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EXTERNAL RECYCLE CHROMATOGRAPHY: A PRACTICAL METHOD FOR PREPARATIVE PURIFICATIONS

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SUMMARY

This report describes a new recycle concept for use in preparative liquid chromatography (PLC). External recycle is a technique that involves the reinjection of unevaporated impure eluent fractions, as a single injection, back onto the original column to enhance the total recovery and purity of components from a separation. Results of this study indicate that resolution is more dependent upon sample load and on the weight distribution of components in the mixture than upon injection volume. The application of this technique to PLC was demonstrated using crude reaction mixtures of synthetically prepared compounds of pharmaceutical interest.

INTRODUCTION

Preparative chromatography is the application of chromatographic methods for the purpose of isolating significant amounts of pure compounds from a mixture. Generally, these methods are based on analytical high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) systems.

The objectives for PLC separations are different from HPLC or TLC separations in that productivity should be emphasized rather than resolution[1]. To maximize productivity, (the weight of pure product resolved per gram of adsorbant), PLC columns are typically operated at sample overloaded conditions which often results in the incomplete resolution of the components.

The effect of sample overload on band broadening and resolution has been the subject of a number of investigations[2–9]. Knox and Pyper[5] discussed the effects of both injection "volume overloading" and "concentration overloading" on band broadening in preparative chromatography. "Volume overloading" for a single component is insignificant as long as the injection volume remains less than half the volume of a compound's eluting band. "Concentration overloading" is a result of applying sample concentrations that are higher than the linear portion of the adsorption isotherm.

In addition to understanding the effects of overloading on the resolution of components for single-pass separations, there are a variety of methods, such as conventional recycle chromatography[10–14], to enhance the recovery and purity of

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single-pass separations where the resolutions are not complete. In conventional recycle chromatography, the eluent is routed from the column outlet through the pump and directly back to the head of the column. The effect of recycle chromatography is that the components of a mixture are further resolved with each additional pass through the column. There are also variations on the conventional recycle method[15,16] to improve the performance of separations, however, conventional recycle systems are typically limited to the isolation of two component mixtures, and to separations where the total band width is less than the recycle volume.

This report describes an external recycle technique for preparative purifications that fills an operational gap between single-pass purifications and conventional recycle methods. This technique is useful after an initial single-pass purification, where a crude mixture has been applied to a column and eluted from the column using the appropriate solvent system. Some of the eluted fractions may still contain an impure mixture of adjacent eluting compounds. These impure mixture fractions can then be sequentially reinjected back onto the column, in a "volume overload" condition, to further resolve the desired components. Sequential reinjection of fractions refers to a process by which any number of unevaporated fractions are recycled back onto the same column, in the same order in which they had eluted from the column during the previous single-pass. This process is, in effect, a recycle rechromatography of only the unresolved portion of the initial sample. This reinjection process by itself or in conjunction with conventional recycle techniques provides another approach for improving the quality and speed of purifications.

EXPERIMENTAL

Apparatus

Preparative chromatography was performed using either a Beckman 421 controller and 100A pumping system or a Waters PrepLC/500A system. The elution profiles were monitored by either a Hitachi Model 100-10 UV–VIS detector, or a Waters 500A refractive index detector.

Chemicals

Androst-4-ene-3,17-dione (AD) and $\Delta^{1,4}$-androstene-3,17-dione (ADD) were obtained from Searle Chemical. Diastereomers of a *tert.*-butyloxy-carbonyl (Boc) protected peptide and an isoquinoline derivative were obtained as synthetic intermediates from reaction mixtures (G. D. Searle).

Methods

All resolution studies were performed using mixtures of AD and ADD on a LiChrosorb Si-60, 10 μm, 500 mm × 10 mm silica column and a mobile phase of 10% ethyl acetate in methylene chloride. The eluent was monitored by TLC, and by UV adsorption using a Hitachi Model 100-10 spectrophotometer at a wavelength of 286 nm so as to obtain equal responses for AD and ADD.

