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APPLICATION OF CYCLING ZONE SEPARATION TO PREPARATIVE HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

Cycling zone separation techniques are applied to standard high-pressure liquid chromatography equipment to develop a new preparative separation technique. Results are presented for the continuous removal of glycyl-L-phenylalanine and glycyl-L-tyrosine from water using dichloroacetic acid to force the separation. The effect of operating conditions, comparison with elution chromatography and preparative applications are discussed.

INTRODUCTION

Cycling zone separation is a technique that has developed as a preparative separation using chromatography, adsorption or counter-current distribution equipment. The technique separates a continuous feed by periodically changing a thermodynamic variable which affects the distribution of solutes between the stationary and mobile phases. The much larger throughput that can be achieved with continuous feed when compared with pulse feed makes this process an interesting alternative for preparative chromatography.

Pigford *et al.*[1] and Baker and Pigford[2] developed cycling zone adsorption as a method for removing all of the solutes in the feed. In the "traveling-wave mode" of operation the entering streams were heated and cooled periodically. The temperature variation changed the distribution coefficients and thus forced the separation. Both liquids and gases were separated[1,2] and a mathematical model of the separation was presented[2]. Busbice and Wankat[3] extended the technique to low-pressure liquid chromatography, utilizing pH as the thermodynamic variable. A theoretical method for multicomponent fractionation was developed by Wankat[4]. Theoretical and experimental results are covered in detail in a recent tutorial review[5].

In this paper cycling zone separation is extended to high-pressure liquid chromatography (HPLC) using the concentration of an added chemical as the thermodynamic variable which forces the separation. Specifically, glycyl-L-phenylalanine and glycyl-L-tyrosine were removed from water using dichloroacetic acid concen-

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tration as the thermodynamic variable on a reversed-phase C_{18} bonded packing. The effect of operating variables, comparison with elution chromatography and possible use for preparative separations are discussed.

CYCLING ZONE SEPARATION

Since the experimental and theoretical aspects of cycling zone adsorption are covered in detail elsewhere[1–5], only a brief description will be presented. The basic idea of cycling zone separation is to use a thermodynamic variable to force the solutes to concentrate in certain portions of the fluid as it flows through the column. Since dichloroacetic acid is known to increase the retention of dipeptides on nonpolar packings[6], concentration waves of dipeptides can be developed in the column by periodically varying the inlet dichloroacetic acid concentration. This is accomplished by inputting square waves of dichloroacetic acid varying between two concentration levels. When the exiting fluid contains low concentrations of dichloroacetic acid, the dipeptide concentration will be high and vice versa. With this technique it is possible to concentrate the dipeptides in part of the water stream, and remove the solutes from the remainder of the fluid. Thus a continuous feed can be treated to produce a product whose concentration varies periodically. The cycling zone separation differs from gradient elution chromatography in that the material to be separated is fed continuously, the solutes are concentrated instead of diluted, and the thermodynamic variable, dichloroacetic acid in this case, forces the separation instead of merely speeding up or improving the separation.

The simple cycling zone separation technique described above can give large separation factors if "amplification" and/or "trapping" occur. Amplification occurs when very small changes in the thermodynamic variable cause large changes in the distribution coefficients. Trapping will occur if the dichloroacetic acid moves through the column at a velocity that is greater than the velocity of the dipeptide at high dichloroacetic acid concentrations, but less than the velocity of the dipeptide at low dichloroacetic acid concentrations. Then dipeptide in the region of low dichloroacetic acid concentration will overtake the dichloroacetic acid wave front but will be unable to pass this front. As a result the dipeptide is "trapped" and a large peak of dipeptide will exit at the point where the dichloroacetic acid concentration drops to the lower level. "Amplification" appears to be illustrated in the results shown later, but "trapping" does not.

