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RECYCLING LIQUID CHROMATOGRAPHY USING MICROBORE COLUMNS

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SUMMARY

A study of recycling in high-performance liquid chromatography was carried out using 50 cm × 1 mm I.D. microbore columns. It is demonstrated that, although there are stricter requirements for the recycling microbore column system design than for larger diameter columns, the system can be designed to be highly efficient and can be successfully employed for analytical work. Theoretical aspects of an alternate pumping technique are considered. Equations relating resolution between two peaks of interest and limiting cycle number to various chromatographic parameters are derived. The experimental design of a computerized system operating in an automated mode is described. Several applications demonstrating how closely eluting solutes can be separated are shown and advantages and disadvantages of recycling using scaled down chromatographic systems are discussed.

INTRODUCTION

The technique of recycling liquid chromatography was introduced by Porath and Bennich[1] in 1962 for gel filtration separations. In this method, partially resolved sample components eluted from the column were diverted back onto the column using a specially designed column switching valve. Three basic recycling designs were reported in the literature. An alternate pumping system based on two columns and an eight-port switching valve was designed by Kucera[2] in 1967 and later on studied by Biesenberger and co-workers[3,4] and others[5,6]. A one-column "closed-loop" system was also reported[7–9]. In this case, however, the solute mixture is allowed to pass through the chromatographic pump into the column repeatedly, and since, in most cases, the pump makes a significant contribution to solute band broadening, this system is usually not preferred for analytical work and is only used for preparative scale purposes[10]. An excellent theoretical treatment of "closed-loop" recycling is given by Martin *et al.*[11]. Snyder and co-workers[12,13] recently revived another form of recycling to perform boxcar-recycle chromatography. A novel concept of the recycle combining the advantages of the previous two systems was designed in 1981 by Minarik *et al.*[14].

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It has been suggested in the literature[11] that a relatively strong dependence of recycling resolution on the column diameter makes recycling interesting mainly for the purpose of preparative scale liquid chromatography. If very low dead volume can be achieved, however, favorable analytical use can be made of recycling narrow-bore columns. It has been shown previously[15] that coupling microbore columns together can produce extremely high efficiencies and, generally, efficiency increases linearly with column length[15]. When wider bore columns are joined together, about 40% efficiency is lost during each coupling step, and this has been attributed to interstitial porosity variations across the column and different column heat transfer processes at higher volumetric flow-rates. Thus, all the advantages of the microbore column systems reported previously[16] can be fully utilized and recycling efficiency can be increased. Furthermore, column detector and column switching valve connecting tubes can be completely eliminated because of the 1/16 in. O.D. of the column. A properly designed microbore column recycling system would also be ideally suited for an automated operation using computer data handling.

The purpose of this paper was to investigate an alternate pumping recycling approach using relatively short microbore columns to demonstrate the feasibility of practical analytical operation and to study recycling parameters that affect the desired chromatographic resolution.

THEORETICAL

Chromatographic resolution in recycling

Any analytical chromatographic system can be characterized in terms of three basic attributes: resolution, speed and scope. For the practicing chromatographer, the desire to improve the separation is the main reason to use the recycling technique. However, when using a repeated operation in a microbore loop-type system, such as depicted in Fig. 1, a condition is reached where the retention volume difference between the first and the last eluted peak is greater than the total column dead volume, and any further recycling will involve the loss of part of the sample since this would exit from the loop. Thus, in recycling, we are effectively trading speed and scope or peak capacity for an increase in resolution and the number of theoretical plates. Let us assume that n is the number of passages of the sample through one column; then, at a constant flow-rate of the mobile phase, the retention volumes, $(V_A)_n$ and $(V_B)_n$, of the two solutes A and B after the nth cycle would be proportional to the respective retention volumes in a one-cycle system, V_A, V_B:

$$(V_A)_n = n(V_A + V_S)$$

$$(V_B)_n = n(V_B + V_S)$$

Since microbore columns can be coiled to a relatively small coil diameter[17] without impairing the column efficiency significantly, no connecting tubing between the column and the switching valve is necessary and the only band dispersion would occur in the internal volume of the switching valve, V_S. (V_S represents two internal loop volumes of the switching valve.) Furthermore, using the principle of summation

