just prior to testing in biological models, the Nakanishi group routinely uses preparative LC to isolate these as end-products from synthetic sequences just before use (17). Also the technique is used to isolate key, but labile, synthetic intermediates, e.g. the 9-cis and all-trans compounds produced from one synthetic step and shown in fig. 2. The LC system was 11% diethyl ether in hexane (18).

In studying synthetic routes to certain insect pheromones, Nakanishi and coworkers were faced with the problem of resolving

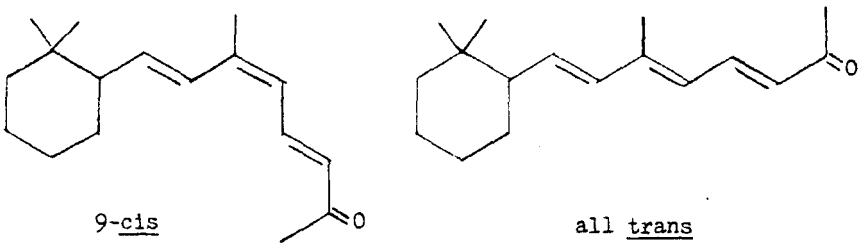


FIGURE 2

the cis and trans isomers of a key intermediate (cf. fig. 3)(18). This mixture would not resolve on silica alone. However, silver impregnated silica has long been used to separate olefins. The Nakanishi group solved the problem by removing the frit on a PrepPAK and replacing its contents with silica treated with 10% silver nitrate. The process of replacing the PrepPAK contents had about a 25% success rate (19), but with the same 11% diethyl ether in hexane system used for the retinal intermediates, the isomers were cleanly resolved. Recently a procedure for on-column impregnation of silica with silver has been published (20) and could possibly be applied to prepackaged columns.

The same group has also explored the direct isolation of pure natural products from crude plant extracts by preparative LC (21). Two solvent stages were used on a crude plant extract, first 10% diethyl ether in hexane and secondly 25% ethyl acetate in hexane. Several pure compounds including a new alkaloid and an unknown were obtained in crystalline state.

Generally some preliminary work is needed to concentrate the desired components from plant extracts, but some fairly crude materials can be resolved by preparative LC. In our experience at Bristol Laboratories this was the case in isolating the antitumor

$$THP\text{-}O\text{-}(CH_2)_5CH{=}CH(CH_2)_3CHO$$

FIGURE 3. THP = tetrahydropyranyl.

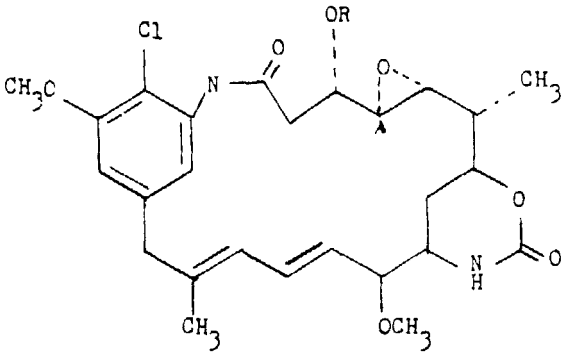


FIGURE 4. General structure of the maytansinoids and the ansamitocins. For the maytansinoids, R = C(:O)-CH($CH_3$)-N($CH_3$)-R' where R' is various various lower alkyl acyl groups. For maytansine, R' is acetyl. For the ansamitocins, R is various lower alkyl acyl groups the N-methyl alanyl moiety being missing in this series.



agent maytansine and other maytansinoids (22) (cf. Fig. 4). The source was far richer than any found before for these compounds, but even after acid and base washes and a batch silica process, the crude material contained enough oils and waxes to foul columns in the preparative system making them unusable after a few runs. Fortunately the products could be crystallized from the fractions which still contained oil.

It is interesting to note that water was needed in the system used to resolve the maytansinoids, it being 5% iso-propanol in methylene chloride equilibrated with 0.5% water. Later a persistant impurity was found in the maytansine product which could not be resolved in the halocarbon system. Substitution of ethyl acetate as the carrier with the same proportion of the alcohol afforded good resolution of the mixture. In this case water was not needed. Not infrequently ethyl acetate may be substituted for a halocarbon carrier solvent with improvement in resolution.