The resolution values were calculated using the standard equation

$$R_s = 2(V_{R,2} - V_{R,1})/(W_1 + W_2)$$

where R_s is the resolution, $V_{R,1}$ is the retention volume (ml) of the AD peak, $V_{R,2}$ is the retention volume (ml) of the ADD peak, W_1 and W_2 are the eluting band widths (ml) for AD and ADD. In all cases, the skewed peak retention volumes could be measured from the chromatogram that was obtained during each run. The band widths were often completely obscured due to mass overloading. For this reason the band widths for AD and ADD were determined by the number of fractions that contained each of the compounds and multiplied by 10 ml per fraction. Therefore, the base width measurements have a precision that is related to the fraction size.

Method development for the separation of the crude sample mixtures was performed using precoated Merck Kieselgel 60, F_{254}, 0.25-mm TLC plates, and the resulting separations were scaled directly to column purifications using the same mobile phases. All preparative column purifications were performed using Merck Kieselgel 60, 230–400 mesh silica dry packed into stainless-steel columns. The selection of an appropriate column size was determined by the sample weight, typically being on the order of 0.02 g sample/g silica. Columns were then preconditioned with 1–2 void volumes of mobile phase prior to sample injection. For practical considerations, a column void was determined as the volume of solvent residing in the preparative column. These provide an estimate of the amount of solvent necessary to saturate a dry packed column, or to displace a solvent previously occupying the column.

Weights of reinjected samples were not determined directly but were estimated by integrating the areas under the peaks and by the final weight of products. This is because the compounds purified during the reinjections had been combined with the same pure compound produced from the first single-pass run through each column.

Samples were typically dissolved in the appropriate mobile phases at concentrations of 0.2–0.3 g/ml, and injected onto the column. Eluent fractions ranged from 0.05 to 0.20 void volumes, with the fraction purity being determined by TLC. The wet unevaporated fractions containing the unresolved components were sequentially reinjected onto the head of the column using the solvent inlet lines to the pump. The reinjections were made from test tubes or other vessels containing the impure fractions. Generally, each vessel was washed with a small amount of mobile phase to ensure a quantitative transfer onto the column. A three-way valve was used to momentarily cavitate the pump while the solvent line was being moved between fractions to eliminate the introduction of air into the system.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of sample injection volume on resolution. An amount of 16 mg of a mixture containing equal proportions of AD and ADD was applied per injection onto a column having a 28.5-ml void volume. The resolutions measured ranged from 1.33 to 0.87 when the injection volume applied to the column was varied from 0.160 to 40.0 ml. These results are difficult to compare to the Knox model for "volume overloading" since, in the present study, the sample weight was kept constant and the effect of injection volume on the resolution of a two component mixture was evaluated. It was observed that even though the 40.0-ml injection volume was equal to the elution band width for AD during the 0.16-ml injections, the resulting peak shapes were not flat, but showed only a slight increase in peak broadening. It seems that even at the sample weight used in this study, a "concentration overloading" effect was still

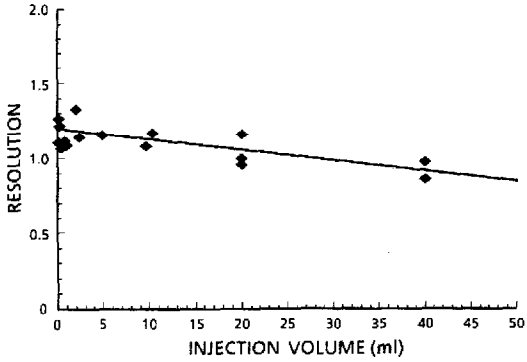

Fig. 1. Influence of injection volume on the resolution of a 50% mixture of AD and ADD. Column 50 × 1.0 cm I.D., LiChrosorb SI-60, 10 μm; column void, 28.5 ml; solvent, 10% ethyl acetate in methylene chloride; flow-rate, 8 ml/min; sample weight, 16 mg; detector, 286 nm for equal AD and ADD response. Resolutions are calculated based on detector response and TLC profile analysis.

observed. These results indicate that the injection volume may not be the most significant factor in determining the resolution of a multi-component separation as long as the total weight of sample mixture being injected remains constant.

Fig. 2 illustrates the effect of increased sample weight on resolution when a 50% mixture of AD and ADD was applied in a "concentration overloaded" condition. In this example, sample weights greater than 0.4 g reduced the yield and purity of AD and ADD to make preparative purifications of larger samples impractical on this column. Although the effect of sample weight on resolution is dependent on a number of chromatographic parameters, this example illustrates that sample load is typically the dominant factor influencing resolution. The Knox model for "concentration overloading", where the peak mass distributions are skewed toward earlier elution volumes as band spreading increases, fits the elution profiles obtained for AD and ADD during these trials.