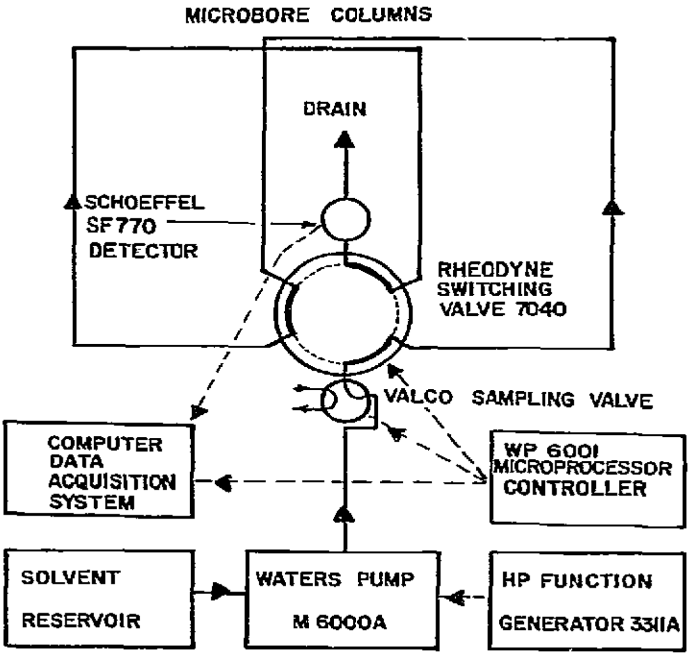

Fig. 1. Block diagram of the recycling microbore column system.

of variances and assuming that both solutes have similar band widths ($\sigma_A = \sigma_B = \sigma$) in a one-cycle system

$$(\sigma)_n = (\sigma_A)_n = (\sigma_B)_n = \sqrt{n(\sigma^2 + \sigma_v^2)}$$

where σ_v^2 is the volume variance resulting from the switching valve, and $(\sigma)_n$ is the solute band width after the nth cycle. Resolution after one cycle, $R(1)$, can now be related to the difference in solute capacity factors, k', on one column

$$R(1) = \frac{(V_B - V_A)}{4\sigma} = \frac{V_0\,(k'_B - k'_A)}{4\sigma} \tag{1}$$

where V_0 = column dead volume. For the nth cycle:

$$R(n) = \frac{(V_B)_n - (V_A)_n}{4\sqrt{n(\sigma^2 + \sigma_v^2)}} = \sqrt{\frac{n}{\left[1 + \left(\frac{\sigma_v}{\sigma}\right)^2\right]}} \cdot \frac{(V_B - V_A)}{4\sigma} \tag{2}$$

Thus:

$$R(n) = R(1)\sqrt{\frac{n}{\left[1 + \left(\frac{\sigma_v}{\sigma}\right)^2\right]}} \tag{3}$$

Eqn. 3 is a similar function to that described by Martin *et al.*[11] for closed-loop systems. However, for an alternate pumping system given here, a different function for the relative band spreading is obtained. Assuming now that the solute peak width is increased by $p\%$ during each solute passage through the switching valve

$$\sigma_v = p\sigma$$

and:

$$(\sigma)_n = \sqrt{n(1 + p^2)\sigma^2}$$

We can arbitrarily define efficiency of recycling, η, as:

$$\eta = (1 - p)100 \tag{3a}$$

Eqn. 3 can now be written as:

$$R(n) = R(1)\sqrt{\frac{n}{(1 + p^2)}} = R(1)\sqrt{\frac{n}{\left[1 + \left(1 - \frac{\eta}{100}\right)^2\right]}} \tag{4}$$

It can be seen that the resolution after the nth cycle will be a function of the cycle number, n, efficiency of recycling, η, and $R(1)$. η can be experimentally determined from the slope of the $R(n)/R(1)$ *versus* the $\sqrt{n}$ plot. Assuming 100% efficiency of recycling, the relative resolution would be equal to the square root of the cycle number. The volume variance due to the switching valve, σ_v^2, would be proportional to the volume of the switching valve[16], V_S

$$\sigma_v^2 = KV_S^2 \tag{5}$$

where K is a constant.