A Japanese group from Takeda Chemical Industries, Ltd. used a 7:1 ethyl acetate:hexane system saturated with water at 22° to resolve ansamitocin mixtures (23), these being another class of maytansinoids produced by fermentations of a Nocardia sps. In their

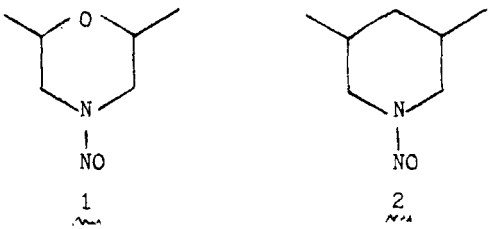


FIGURE 5

experience half gram samples could not be resolved on silica Prep-PAKs but were well resolved when they packed an empty cartridge with Kieselgel 60 from E. Merck and used it.

Workers at Frederick Cancer Research Center found ethyl acetate superior, in this case, as a modifier compared to chloroform. The problem was the separation of some conformational isomers of morpholine and piperidine nitrosamines (cf. Fig. 5), of interest as potential carcinogens (24). With 70% chloroform in hexane the cis and trans isomers of the morpholine nitrosamine, 1, were not well resolved although some enrichment was observed. With 20% ethyl acetate in hexane retentions were shortened and resolution was greatly improved. With recycle gram quantities of each isomer were prepared. In the piperidine series, 2, serious tailing occurred with the 10% ethyl acetate in hexane system predicted by analytical HPLC, but this was solved by increasing the modifier content to 15%.

The natural farnesol isomers, 3 and 4 (cf. Fig 6), used in perfumery, have been resolved on compressed silica beds using a system of 10% diethyl ether in iso-octane (25) by a U. S. Dept. of Agriculture investigator at Beltsville, Md.

Several difficult aromatic mixtures have been nicely resolved. A Czech group has prepared analytical reference samples of a variety of monoaromatic hydrocarbons from crude oil cuts using n-pentane as the running solvent. Note here the lack of any need for a modifier. Pure quinoline and several methylquinoline positional isomers, obtained from commercial sources in purities of 90 - 99.5%,

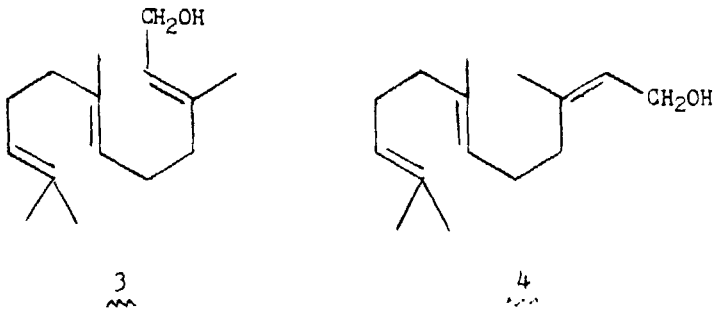


FIGURE 6

were brought to 99 - 100% purity by workers at the American Health Foundation in Valhalla, N.Y. (27). Quantities of 10 - 40 g were prepared using a 1% ethanol in chloroform system. These compounds were needed in large amounts and without impurities for animal carcinogenicity testing.

A team at St. Louis Univ. School of Medicine used preparative LC to purify a steroid from carp bile, 5$\alpha$-anhydrocyprinol, in gram quantities to the extent that they were able to crystallize it for the first time. Two systems were used, 1:14 methanol:chloroform and 1:1 acetone:benzene. The substance is used as a starting material for the synthesis of various 5$\alpha$-steroids (28).

There are more examples that could be cited, but these should suffice to show the parameters one may expect to find on silica in a compressed bed. Where solute functionality is limited, finding a separation is usually fairly straightforward. The exceptions generally are found when the differences between solute components are very small. Where there is a multitude of functionalities, more work will often be needed in developing the proper modifiers.

Alumina Chromatography

Once at the height of steroid chemistry, alumina was as popular, if not more so, as silica. Now one scarcely hears mention of it and, in fact, may be hard pressed to find suppliers of it. Many researchers claim it is too active, binding materials irreversibly.

While this has occurred, there is little modern evidence that this is any different from effects of high activity silicas. The role of various modifiers has received very little research, and it can not be said that alumina's adsorptive properties are uncontrollable.

There have been no reports in the literature of preparative LC using compressed alumina beds. However, several years ago Waters Associates made a few experimental alumina PrepPAKs available, two of which we received for evaluation. Since Bristol Laboratories markets mitomycin C, which is traditionally purified by alumina chromatography, we studied the separation of this agent from mitomycin A (cf. Fig. 7), using some side fractions from a commercial run (29).

In evaluating systems on a Lichrosorb Alox T, 10 $\mu$, (E. Merck) column we found that resolution was poor until water was added to the system, much in the same way as our experience with antitumor agents on silica. Excellent separation was found in a 92:8:0.5 methylene chloride:iso-propanol: water system, mitomycin A eluting near the front ($k' = 1.0$) and mitomycin C being well retained ($k' = 3.8$). This system translated smoothly to the preparative run.