Fig. 3 illustrates a third major factor in determining the overall resolution of a sample mixture. In this case, the sample weight ratio of AD and ADD is shown to have a major influence on the resulting resolutions. Fig. 3a shows a separation using

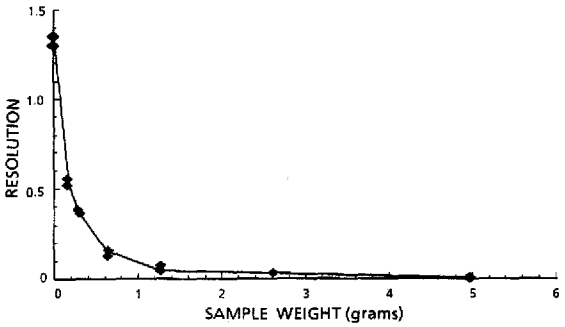

Fig. 2. Effect of sample loading weight on resolution of a 50% mixture of AD and ADD. Conditions are the same as in Fig. 1.

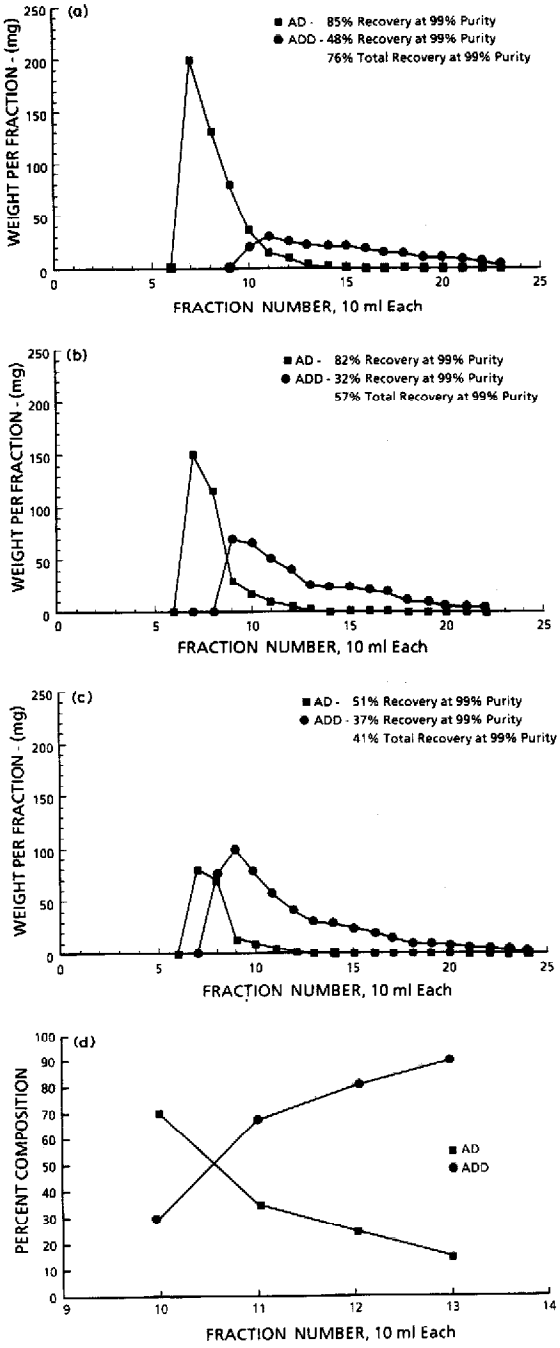

Fig. 3. Effect of weight composition of a mixture of AD and ADD on resolution. Sample: 640 mg. Conditions are the same as in Fig. 1, except the flow-rate was 2.0 ml/min. (a) 75% AD and 25% ADD; (b) 50% AD and 50% ADD; (c) 25% AD and 75% ADD; (d) percent AD (■) and ADD (●) composition of overlapping fractions 10–13 from a.