Substituting for the solute band width, σ, in a one-cycle system

$$p = \left(\frac{\sigma_v}{\sigma}\right) = \frac{4\sqrt{NK}\,V_S}{\pi D^2 L\varepsilon(1 + k')} \tag{6}$$

where N, L and ε are the column efficiency, column length and the total column porosity, respectively. It can be seen from eqn. 6 that, for columns of larger diameter, D, a much greater internal volume of the switching valve can be tolerated in order to operate the system at the same recycling efficiency. It will be seen later on that, even with a relatively large volume of the switching valve ($V_S = 2$ µl), up to 80% recycling efficiency of the microbore column system can be achieved.

Limiting cycle number

The maximum number of theoretical plates that can be achieved when coupling microbore columns together in series is eventually limited by the pressure rating of the chromatographic system operated at a given flow-rate. This has been discussed recently[15]. Similarly, in length-programmed systems, the volume of the recycling system, the maximum solute concentration–detector sensitivity relationship and the overall column stability to recycling pressure fluctuations will ultimately limit the number of cycles that can be employed. As the number of cycles increases, the solute peaks broaden and the maximum solute concentration, X, corresponding to the solute peak height decreases

$$(X_A)_1 = m/\sqrt{2\pi}\cdot\sigma \qquad (X_A)_n = m/\sqrt{2\pi}\cdot(\sigma)_n$$

where m is the total solute mass introduced into the system.

$$\frac{(X_A)_n}{(X_A)_1} = \frac{\sigma}{(\sigma)_n} = \frac{1}{\sqrt{n(1+p^2)}} = \frac{K_1}{S} \tag{7}$$

For the given solute mass, the limiting cycle number, n, will depend on the detector sensitivity, S, expressed as the solute concentration equivalent to twice the noise level, constant K_1 and the efficiency of recycling, or factor p. However, in practice, this condition is usually overridden by the limited volume capacity of the recycling loop system, given by the column dead volume, V_0, and the switching valve internal volume, V_S. Assuming that both peaks will spread to the same extent after the nth cycle

$$(V_B)_n - (V_A)_n + 4(\sigma)_n \leqslant V_0 + V_S \tag{8}$$

and the following second-order equation for the limiting cycle number, n, can be obtained

$$nV_0\cdot k'_A(\alpha - 1) + 4\sqrt{n}\cdot\frac{V_0(1+k'_A)}{\sqrt{N}}\cdot\sqrt{1+p^2} - V_0 - V_S \leqslant 0 \tag{9}$$

where α is the selectivity coefficient:

$$\alpha = k'_B/k'_A$$

Eqn. 9 leads to the following condition for the maximum cycle number

$$n_{max} \leqslant \left(A - \sqrt{A^2 + B}\right)^2 \tag{10}$$

where

$$A = \frac{2}{\sqrt{N}}\left(\frac{1+k'_A}{k'_A}\right)\frac{\sqrt{(1+p^2)}}{(\alpha - 1)} = \frac{1}{2R(1)}$$

and:

$$B = \frac{(V_S + V_0)}{k'_A\,V_0\,(\alpha - 1)} = \frac{V_t}{(V_B - V_A)}$$

Eliminating negligible terms, eqn. 10 can be approximated by:

$$n_{\max} \leqslant B \cdot [1 - \sqrt{(16B/N)}] \tag{11}$$

Obviously, a good separation after a given number of cycles requires a resolution factor greater than one. Solving eqn. 2 will yield the relationship for the minimum cycle number:

$$n_{\min} \geqslant 4A^2 \tag{12}$$

The maximum theoretical cycle number plotted against the selectivity α for different values of k' of the first eluted peak is shown in Fig. 2 ($\eta = 100\%$). It can be seen from eqns. 10 and 12 and Fig. 2 that the smaller the α, the higher the column efficiency, and the less spreading in the switching valve, the greater the number of cycles possible. Also, the larger the k', the broader the peaks that will be obtained and the total volume capacity, V_t, of the system will be exhausted faster.