In exploring some other systems having methanol as the polar modifier, we had a rather curious experience with this mixture. The resolution of the two mitomycins was quite good, but noting a fishy odor to the methanol being used, we replaced this with another lot from the same supplier. Immediately the resolution deteriorated significantly. It was restored by adding a small concentration of ammonia to the system.

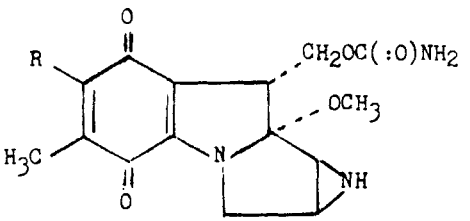


FIGURE 7. General structure of the mitomycins. For mitomycin A R = $CH_3O$; for mitomycin C, R = $H_2N$.

Neutral alumina is not normally thought to have acidic functions like the silanols. Furthermore, the nitrogens on the mitomycins are not very basic. In fact the quinone amino function of mitomycin C is actually a vinylagous amide and behaves as one. One has to believe that there are a lot of fascinating things yet to be learned about alumina chromatography.

Reverse Phase Chromatography

Just in recent years there has been an enormous surge of interest in and use of reverse phase packings. This is quickly extending to the preparative area. To date almost all the work reported has been on octadecylsilyl (ODS) bonded silicas, but with a variety of bonded supports becoming available in bulk form one can expect a broadening of the scope of the whole area.



Reverse phase chromatography is particularly valuable for materials having only water solubility. A New Zealand group has purified an underivatized tetrapeptide, L-leucyl-glycyl-glycyl-glycine in up to 10 g lots using a degassed 95:5:0.5 water:methanol:trifluoroacetic acid system. The peptide was prepared synthetically (30).

Leucine, the N-terminal amino acid, has an aliphatic chain which must play a role in the retention of this compound. Many peptides, and even some proteins, assume conformations whereby their polar function bond internally to one another, thus causing the molecules to present a relatively lipophilic exterior. This is not sufficient to make them solvent soluble, but it seems likely that this would enhance their retention on a reverse phase adsorbant. Insofar as the porosity of the packing allows, one might expect reasonable retentions of such compounds, provided they are stable in the systems used.

Nakanishi's group the direct purification of the insect antifeedant sesquiterpenes, schkuhrins I and II, directly from methanol extracts of the whole plant, Schkuhria pinnata (31). A 53:47 methanol:water system was used, 6.6 g of crude material being run on 1 kg ODS bonded silica, 20 $\mu$.

H. J. Segall of the Univ. of California has reported the preparative isolation of pyrrolizidine alkaloids from Senecio vulgaris, these being a common cause of livestock poisoning (32). In a 60:40 methanol:0.005 M $KH_2PO_4$ (pH 6.3) system retrosine eluted first followed by seneciphylline and senecionine which were resolved by recycle in a rather elegant process.

A team at Mississippi State Univ. separated several dyes including Rose Bengal in the form of the potassium or sodium salt and rhodamine B which exists as an iminium salt (33). The separation of ionized species is yet another valuable potential for preparative reverse phase chromatography.

The cost and availability of bulk quantities of reverse phase packings have been a drawback to advances in their preparative use. However, as noted at the beginning of this section, the situation is changing, and one can expect much greater progress in this area in the near future.



Gel Permeation Chromatography

In this area one can only speculate since there are no rigid packings available in bulk with which to attempt preparative molesizing. In the section discussing the scope and limitation of the operation of preparative LC, I commented on the controlled pore size glass beads once marketed by Corning and current possibilities for controlled pore size silicas. These may yet bear fruit.

## FUTURE POTENTIALS

One can speculate a great deal on what the future will bring, and in fact I have already included some of this type of thought in previous sections. Certainly the variety of things that one can put into preparative systems of all kinds will increase. Bonded phases will offer many unusual properties for adsorbants and presumably more uniform ones. Smaller particles and higher pressures will also be in the picture, but only as far as the pressures can be handled safely and the advantages make it worth while.

It seems unlikely that additional ways to compress beds will be found, there being a geometrical limit to this sort of thing. Certainly spherical packings should largely replace irregular ones.

Large contributions to all preparative chromatography will come from the physical and physical organic chemists studying its processes. Major benefits will be reaped when these researchers learn the mechanics of surface interactions under overload, since this will allow the design of surfaces specifically for preparative work.

