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LARGE-SCALE PREPARATIVE COUNTERCURRENT CHROMATOGRAPHY FOR SEPARATION OF POLAR COMPOUNDS

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SUMMARY

Gram quantity separations of polar compounds (tryptophyl-leucine and valyl-tyrosine) have successfully been accomplished with the use of a horizontal coil planet centrifuge. Two columns of different length fluorinated ethylene propylene tubing but of same internal diameter (0.55 cm) were coaxially coiled around a holder 7.5 cm or 15 cm in diameter and used to assess the preparative capabilities of the apparatus in terms of stationary phase retention and sample peak resolution. Optimal operating conditions derived from preliminary studies with the short column were applied to a column 7 times in length and volume. Volume capacities were 114 ml and 750 ml respectively. A hydrophilic solvent system of *n*-butanol, acetic acid and water (4:1:5) was used with both the aqueous and non-aqueous phases being used as the mobile phase. Preliminary studies revealed that the hydrodynamic distribution of the two phases was independent of the helical diameter while peak resolution was sensitive to both helical diameter and rpm setting.

INTRODUCTION

In the past several schemes have been presented that demonstrate the preparative capabilities of the horizontal flow-through coil planet centrifuge in the separation of moderately hydrophobic compounds using a two-phase solvent system of intermediate hydrophobicity[1,2]. This paper demonstrates the same preparative capabilities (gram quantities) of the apparatus but with the separation of polar compounds using a hydrophilic two-phase solvent system.

The solvent system of *n*-butanol, acetic acid and water (4:1:5) was used in the separation of two dipeptides, tryptophyl-leucine (trp-leu) and valyl-tyrosine (val-tyr). Traditionally, this solvent system is most commonly used in the separation of peptides but has been very difficult to work with. The additive properties of low interfacial tension and high viscosity tend to produce an emulsification and result in the concomitant loss of the retained stationary phase and peak resolution. The use of slower flow-rates lessens this emulsification as well as decreasing the pressure build-up associated with highly viscous systems.

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With droplet countercurrent chromatography, high retention of the stationary phase is achieved with this solvent system but at a low flow-rate of 4.2 ml/h[3]. The Kontes model horizontal flow-through coil planet centrifuge equipped with eccentrically mounted columns with 2.6-mm I.D. tubing was able to maintain an adequate level of retained stationary phase (40%) at higher flow-rates of 24 ml/h while sample loading capabilities were in the range of 200 to 300 mg[4].

This paper deals with a horizontal flow-through coil planet centrifuge with a coaxially mounted column. Separation of dipeptides using the same solvent system were accomplished at significantly higher flow-rates of 120–240 ml/h while maintaining stationary phase retention values of 50% and greater and at a sample loading capacity of 1 g. Effects of sample size, flow-rate, rpm, and helical diameter were studied in order to optimize operational conditions.

PRINCIPLE

As described in previous papers[2,5] partitioning between the two phases of a two-phase immiscible solvent system provides an excellent modality upon which to separate chromatographically mixtures of different compounds. Countercurrent chromatography provides such a modality quickly and efficiently. In a slowly rotating helical coil filled with water, all objects placed within the coil will move towards one end. This is called the head end while the other end is called the tail. This is in accordance with the Archimedean screw principle. When the coil is subjected to a particular type of synchronous planetary motion, a hydrodynamic equilibrium is established whereby one of two phases of a two-phase solvent system is permanently retained within the column without the use of solid supports. Samples introduced with the mobile phase pass through the stationary phase within the column and are separated according to their partition coefficients. This system provides for the continuous elution of the sample via the mobile phase.

In Fig. 1 the synchronous planetary motion is achieved by mounting a column holder with a planetary gear coupled to an identical stationary sun gear coaxially mounted along the central axis of the apparatus. The column holder of radius r essentially rotates about its central axis once with respect to the apparatus and twice with respect to gravity and always with its axis parallel to and at a distance R from

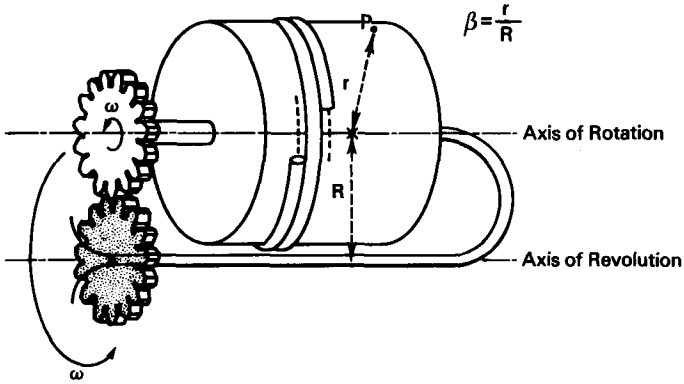

Fig. 1. Synchronous planetary motion of the coil holder.