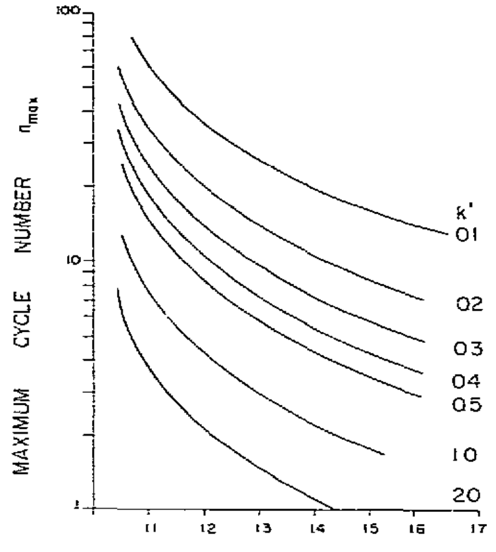

Fig. 2. Graphs of the maximum cycle number *versus* the selectivity for different capacity factors.

It is interesting to note that the constant A is related to the reciprocal of the resolution factor in a one-cycle system where $k'_B \approx k'_A$ and the constant B has the meaning of the relative retention volume difference in a one-cycle system, $V_t/\Delta V$.

Band spreading can be controlled by the ratio V_S/V_0, which should be as small as possible. Thus, recycling should preferably be carried out within the limits given by

$$n_{\min} \leqslant n \leqslant n_{\max}$$

and the conditions for the special case where $n_{\max} = n_{\min}$ can be obtained by solving eqns. 10 and 12.

EXPERIMENTAL

Recycling apparatus

Fig. 1 shows a diagrammatic scheme for the recycling system which consists of a Rheodyne six-port, 1-μl internal loop switching valve (Model 7040); two identical 50 cm × 1 mm I.D. microbore columns packed with DuPont Zorbax ODS 8-μm particles; a Valco sampling valve with a fixed internal loop of 0.5 μl (Model ACV-4UHPa-N60); and a Waters Model 6000A pump driven by a Hewlett-Packard 3311A function generator. A Minarik programmable microprocessor controller (Model WP 6001) was used to initiate sample injection, computer acquisition and column switching. A Schoeffel SF770 UV/VIS spectrophotometer equipped with a 0.5-μl cell was operated at 224, 254 or 350 nm, depending upon the sample application. The detector was connected to the CIS data handling system (Computer Inquiry Systems, Waldwick, NJ, U.S.A.) and to a potentiometric recorder. Assuming port 1 to be the input to the valve and port 4 to be the exit to the detector, the columns are connected to the valve from port 6 to port 3 and from port 2 to port 5, respectively. Column ends are inserted directly into the port openings, butted against 2-μm frits seated within the ports, and sealed with Rheodyne low dead volume fittings. The connection from the sampling to the switching valve is made with 2 cm × 0.005 in. I.D. tubing, and from the exit port to the 0.5-μl detector cell with a short section of packed microbore column containing the same packing material as the recycling columns.

Ideally, the detector cell should be interposed between each column and the switching valve. This arrangement, however, performed very poorly and, thus, the recycling was carried out in a blind fashion. Only the peaks exiting from the loop system were monitored by the detector. A closeup picture and connections is shown in Fig. 3.