EXPERIMENTAL

As shown in Fig. 1, HPLC system consisted of standard equipment arranged for cycling zone separation. Two stainless-steel feed reservoirs contained equal dipeptide concentrations but different concentrations of dichloroacetic acid. An arrangement of Asco solenoid valves allowed for alternate feed to a Milton Roy (Philadelphia, Pa., U.S.A.), Mini-Pump. A Chromatronix (Santa Clara, Calif., U.S.A.) HPSV-20 sample injection loop valve was installed between the pump and column so that pulses could be fed to the column. The injection valve was not employed in the cycling zone mode of operation. The stainless-steel column was 2 ft. × 1/8 in. O.D. (2.1 mm I.D.) (No. 98151, Waters Assoc., Milford, Mass., U.S.A.), and was

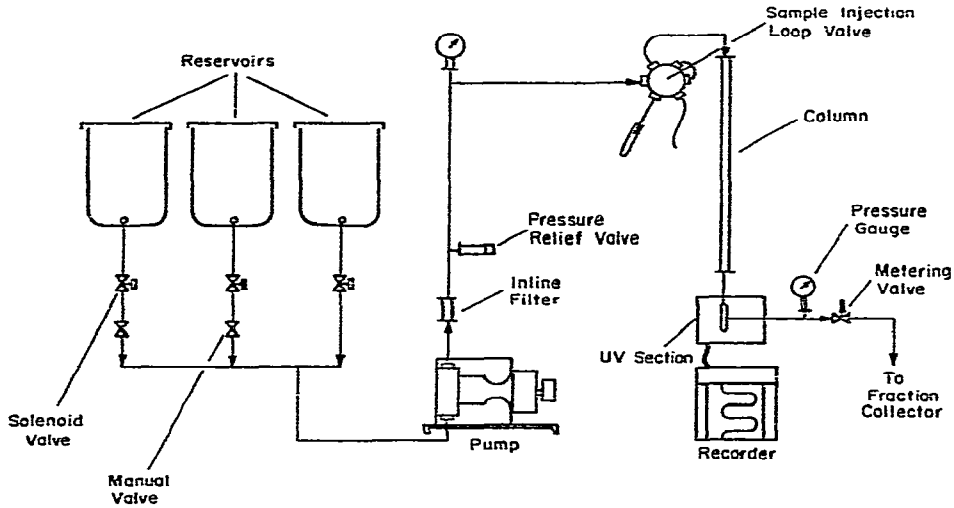

Fig. 1. Schematic diagram of apparatus.

packed with Waters Bondapak C_{18}/Porasil B which is a C_{18} hydrocarbon bonded to a porous silica support. Since preparative separations were being investigated a 37–75 μm packing was used. The "tap–fill" method of packing the column was employed[7]. A Chromatronix Model 230 dual-channel mixed-wavelength UV detector (254 and 280 nm) was used with a Houston Instrument (Houston, Texas, U.S.A.) Model 5211-11 two-channel integrating recorder.

The dipeptides were purchased from Cyclo Chem. (Los Angeles, Calif., U.S.A.) and the dichloroacetic acid was from Aldrich (Milwaukee, Wisc., U.S.A.). The pulse experiments showed two minor impurities in both dipeptides, although the glycyl-L-phenylalanine was considerably purer.

Pulse experiments used standard chromatographic operating techniques and methods of analyzing the data. The cycling zone experiments consisted of periodically switching feed reservoirs until a repeating state was reached where each cycle was a copy of the previous cycle. This usually required four or five cycles. To separate the UV signals of the dipeptide and dichloroacetic acid a blank cycling zone experiment was done with dichloroacetic acid alone. The results from the blank experiment represented the dichloroacetic acid wave leaving the column, and this signal was subtracted from the total signal to give the dipeptide signal. The 254-nm signal was used for the results presented here since neither dichloroacetic acid nor glycyl-L-phenylalanine absorbed at 280 nm. Mass balances on the dipeptide and the dichloroacetic acid were used as a check on the results. Complete details of the experimental equipment and procedure are given by Nelson[8].

RESULTS

Pulse experiments (elution chromatography) were run to study the column efficiency and the effect of dichloroacetic acid on retention. These experiments showed that glycyl-L-phenylalanine and glycyl-L-tyrosine were very clearly separated

TABLE I

DIPEPTIDE RETENTION TIMES

Dichloroacetic acid (%)	*Glycyl-L-phenylalanine retention time (min)*	*Glycyl-L-tyrosine retention time (min)*
0.0	5	2.5
0.005	31.5	14
0.01	35	18

at a linear velocity of 0.25 cm/sec with distilled water as the solvent. Under these conditions the column contained approximately 125 theoretical plates. Addition of very small amounts of dichloroacetic acid to the solvent greatly increased the retention time. This is shown in Table I. Since the dichloroacetic acid concentration does have a strong effect on the retention times, it is reasonable to expect that cycling zone separations will be successful.