TABLE I

THE EFFECT OF AD AND ADD WEIGHT DISTRIBUTION ON RECOVERY AND RESOLUTION

*AD:ADD composition**	*Totals at 99% purity*			*Resolution*
	AD (mg)	*ADD (mg)*	*Percent***	
75:25	408	76	76	0.29
50:50	262	103	57	0.15
25:75	82	177	41	0.05

* 640 mg total weight per injection.
** Percent total recovery at 99% purity.

0.64 g of a mixture containing 75% AD and 25% ADD. Fig. 3b shows a similar profile for 0.64 g of 50% AD and 50% ADD, while Fig. 3c shows a profile for 0.64 g of 25% AD and 75% ADD. The effect of sample weight distribution on the resolution and recovery of AD and ADD is shown in Table I. This data indicates that for weight overloaded conditions the total resolution of a mixture of components can be enhanced if the component distributions are altered.

The separation of the fastest eluting component of any mixture is significantly improved as its relative weight in the sample mixture is increased. This is because the tail of each peak remains at a relatively constant tail capacity (k'_t) value that is equivalent to the same component's peak capacity (k'_p) for small analytical injections[6]. As the weight of a component in a mixture is increased, the peak capacity (k'_p) gets smaller and comes at an earlier elution volume due to greater "concentration overloading" effects[4]. This band broadening effect improves the resolution of a separation if the first eluting component of a mixture has the highest weight ratio. This is because as this band broadening occurs, it cannot spread forward to contaminate any other eluting compounds. Later eluting components can overlap the faster eluting components as their relative weight in the mixture increase, and the overall resolution of a separation decreases dramatically.

These studies indicate that injection volume, sample weight, and sample distribution all have an effect on resolution. However, the change in sample weight distribution can outweigh the disadvantages of larger injection volumes required to apply wet fractions directly back onto the column. This significantly enhances the advantages of injecting the unevaporated impure fractions directly back onto the column, as a single injection, rather than combining, drying, and rechromatographing them as a new mixture to obtain the desired purity and recovery. Fig. 3d illustrates the region of AD and ADD overlap from Fig. 3a. Although the AD and ADD contained in these fractions has not been purified to the desired level, the distribution of AD and ADD in these fractions has been changed dramatically. These fractions are ideal for reinjection because the weight distribution of AD and ADD has been altered.

Fig. 4a shows a single-pass normal phase purification of a mixture of diastereomers of a Boc protected dipeptide using a mobile phase of 3% ethanol in methylene chloride. TLC analysis of the eluted fractions indicated that fractions 28–33 still contained the first diastereomer and some of the faster eluting impurities, while fractions 45–51 contained a mixture of both diastereomers. The column was then eluted with 100% ethanol to remove any residual impurities remaining on the column,

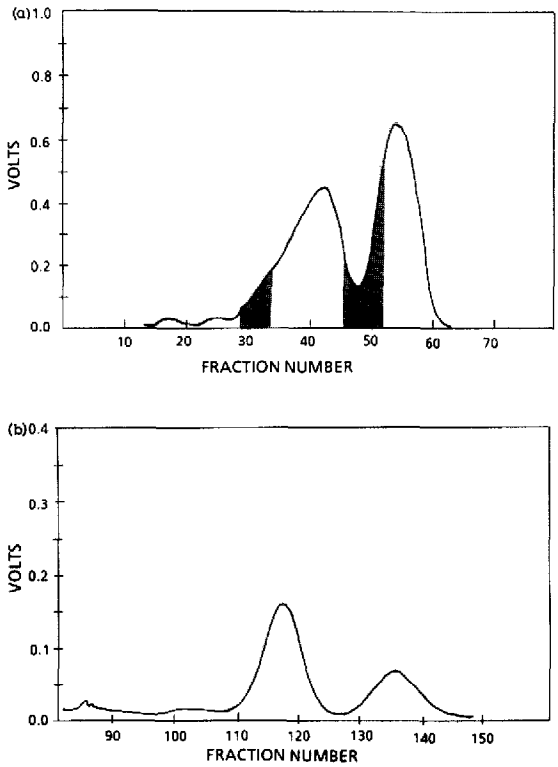

Fig. 4. (a) Single-pass separation of a crude mixture of Boc-protected dipeptide. Column: 100 × 2.5 cm I.D.; dry packed with Merck Kieselgel 60 silica; column void: 400 ml; solvent: 3% ethanol in chloroform. Sample: 5.29 g of a mixture of Boc-protected dipeptide diastereomers; flow-rate: 25.0 ml/min; fraction volume: 25.0 ml; detector: 254 nm. (b) Re-injection of fractions 28–33 and 45–51 from a onto the same column using the same conditions. Sample: 1.07 g. The purified diastereomers were found to constitute 94% of the crude sample mixture weight.