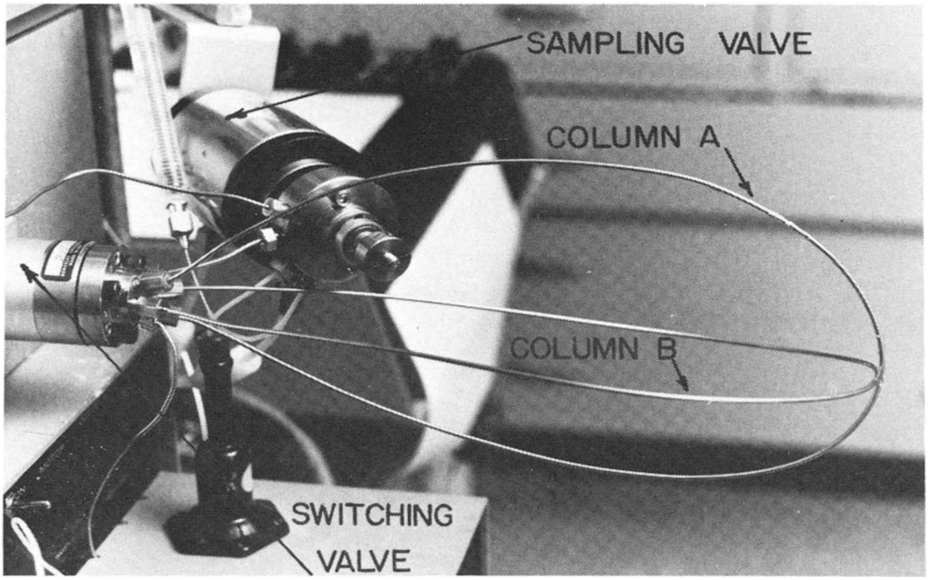

Fig. 3. Detailed picture of the sampling valve/switching valve arrangement.

Determination of switching times

Switching time for a given sample for *n* number of cycles can be determined by first running the sample with no switching input (two cycles) and then with a single switching input (one cycle). Sample residence time per column, as well as the residence time in the exit line, can then be determined, and programs can be constructed for multiple-cycle operations. However, the increase in the number of theoretical plates and retention times due to the detector connecting tubing was negligible, especially for higher numbers of cycles.

Mobile phases and samples

Glass-distilled solvents (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and deionized water were used to prepare mobile phases. Sample solutes were from commercial sources, except retinoic acids obtained in-house from Hoffmann-La Roche, and were used without further purification. The samples were dissolved in the mobile phase and were placed on the column using the 0.5-μl Valco air-actuated sampling valve.

RESULTS AND DISCUSSION

Since the peak capacity of the recycling system is low, recycling is ideally suited for the separation of closely eluted compounds exhibiting only small differences in their molecular structure. Such would be the case for the separation of fully deuterated benzene and H-6 benzene, which can be seen in Fig. 4. With Zorbax ODS reversed-phase columns and a flow-rate of 30 μl/min, baseline-to-baseline separation between both isotopes was obtained after five cycles; 75,660 theoretical plates for both solutes was achieved, which is exactly five times the column efficiency obtained in a one-cycle system ($k'_{D\text{-}6} = 2.16$; $k'_{H\text{-}6} = 2.25$). The peaks eluted after five cycles

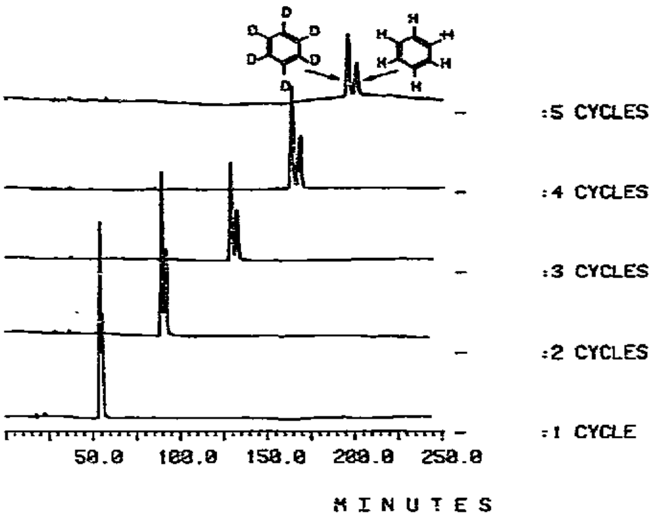

Fig. 4. Separation of benzene isotopes on the recycling microbore column system. Conditions: two Zorbax ODS columns, 50 cm × 1 mm I.D. each; eluent, methanol–water (75:25), flow-rate 30 μl/min; detection at 254 nm.

were extremely symmetrical, and this can be seen in Fig. 5. The asymmetry factor arbitrarily measured at 20% of the peak height was 1.03 for both solutes. Throughout this work, the determination of the number of theoretical plates as well as baselines, asymmetry factors, k's and retention times were done by the CIS computer using special external BASIC programs operating on the digital chromatographic data, and thus all possible errors introduced by the operator were eliminated. Fig. 6 shows another application of the recycling microbore system. Light isomerization of retinoic acid provides within minutes a mixture of 13-*cis*-retinoic acid and all-*trans*-retinoic acid (tretinoin). These solutes could easily be resolved in five cycles with a 5% v/v chloroform–methanol mobile phase modified by the addition of 0.3% ammonium acetate.