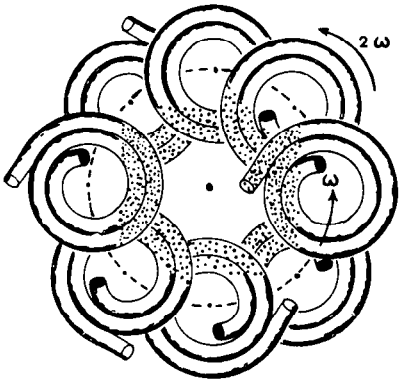

Fig. 2. Droplet zone motion in the concentric coil planet centrifuge.

the central axis of the apparatus. This planetary motion produces a hydrodynamic equilibrium that is capable of retaining large amounts of stationary phase at high flow-rates. In addition, earlier studies revealed that this synchronous planetary motion produces a centrifugal force field pattern that varies in both magnitude and direction[6]. This force field is highly dependent upon the location of a point P located on the column holder. Location of the point P may be expressed in terms of $\beta = r/R$. At a β value of 0.5 or greater, the force vectors are always pointing outwardly from the coil and fluctuate in both magnitude and direction during each revolutional cycle. Recent studies conducted by Conway and Ito[7] indicated that the rotating coil exhibits a localized region of intense droplet formation (Fig. 2). This mixing zone is located in those helical turns closest to the center of rotation of the column. The remaining area of the column represents the coalescence of the droplets and the reformation of two distinct phases such that the heavier phase occupies the most peripheral aspect of each turn of tubing while the lighter phase occupies the inner most half. This may be explained on the basis of the centrifugal forces exerted on the coil. The mixing zone is propagated through the coil in an analagous fashion to that of waves moving through the ocean. When the rpm level is increased, this mixing zone formation increases per unit time and probably contributes greatly to the high efficiency of separation obtained with this system.

APPARATUS

The apparatus illustrated in Fig. 3 is a modified version of a horizontal flow-through coil planet centrifuge used in previous reports[8]. Modifications dealt mainly with the column holder. The holder was made removable by altering the bearing assemblies such that they were now held to the rotary frame by a pair of screws. The holder itself was modified with a pair of flanges that support the tubing at either end of the holder.

The motor drives the rotary frame around the central stationary shaft via a pair of toothed pulleys and a toothed belt. The rotary frame consists of two symmetrically rotating holders each with their central shafts parallel to the central axis of the apparatus and held to the rotary frame with sealed ball bearings. The rotating

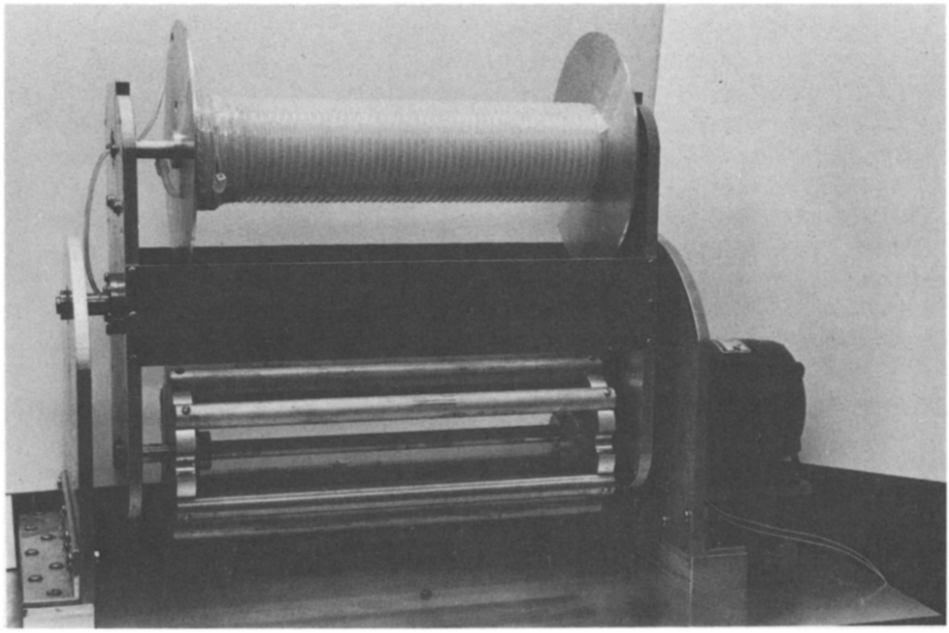

Fig. 3. Photograph of the apparatus.