and reconditioned with the initial mobile phase. The unevaporated factions 28–33 and 45–51 were then sequentially reinjected back onto the original column as a single volume overloaded injection. Each of these 13 fractions was 25.0 ml for a total single injection volume of 325 ml or 0.81 column void volumes. It is critical that these fractions be reinjected back onto the column in the same order in which they had initially eluted, so that the faster eluting components are the first to be reinjected back onto the column. The results of this external recycle are shown in Fig. 4b, where the reinjected impure fractions were completely resolved. The final result is that instead of approximately 75% total recovery of both diastereomers after one single-pass through the column, there is now almost complete recovery at 100% purity. This technique of externally recycling fractions need not be limited to the overlap of only two adjacent components, but can be applied to any number of adjacent overlapping peaks from the same separation.

The major criteria used to decide when a reinjection of eluent fractions should be performed can best be determined by examining the tail retentions of the eluting peaks

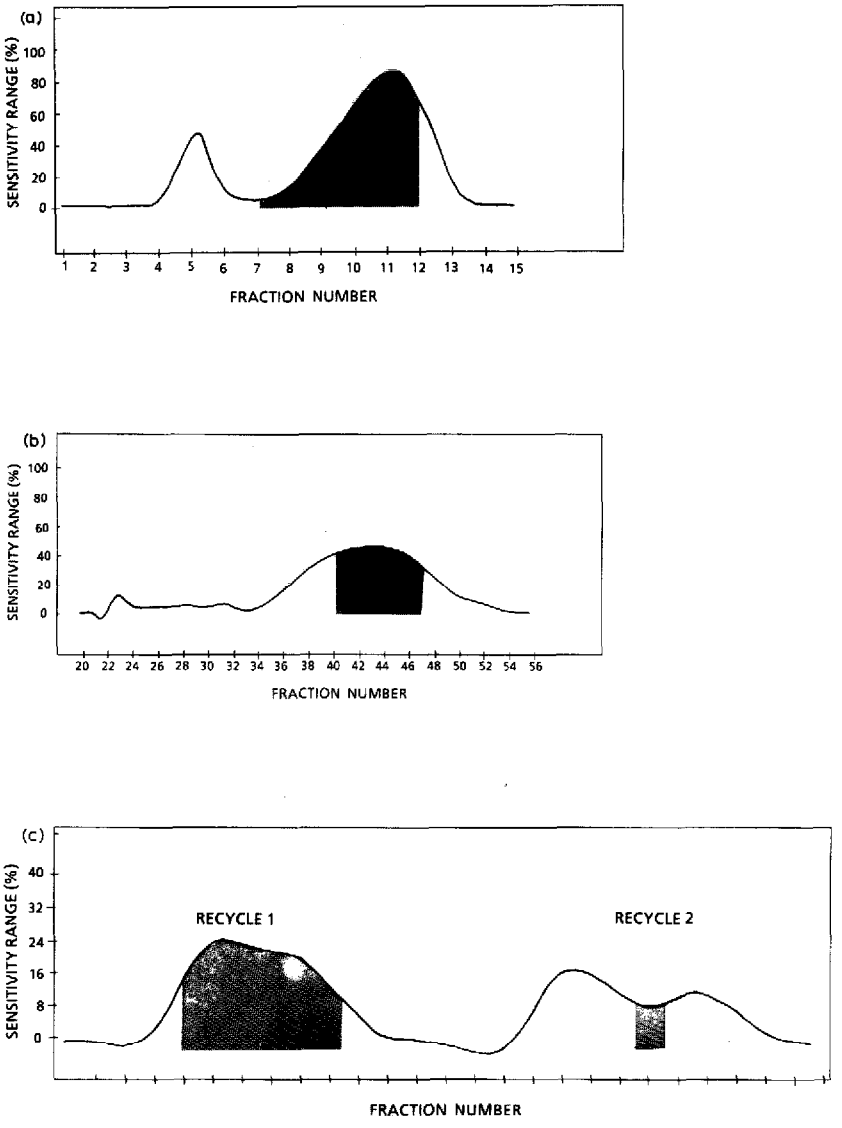

Fig. 5. (a) Single-pass separation of a crude mixture of tetrahydral isoquinoline diastereomers. Column: 150 × 5.0 cm I.D.; dry packed with Merck Kieselgel 60 silica; column void: 2400 ml; solvent: 3.0% isopropanol in hexane. Sample weight: 20.7 g; flow-rate: 500 ml/min; fraction volume: 500 ml; detector: refractive index with a range of 2. (b) Re-injection of fractions 7–11 from a onto the same column using the same conditions, except for the fraction volume being 250 ml. Sample: 11.5 g. (c) Re-injection of fractions 40–46 from b onto the same column, then recycled for two passes using the same conditions, except for the refractive index range being 5. Shaded areas were recycled or collected as a mixture. Sample: 6.5 g. The purified diastereomers were found to constitute 73.4% of the crude sample weight.

of interest. Large differences in the tail retentions of eluting compounds could indicate that a reinjection will be useful. When an initial separation has been partially successful, improvements in recovery and purity can generally be expected for all components. Reinjections are usually performed with only a portion of the initial sample weight. This reduced total sample weight does not seem to be a significant factor in determining the resolution of the components for reinjected fractions, but it appears to be more dependent on the altered weight distribution of the mixture components in the fractions.

Another example of the advantages of external recycle is shown in Fig. 5a. The initial single-pass separation isolated a mixture of tetrahydral isoquinoline diastereomers from a crude reaction mixture using a mobile phase of 3% isopropanol in hexane. The column was then eluted with 50% isopropanol in hexane to remove the more polar impurities, and reconditioned with the initial mobile phase. TLC analysis of all eluting fractions indicated that fractions 7–11 contained a mixture of both diastereomers. Fractions 7–11 were then sequentially injected back onto the same column as a single injection. The results of the reinjection in Fig. 5b indicate that a complete separation was still not obtained.