A typical cycling zone experiment with glycyl-L-phenylalanine is shown in Fig. 2B. The inlet and outlet dichloroacetic acid concentration waves which caused this separation are shown in Fig. 2A. Comparison of these two figures shows that, as expected from the retention times, glycyl-L-phenylalanine elutes from the column when the outlet acid concentration starts to drop, and is held up in the column when the outlet acid concentration rises. The "shoulder" or flat portion of the dipeptide curve at the feed concentration is an indication that the cycle time is too long. The dipeptide exiting in this region passed through the column without experiencing a change in acid concentration. The outlet dichloroacetic acid wave shown in Fig. 2A exhibits a lag of about 3 min from the inlet wave. About 45 sec of this lag is due to the column dead volume while the remainder is caused by the dead volume between the switching valves and the column. This dead volume should have little effect on the separation. The peak-to-peak separation factor of 5.5 shown in Fig. 2B was improved considerably in later experiments.

A series of cycling zone experiments were run on glycyl-L-phenylalanine to study the effect of the operating variables and to optimize the separation. Complete details are given by Nelson[8]. Lowering the flow-rate from 2 ml/min to 1 ml/min with constant cycle time, measured in volume, increased the maximum separation factor to 7.05 and sharpened the high concentration peak. The locations of the peaks and shoulders were not changed. To remove the shoulders the cycle time was cut in half. This change successfully removed the shoulders and improved the average separation, but the maximum separation factor was decreased slightly since less dipeptide was available to be dumped into the high concentration peak. The separation was also increased if the low dichloroacetic acid concentration was decreased or the high dichloroacetic acid concentration was increased. This result was expected since these changes cause larger changes in the dipeptide distribution coefficient and thus increase the driving force for separation. Decreasing the dipeptide feed concentration resulted in larger separation factors with less tendency for shoulders to form. Again this was the expected result since it agrees with previous experimental and theoretical results[2,3], and since the packing will be less overloaded. When the cycle time was increased to 1½ times that shown in Fig. 2, the average separation deteriorated even more and