column holders are positioned 15 cm from the central axis of the apparatus and are coupled to a stationary gear of the central shaft via two identical gears fixed to the holders. One of the two holders serves as the experimental column while the other functions as a counterbalance.

Two studies were conducted using two different length columns with the same internal diameter of 0.55 cm. Preliminary studies employed a short column consisting of a single layer of fluorinated ethylene propylene (FEP) (Galtek) tubing, 420 cm long and 114 ml in capacity. The second study utilized a long column consisting of 2 layers of the same FEP tubing but 40 m long and 750 ml in capacity. The apparatus has a 42-cm wide holder with a 7.5-cm diameter core held 15 cm from the center of the apparatus. This results in a β value of 0.25. The β value may be increased by adding several plastic cylinders, 42 cm in length, around the core of the holder so that it was possible to increase the holder diameter to 15 cm to obtain a β value of 0.5. These two β values were tested during the preliminary studies, the original value of 0.25 and a larger value of 0.5. Flow tubes from the column are passed through the hollow central shaft to the monitoring system. The planetary motion of the apparatus prevents twisting of the flow tubes and thus eliminates the need for rotating seals and the associated problems of leakage and contamination. Revolutional speed was regulated by a Bodine Electric Co. speed control unit. Solvent was pumped through the column using a Beckman ACCU-FLOW pump. The effluent was monitored by an LKB Uvicord S at 280 nm and collected into fractions with an LKB Ultrorac fraction collector.

EXPERIMENTAL

Reagents

The dipeptides used in this study, L-tryptophyl-L-leucine (trp-leu) and L-valyl-L-tyrosine (val-tyr), were obtained from Sigma, Saint Louis, MO, U.S.A. The two-phase solvent system was composed of *n*-butanol, acetic acid and water at a volume ratio of 4:1:5. Glass distilled *n*-butanol was obtained from Burdick and Jackson Labs., Muskegon, MI, U.S.A. and the acetic acid from J. T. Baker, Phillipsburg, NJ, U.S.A.

Solvent system and sample solution

The two-phase solvent system was prepared by mixing the *n*-butanol, acetic acid and water in a 2-l separatory funnel. The phase system was equilibrated at room temperature in the separatory funnel and separated before use. The sample solution for the preliminary studies was prepared by dissolving 200 mg of trp-leu and 80 mg of val-tyr in 50 ml of the lower phase and in each separation 1.0 ml was charged into the column for a net sample size of 5.6 mg. With the long column, a variety of sample doses was used. Equal amounts of each dipeptide were dissolved in equal volumes of both upper and lower phases for a final sample volume of 20 ml and net sample sizes of 100, 250, 500, and 1000 mg were tested.

Separation procedure

The column was first filled entirely with the stationary phase which was then followed by the introduction of the sample mixture through the sample port. Following sample injection the apparatus was rotated while the mobile phase was introduced into the column at the desired flow-rate of either 120 ml/h or 240 ml/h. Different elution modes (head to tail or tail to head) were selected for each run as well as the choice of the mobile phase and the rpm setting. The effluent was continually monitored at 278 nm and collected with a fraction collector. An aliquot of each fraction was diluted with methanol and analyzed with a Zeiss PM6 spectrophotometer at 280 nm. Following the separation, the column contents were voided with nitrogen gas at 80 p.s.i. while the column was slowly being rotated in a tail to head elution mode to facilitate emptying the column. Column contents were collected in a graduated cylinder to calculate the volume of the stationary phase retained in the column.

RESULTS AND DISCUSSION

The effect of the helical diameter of the column, the rpm level, flow-rate, and choice of mobile phase were studied on the retention and peak resolution of the pair of dipeptides in a solvent system composed of *n*-butanol, acetic acid and water.

Fig. 4 illustrates the phase distribution patterns using the short column. Along the left hand margin are the β values tested, 0.25 and 0.5. The flow-rate and choice of mobile phase are located at the top of each set of graphs. The ordinate depicts the percent stationary phase remaining in the column while the abscissa indicates the rpm setting. Elution modes are indicated by either a solid or broken line.