The initial injected sample weight could have been decreased, or a larger column size could have been used to give a complete separation of the mixture with one single-pass separation. The major drawback with this approach to preparative chromatography is that the amount of work and materials required to achieve the purification of the total sample increases with the number of runs and the size of the column used for the purification. Hence, in most instances, sample weight overloading is a more prudent way to maximize the throughput efficiency of preparative purifications[1].

TLC analysis of the fractions in Fig. 5b suggested that the weight distribution of the components had been altered significantly. This indicated that the reinjection process was working and only needed to be continued in order to further resolve the diastereomers. A second reinjection was made using fractions 40–47. This process of reinjecting fractions could have continued until the sample was completely resolved, however, because the band width of the two diastereomers was less than the recycle eluent volume, the system was run in a conventional recycle mode for two passes through the column. The resulting separation profiles are shown in Fig. 5c, where the pure portion of each eluting diastereomer was removed with each recycle pass through the column. The final outcome of using this technique is that a 96.4% recovery of the diastereomers at better than 95% purity was obtained in one column run using two reinjections and two conventional recycle passes.

This example illustrates how a purification can evolve from a clean-up mode into a highly efficient conventional recycle mode of operation by incorporating an external recycle operation into the process. Conventional recycle is the easiest method of performing recycle chromatography, but although it has many practical applications its use is often limited. When a mixture containing more than two components is being separated, or a two-component eluent band volume is larger than its conventional recycle eluent volume, external recycle chromatography can be more practical than conventional recycle methods.

CONCLUSION

External recycle is a technique where significant improvements can be achieved in the recovery and purity of valuable samples when an initial single-pass separation through a column does not give adequate resolution of the components of interest. This operation can be performed using multiple fractions in an eluting stream, provided they are sequentially reinjected back onto the column in the same order in which they had initially eluted. Further, reinjections can be performed in a series of cycles with most separations, to stepwise isolate the components of interest. This method is less laborious and more efficient than combining and drying the unresolved fractions to reduce the injection volume, and then rechromatographing them as a new mixture. This technique has been applied to reversed-phase systems, and should be applicable to other modes of liquid chromatography when using isocratic conditions and where the lack of resolution is due to sample weight overload on the column.

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