I could speculate further and probably at excessive length, but I feel this might best be left for the end of the second part of this review. After all, compressed beds are only one facet of this area of research and production.



## REFERENCES


1. DeStefano, J. J. and Kirkland, J. J., Anal. Chem., 47, 1103A, 1193A (1975).
2. Snyder, L. R. and Kirkland, J. J., Introduction to Modern Liquid Chromatography, second edition, John Wiley & Sons (1979).
3. Godbille, E. and Devaux, P., J. Chromatogr., 122, 317 (1969).
4. High-Performance Preparative Liquid Chromatography Systems, technical bulletin from Instruments SA, Inc., J.-Y. Optical Systems Division.
5. Karasek, F. W., Res. Dev., 28, 32 (1977).
6. Rahn, P. C., Woodman, M., Beverung, W., and Hechendorf, A., Preparative Liquid Chromatography and Its Relationship to Thin Layer Chromatography, Waters Associates.
7. Fallick, G. J. and Rausch, C. W., Amer. Lab., 11, 87 (1979).
8. Radial Compression Separation System, data brochure from Waters Associates (1979).
9. Little, J. N., Cotton, R. L., Prendergast, J. A. and McDonald, P. D., J. Chromatogr., 126, 439 (1976).
10. Coq, B., Cretier, G. and Rocca, J. L., J. Chromatogr., 186, 457 (1979).
11. de Jong, A. W. J., Poppe, H. and Kraak, J. C., J. Chromatogr., 148, 127 (1978)
12. de Jong, A. W. J., Kraak, J. C., Poppe, H. and Nooitgedacht, F., J. Chromatogr., 193, 181 (1980).

13. Nettleton, D. E., Jr., Balitz, D. M., Doyle, T. W., Bradner, W. T., Johnson, D. L., O'Herron, F. A., Schreiber, R. H., Coon, A. B., Moseley, J. E. and Myllymaki, R. W., J. Natl. Prods., 43, 242 (1980).

14. Nettleton, D. E., Jr., Bradner, W. T., Bush, J. A., Coon, A. B., Moseley, J. E., Myllymaki, R. W., O'Herron, F. A., Schreiber, R. H. and Vulcano, A. L., J. Antibiot., 30, 525 (1977).

15. Doyle, T. W., Nettleton, D. E., Jr., Grulich, R. E., Balitz, D. M., Johnson, D. L. and Vulcano, A. L., J. Am. Chem. Soc., 101, 7041 (1979).

16. Adams, M. A. and Nakanishi, K., J. Liq. Chromatogr., 2, 1097 (1979).

17. Arnaboldi, M., Motto, M. G., Tsujimoto, K., Balogh-Nair, V. and Nakanishi, J. Am. Chem. Soc., 101, 7082 (1979).

18. Pettei, M. J., Pilkiewicz, F. G. and Nakanishi, K., Tetrahedron Lett., 2083 (1977).

19. Private communication from F. G. Pilkiewicz.

20. Heath, R. R. and Sonner, P. E., J. Liq. Chromatogr., 3, 1129 (1980).

21. Hostettmann, K., Pettei, M. J., Kubo, I. and Nakanishi, K., Helv. Chim. Acta, 60, 670 (1977).

22. Nettleton, D. E., Jr., Balitz, D. M., Brown, M., Moseley, J. E. and Myllymaki, R. W., J. Natl. Prods., in press.

23. Izawa, M., Haibara, K. and Asai, M., Chem. Pharm. Bull., 28, 789 (1980).

24. Singer, S. S. and Singer, G. M., J. Liq. Chromatogr., 2, 1219 (1979).

25. Warthen, J. D., Jr., J. Liq. Chromatogr., 3, 279 (1980).

26. Kuraš, M., Kříž, J., Tříska, J. and Vodička, L., J. Chromatogr., 191, 319 (1980).

27. Dong, M., Schmeltz, I. and Hoffmann, D., J. Chromatogr., 150, 269 (1978).

28. Shaw, R. and Elliott, W. H., J. Chromatogr., 177, 289 (1979).

29. Nettleton, D. E., Jr., Moseley, J. E. and Myllymaki, R. W., unpublished work.

30. Bishop, C. A., Harding, D. R. K., Meyer, L. J. and Hancock, W. S., J. Chromatogr., 192, 222 (1980).

31. Pettei, M. J., Miura, I., Kubo, I. and Nakanishi, K., Heterocycles, 11 471 (1978).

32. Segall, H. J., J. Liq. Chromatogr., 2. 1319 (1979).

33. Peeples, W. A., II and Heitz, J. R., Chromatogr. Sci., 12, 437 (1979).