It is clearly demonstrated that the two solvents are unilaterally distributed

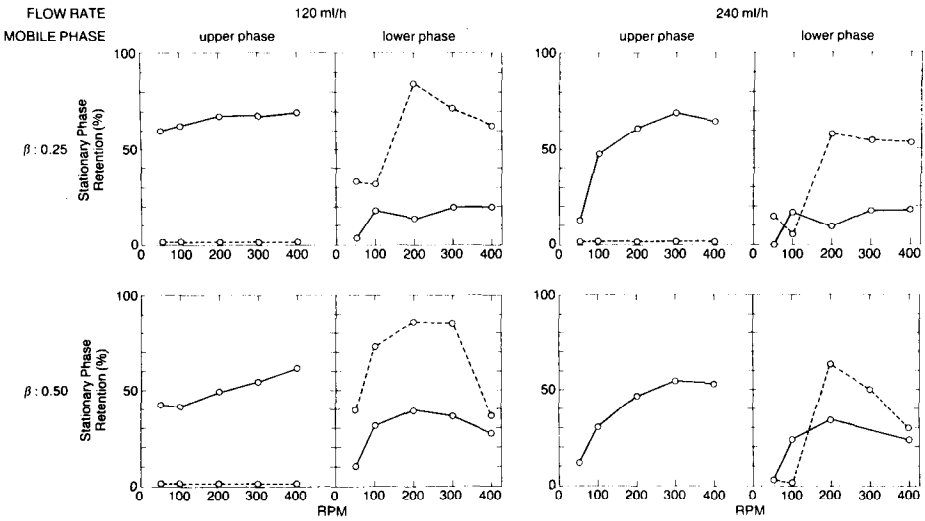

Fig. 4. Phase distribution diagrams of *n*-butanol–acetic acid–water (4:1:5) obtained with the short column at two different β values. ——, head to tail elution; --- –, tail to head elution.

within the column. When the upper phase is used as the mobile phase and introduced through the head end, there is a high retention of the stationary phase at both flow-rates and β values, whereas introduction of the upper phase through the tail results in a total loss of the stationary phase. When the lower phase is used as the mobile phase and introduced through the tail, a high level of the stationary phase remains in the column; however, when the lower phase is introduced through the head end, a significant decrease of the upper stationary phase results. These findings suggest that the upper phase is distributed in the tail end of the column and the lower phase is distributed in the head end regardless of the β values used. This finding is supported by the hydrodynamic observations of the same solvent system in a coil of smaller I.D. tubing of 2.6 mm[9]. At the higher flow-rates retention of the stationary phase decreases somewhat. This occurs especially at the lower rpm levels and is most likely due to an insufficient centrifugal force field needed to maintain a high stationary phase equilibrium. However, by simply increasing the rpm level, satisfactory stationary phase retention can be maintained even at these higher flow-rates. Revolutional speeds exceeding 100 rpm usually provide high levels of retained stationary phase in the proper elution mode while rpm values of 100 and below tend to demonstrate lower retention. In addition, rpm levels in excess of 300 also demonstrated lower retention. This is probably due to an excessive mixing of the two solvents which results in an emulsification and loss of the stationary phase. As indicated in a previous report[10], the peak resolution was found to be increased with the amount of the stationary phase retained in the column.

Studies on the short column revealed that similar stationary phase retention values were obtained at both β values but better peak resolution was observed at the lower β value. With a smaller helical diameter, in a unit length of tubing, it is possible to achieve more helical turns of the tubing as compared to a column holder of larger diameter. Thus, with respect to the mixing zone previously mentioned, there would

be a greater number of mixing zone segments of shorter length per unit length of tubing. An increase in the number of mixing zones would increase the peak resolution. A decrease in the length of each mixing zone would decrease longitudinal mixing so that peak broadening would be minimized.

From the preliminary studies using the short column, optimal operating conditions could now be applied to the long column with a volume and length 7 times as that used in the preliminary studies. A flow-rate of 120 ml/h and a rpm setting of 300 were chosen. When the upper phase was used as the mobile phase, a head to tail elution mode was used; when the lower phase was used as the mobile phase a tail to head elution was used. A β value of 0.25 was exclusively used.