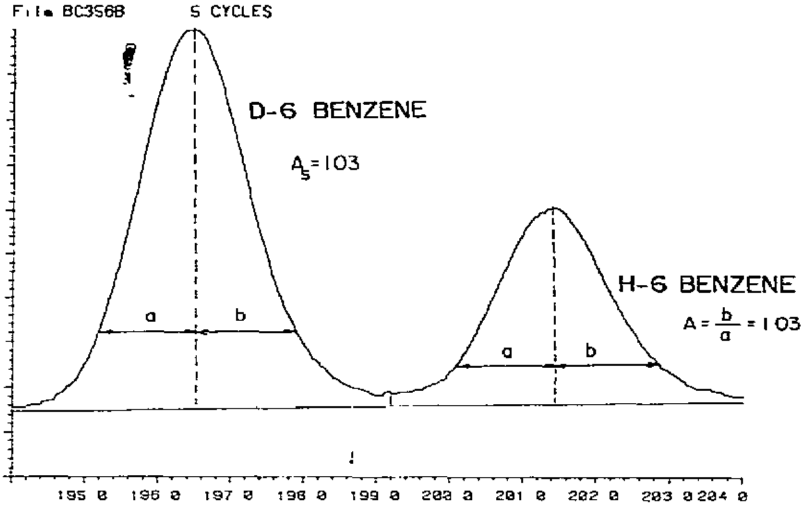

Fig. 5. Demonstration of the symmetry of benzene peaks eluted from the recycling microbore column system.

Since an unmodified Rheodyne switching valve ($V_S = 2$ µl) was employed during this work, it was of interest to determine the efficiency of recycling, η, as arbitrarily defined by eqn. 3a with the given system. The data from the separation of benzene isotopes were used to calculate the relative resolution of recycling as given by eqn. 3. These values were plotted *versus* the square root of the number of cycles, and the resulting curves were fitted to the linear function. The index of determination of the curve fit was 0.99, and the slope obtained in this way was 0.981. The data from these experiments are plotted in Fig. 7 as a double logarithmic plot; also included is the apparent efficiency of fully deuterated benzene in theoretical plates for five cycles. It can be seen from Fig. 6 that excellent linearity between the number of theoretical plates and the cycle number was obtained. This reflects the excellent symmetry of the peaks eluted from the system. It has previously been observed that, when recycling on larger bore columns ($D > 3$ mm), the symmetry of eluted peaks was generally worse and, as a consequence, greater departure from efficiency linearity and system ideality

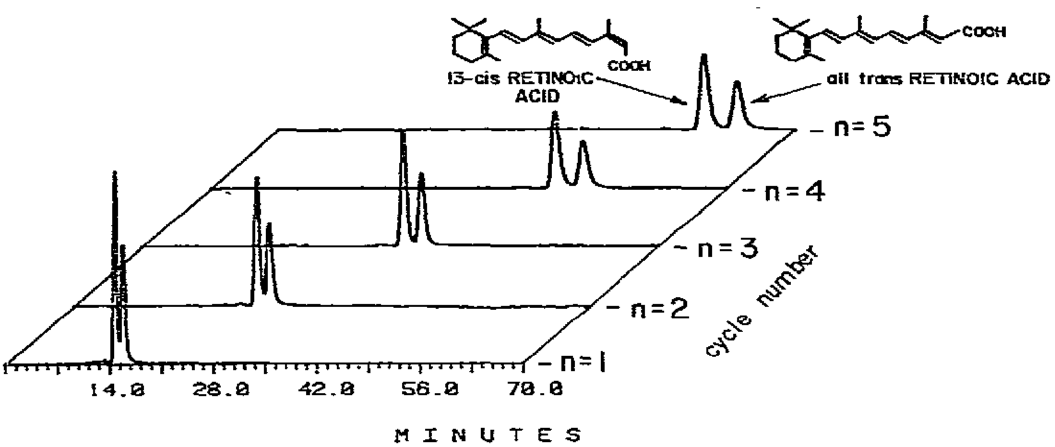

Fig. 6. Separation of *cis/trans* isomers of retinoic acid on the recycling microbore column system. Conditions: two Zorbax ODS columns, 50 cm × 1 mm each; eluent, chloroform–methanol (5:95) containing 0.3% (w/v) ammonium acetate, flow-rate 40 μl/min; detection at 350 nm.