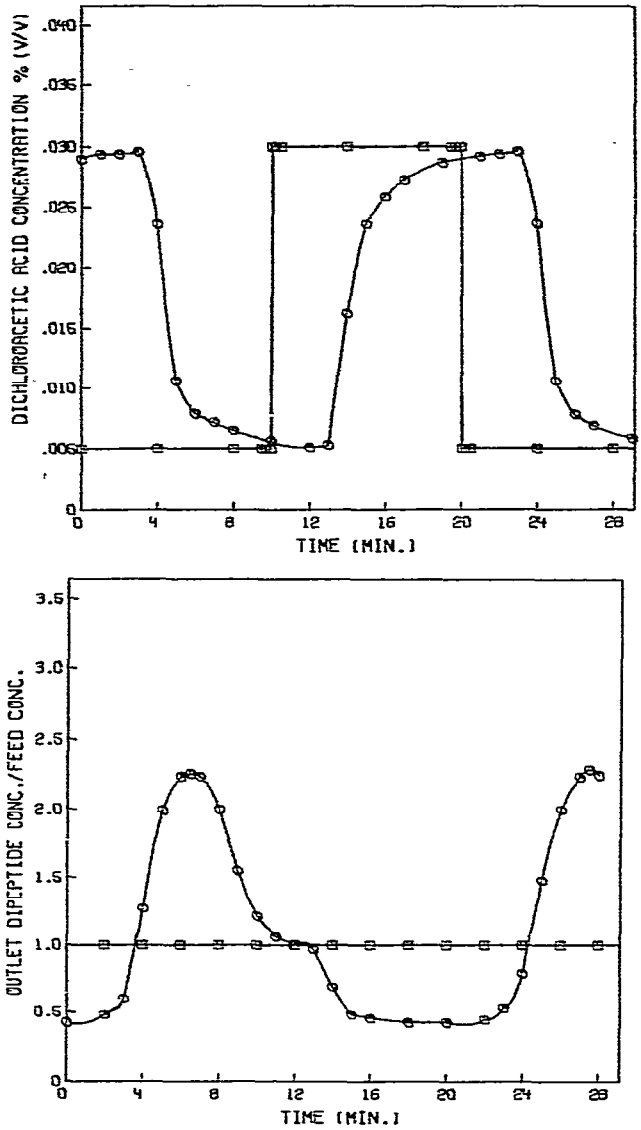

Fig. 2. Cycling zone separation of glycyl-L-phenylalanine from water. (A) Inlet and outlet dichloroacetic acid waves; (B) inlet and outlet glycyl-L-phenylalanine waves. Flow-rate, 2 ml/min; cycle, 20 min (40 ml); dipeptide feed concentration, 80 mg/l; inlet pressure, 640 p.s.i. (average); outlet pressure, 65 p.s.i.; temperature, 30°. □, Inlet; ○, outlet.

shoulders occurred on both the upward and downward portions of the wave. This is an indication that the cycle time is long enough for breakthrough to occur in both halves of the cycle.

Combinations of the above variable changes were also tried and showed that the effects were roughly additive. This required that cycle times be measured in terms of volume per cycle since the amount of dipeptide that has passed through the column

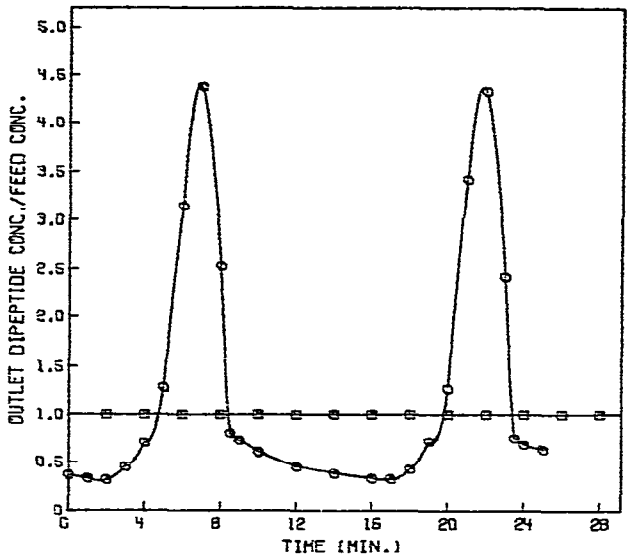

Fig. 3. Cycling zone separation of glycyl-L-phenylalanine from water. Flow-rate, 2 ml/min; feed concentration, 80 mg/l; low dichloroacetic acid concentration, 0.0015%; high dichloroacetic acid concentration, 0.06%; cycle: At low acid concentration, 5 min (10 ml); At high acid concentration, 10 min (20 ml). □, Inlet; ○, outlet. For further conditions see Fig. 2.

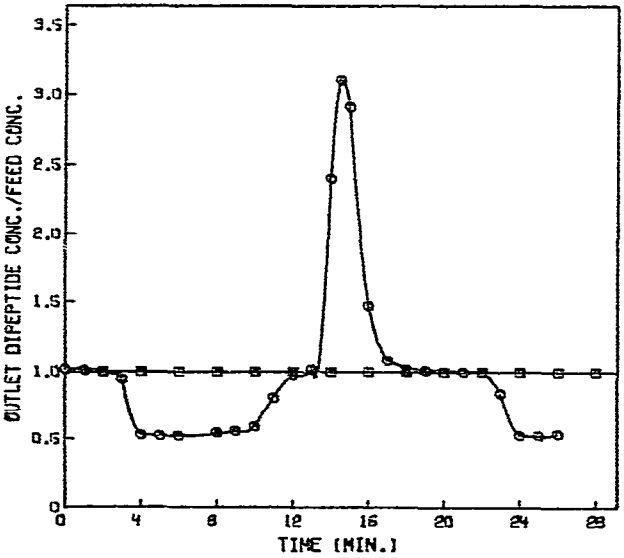

Fig. 4. Cycling zone separation of glycyl-L-tyrosine from water. Flow-rate, 2 ml/min; feed concentration, 40 mg/l; low dichloroacetic acid concentration, 0.0015%; high dichloroacetic acid concentration, 0.03; cycle, 20 min (40 ml), equal cycle halves. □, Inlet; ○, outlet. For further conditions see Fig. 2.

has much more effect than the time required. Thus mass balance considerations are more important than efficiency considerations. These results allowed partial optimization of the purification as shown in Fig. 3. Unequal cycle times were used in this experiment to achieve a peak-to-peak separation factor of 15.5 (the highest obtained) and at the same time eliminate the shoulders.