Fig. 5 shows the stationary phase retention in the long column plotted against sample size. As the sample size is increased, the retention of the stationary phase decreases. The loss of the stationary phase at high sample doses may be attributed to changes that occur in the phase composition. These changes may result in a transient alteration in the phase volume ratio which is followed by the loss of the stationary phase. In addition, a decrease in the interfacial tension due to the high solute concentration may also contribute to this decreased stationary phase retention. At small sample doses up to 250 mg the phase composition is not altered to any significant degree so that high retention of the stationary phase is achieved.

In Fig. 6 the chromatograms obtained with the long column are shown in conjunction with the different sample sizes. Along the left hand column are the various sample sizes used. In each diagram the abscissa depicts time in hours while the ordinate shows the absorbance levels. The absorbance scales have been normalized with respect to the sample size involved. Each of the two phases was used as the mobile phase. Although the peak resolution decreases as sample dose is increased, baseline separation is still maintained, even at the highest sample dose of 1 g. As noted in Fig. 5, when the lower phase is used as the mobile phase, stationary phase retention is decreased as compared to when the upper phase is the mobile phase. This stationary phase retention difference is also reflected in the chromatograms, *i.e.*, better stationary phase retention yields increased resolution and efficiency. Higher stationary phase retention with the upper phase mobile may be attributed to the hydrodynamic stability with respect to each elution mode. When the upper phase is the

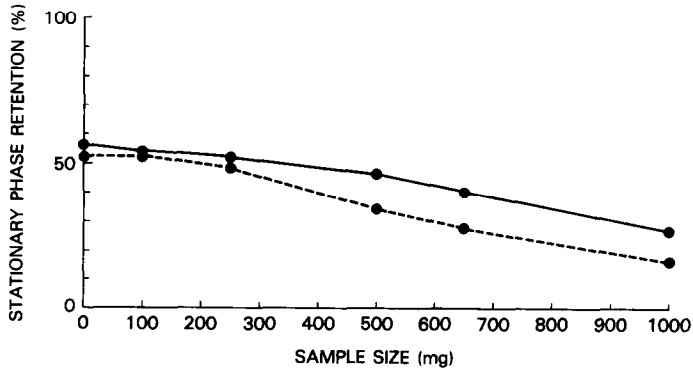

Fig. 5. Effects of sample concentration on retention level of the stationary phase. ——, upper phase mobile; – – –, lower phase mobile.