was obtained. This has been attributed to various trans column band spreading processes and will be investigated in greater detail in the future. From the slope of $R(n)/R(1)$ *versus* the square root of the cycle number, the efficiency of recycling was calculated and was found to be 80%. Certainly, the efficiency of recycling could be improved even further if a 0.5-μl internal volume switching valve were used instead of the 1.0-μl valve.

Experimental verification of the maximum cycle number, given by eqn. 10, was

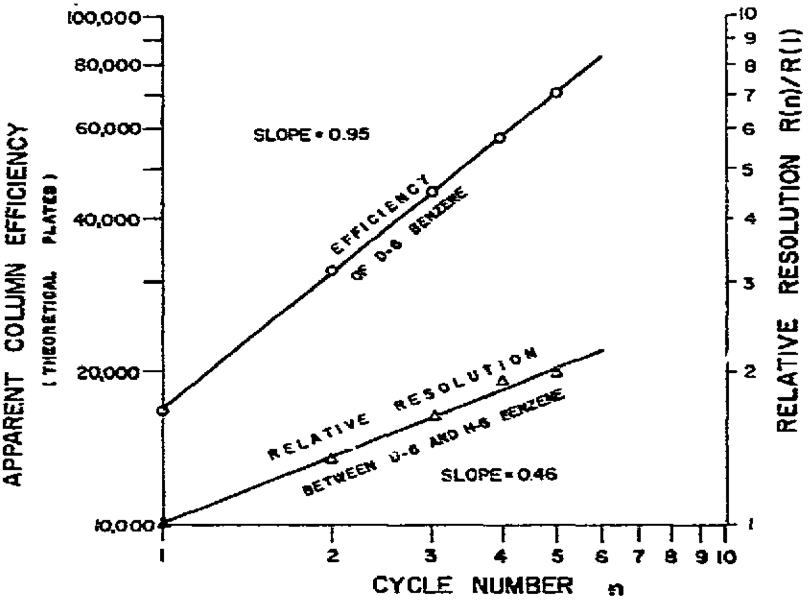

Fig. 7. Graphs of relative resolution and column efficiency against the cycle number.

carried out with a mixture of oligomers present in a non-ionic surfactant, Triton X-100 (isooctylphenoxypolyethoxyethanol). The chromatograms from these experiments can be seen in Fig. 8. The recycling system was operated with a flow-rate of 40 μl/min, 50% acetonitrile in methanol mobile phase and the UV detector was set at 224 nm. The capacity factors of the first and last eluted peaks were 0.52 and 0.78, respectively, which yields $\alpha = 1.5$. Introducing these values into Fig. 2, one can determine that only three cycles should be theoretically feasible. Experimentally, the fourth cycle could not be obtained since part of the solute mass circulating within the recycling loop was always cut off by the switching valve and exited to the detector. Thus, assuming that the peaks are Gaussian in shape, Fig. 2 can be used to predict the maximum cycle number for a given system.