The pulse experiments showed that glycyl-L-tyrosine moved through the column much faster than glycyl-L-phenylalanine, but it was still strongly affected by dichloroacetic acid concentration. A cycling zone separation of glycyl-L-tyrosine from water is shown in Fig. 4. The cycle is clearly too long in this experiment and the shoulders are much more prevalent than in an identical experiment using glycyl-L-phenylalanine. Thus cycle times must be adjusted for the varying retentions of different species.

DISCUSSION

Trace quantities of dichloroacetic acid were required in each half cycle for the cycling zone experiments. When pure water was used as the solvent for half a cycle, multiple peaks occurred in the output dipeptide concentration waves. We hypothesize that very small amounts of the dichloroacetic acid are held up by the nonpolar packing and that the dichloroacetic acid on the packing attracts the dipeptide. Pure water slowly removed the dichloroacetic acid from the column and caused additional dipeptide waves. The multiple peaks that resulted were difficult to decipher and interpret. The upper dichloroacetic acid concentration was also restricted since the packing should not be run at a pH lower than 2.0.

Two theoretical analyses of cycling zone separation have been published[2,3,5] but were not applied since they both require non-linear equilibrium data over a range of dichloroacetic acid concentrations. Since the cycling zone technique greatly overloads the packing compared to an analytical separation, we would expect that the number of equilibrium stages in the system will be significantly less than the 125 found in the pulse experiments. Cycling zone experiments with other systems have shown a small number of theoretical stages[3,8]. This appears to be the case for the dipeptide system also. Because of the preparative nature of the separation we do not expect that a smaller size packing would improve the separations significantly.

The experiments showed that dipeptides can be concentrated by cycling zone operation of HPLC equipment using dichloroacetic acid to force the separation. This type of separation could be useful as a very mild technique for concentrating labile biochemicals. It could also be used for removal of all solutes from a solvent containing minor impurities. Probably of more interest is fractionation of multicomponent mixtures using the cycling zone method. Although this has been demonstrated theoretically[4], it has not been reduced to practice. We are currently starting work on dipeptide fractionation using the system discussed here and the technique developed theoretically[4]. Fractionation places stringent requirements on the wave velocity of the thermodynamic variable, and may require that the dichloroacetic acid wave, which moved at a velocity close to that of the fluid, be slowed down. Slowing down the dichloroacetic acid wave would also increase the separations obtained here since it would allow "trapping" to occur. Successful development of fractionation techniques would be of considerable interest for preparative separations.

The cycling zone technique presented here does not give complete separations, but instead divides the feed into portions of high and low concentration. In some cases[3,5] the purification obtained is much better than that reported here. If a complete separation is desired, then the incorporation of an elution step in the cycle would be helpful. When this is done, the separation approaches preparative liquid chromatography with repeated injection of the feed, which is certainly not a new technique[9]. The cycling zone technique has the advantages of higher throughput of feed and it concentrates the solute, but it has the disadvantage of not providing a complete separation. In some preparative or industrial-scale separations complete separations are not required to meet product specifications and the cycling zone technique should be of considerable interest.

Two examples of possible interest are the production of mixtures of fructose and glucose[3,10], and purification of solvents. Fructose is made commercially from glucose by an isomerization reaction which at equilibrium contains less than 50% fructose. The sweetness and hence the value of the syrup is directly proportional to the fructose content. A separation process which increased the proportion of fructose in part of the product and decreased it in the remainder of the product would be very useful[10]. The low-concentration material could be recycled to the reactor to produce more fructose. The second example is the removal of trace contaminants from high-purity solvents. If complete removal is required standard preparative HPLC can be employed, but this technique will be slow if fairly large quantities of pure solvent are required. If the specifications for the solvent do not require complete removal of the impurities, a cycling zone technique could be employed. The material added to force the separation could be chosen so that it could either be left in the solvent or could be easily removed (*e.g.*, in a rotary evaporator). The cycling zone technique has the obvious advantage of a much higher throughput of the solvent that is to be purified. The choice of separation method to be used will depend upon the purposes of the separation. In cases similar to the two examples discussed here cycling zone techniques may satisfy the goals of the separation more economically than other techniques.

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