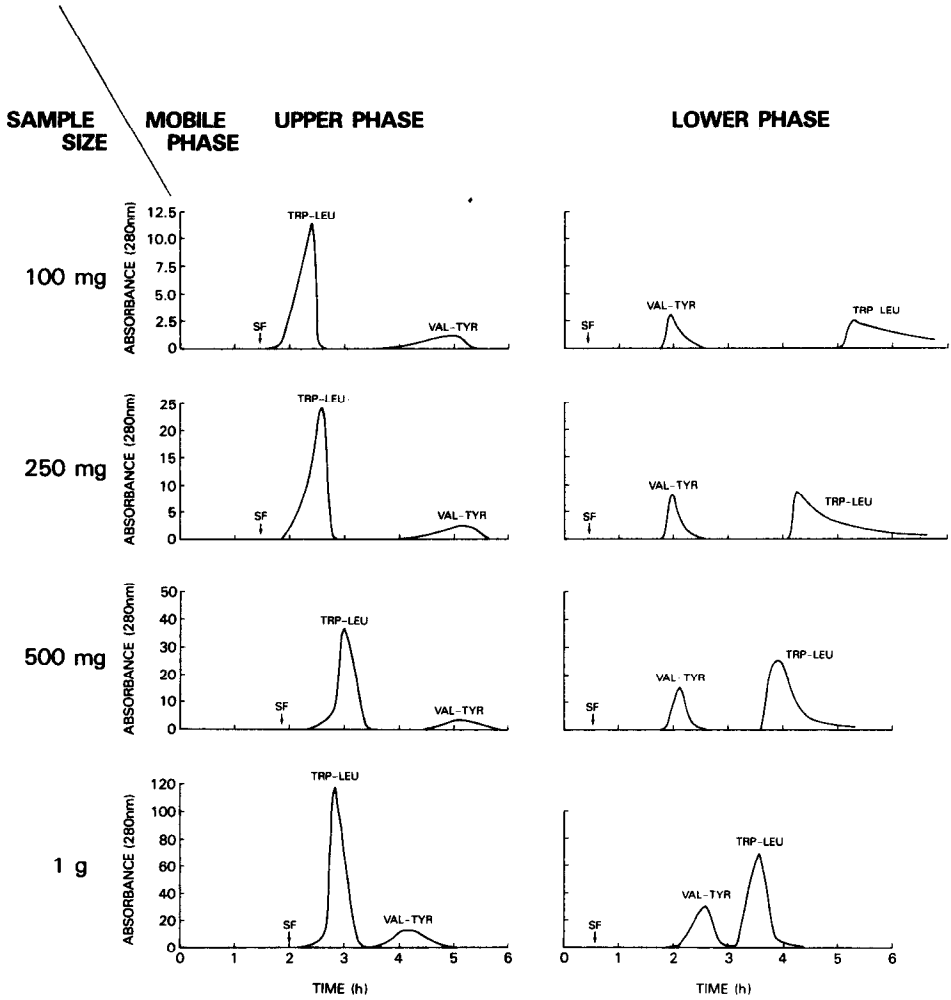

Fig. 6. Effect of sample concentration on separation of a set of dipeptides.

mobile phase a head to tail elution mode is used. This mode produces a very stable hydrodynamic equilibrium such that once the solvent front has moved through the coil, little or no carryover of the stationary phase occurs. This is because any phase in excess, in this case the mobile phase, completely occupies the tail end of the column. However, in the tail to head elution mode with the lower phase mobile, the lower phase dominantly occupies the head end of the coil while a small portion of the stationary upper phase may always remains in the head-end. Hence, continual carryover of the stationary upper phase ensues.

When the lower phase is used as the mobile phase, appearance of the solvent front occurs early in the run, usually within 30 min. At a flow-rate of 120 ml/h, 60 ml of the stationary phase should be displaced as the mobile phase moves through the column. This would result in a stationary phase retention of greater than 90%.

However, experimental observation revealed stationary phase retention values of 52% at the smallest sample size and decreased to 16% at the largest sample size. Thus, even though the solvent front appeared early and displaced very little stationary phase, the hydrodynamic equilibrium associated with the tail to head elution mode resulted in a continual carryover of the stationary phase and eventually yielded a low retention value. In the head to tail elution mode, an initial loss of the stationary phase is greater due to the later appearing solvent front but practically little or no carryover occurred through the remainder of the run since the tail is totally occupied by the excessive mobile phase.

CONCLUSION

In the past it had been demonstrated that the horizontal flow-through coil planet centrifuge is capable of separating gram quantities of DNP-amino acids using a moderately hydrophobic solvent system composed of chloroform, acetic acid and 0.1 *N* hydrochloric acid. Again, the preparative potential of this system is clearly evident in the gram quantity separation of a sample mixture using a different solvent system. Here, the successful separation of polar compounds (dipeptides) was accomplished via a most commonly used hydrophilic solvent system of *n*-butanol, acetic acid and water (4:1:5). The preparative capabilities of this system need not stop at this point but may be further increased by using a longer length of tubing for the column and/or increasing the internal diameter of the tubing. Due to the relatively slow revolutional rate (300 rpm) industrial-scale separations with a larger diameter column may be produced without much compromise to safety.

REFERENCES

1 J. L. Sandlin and Y. Ito, *J. Liquid Chromatogr.*, 7 (1984) 323.
2 Y. Ito, J. L. Sandlin and W. G. Bowers, *J. Chromatogr.*, 244 (1982) 247.
3 H. Yoshida, C. L. Zimmerman and J. J. Pisano, in R. Walter and I. Meienhefer (Editors), *Peptides: Chemistry, Structure, and Biology, Proc. 4th Amer. Peptide Symp.*, Ann Arbor Sci. Publ., Ann Arbor, MI, 1975, p. 955.
4 M. Knight, A. M. Kask and C. A. Tamminga, *J. Liquid Chromatogr.*, 7 (1984) 351.
5 Y. Ito, *J. Chromatogr.*, 207 (1981) 161.
6 Y. Ito, *J. Chromatogr.*, 192 (1980) 75.
7 W. D. Conway and Y. Ito, presented at *1984 Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy*, Abstract No. 472, p. 472.
8 Y. Ito and R. L. Bowman, *J. Chromatogr.*, 147 (1978) 221.
9 Y. Ito, *J. Chromatogr.*, 301 (1984) 387.
10 W. D. Conway and Y. Ito, *J. Liquid Chromatogr.*, 8 (1985) in press.