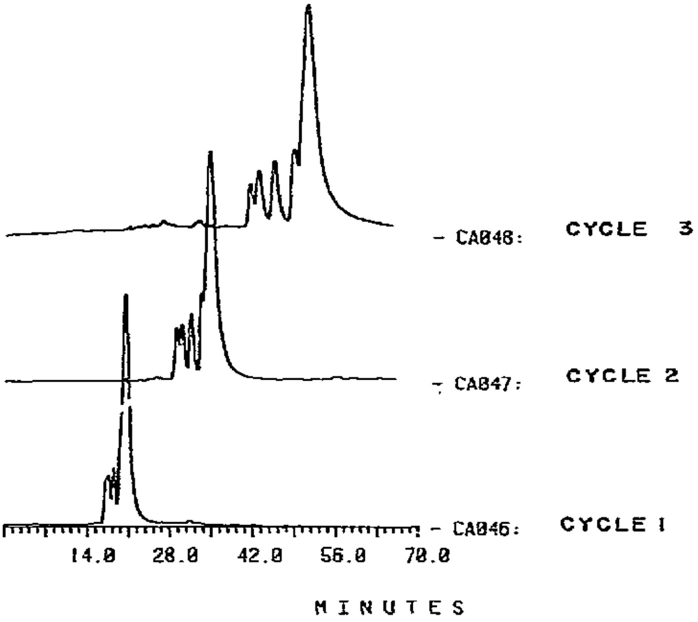

Fig. 8. Experimental verification of the maximum cycle number using Triton X-100. Eluent, acetonitrile–methanol (50:50), 40 μl/min.

CONCLUSIONS

Peaks eluted from microbore columns are, in general, very symmetrical, and, upon connecting microbore columns together in the recycling mode, column efficiencies increase linearly with column length, a result that has been very difficult to demonstrate for larger bore columns. Thus, the recycling microbore column system has distinct advantages over the current conventional recycling systems. Furthermore, the already accepted advantages inherent with the use of microbore columns, such as low solvent consumption of the mobile phase, sample and column material, can be fully utilized. Because the peaks obtained resemble symmetrical Gaussian curves, experimental data are in good agreement with theory and various recycling parameters can be predicted from experimental data obtained in one cycle run.

Chromatographic resolution in recycling depends on the square root of the cycle number and the recycling efficiency, which in turn is related to the solute band spreading in the switching valve. Factors affecting the maximum cycle number can be determined in a one-cycle run and thus the maximum cycle number which can be employed with a given experimental arrangement can be calculated. The system is easily adaptable to total automation. There is no doubt that, at this stage of miniaturization of a liquid chromatograph, the described recycling system can be successfully employed for analytical work. However, the use of an accurate solvent delivery and a low-volume switching valve is very important. Further decrease in the dimensions of the packed column, switching valve volume and sample size is quite feasible. Quite clearly, the alternate pumping principle is superior over other scaled-down designs. If sufficiently low-cell volume, high-pressure rating, high-sensitivity detectors are developed, there is a possibility that the detector cell can be introduced into the recycling loop and the chromatogram development could be monitored during each sample passage through the respective cycle. Using a detector/computer feedback design would then be a highly desirable system for automation.

REFERENCES

1 J. Porath and H. Bennich, *Arch. Biochem. Biophys.*, Suppl. I (1962) 152.
2 P. Kucera, *M. Sc. Diploma Thesis*, Technical University, Prague, 1967.
3 J. A. Biesenberger, M. Tan, I. Duvdevani and T. Maurer, *Polym. Lett.*, 9 (1971) 353.
4 J. A. Biesenberger, M. Tan and I. Duvdevani, *J. Appl. Polym. Sci.*, 15 (1971) 1549.
5 R. A. Henry, S. H. Byrne and D. R. Hudson, *J. Chromatogr. Sci.*, 12 (1974) 197.
6 K. J. Bombaugh and R. F. Levangie, *J. Chromatogr. Sci.*, 8 (1970) 560.
7 K. J. Bombaugh and R. F. Levangie, *J. Chromatogr. Sci.*, 7 (1969) 42.
8 S. Nakamura, S. Ishiguro, T. Yamada and S. Moriizumi, *J. Chromatogr.*, 83 (1973) 279.
9 J. Lesec, F. Lafuma and C. Quivoron, *J. Chromatogr. Sci.*, 12 (1974) 683.
10 H. Kalász, J. Nagy and J. Knoll, *J. Chromatogr.*, 107 (1975) 35.
11 M. Martin, F. Verillon, C. Eon and G. Guiochon, *J. Chromatogr.*, 125 (1976) 17.
12 L. R. Snyder, J. W. Dolan and Sj. van der Wal, *J. Chromatogr.*, 203 (1981) 3.
13 Sj. van der Wal, L. R. Snyder and P. Kucera, *Fifth International Symposium on Column Liquid Chromatography, May 11–15, Avignon, 1981*.
14 M. Minarik, M. Popl and J. Mostecky, *J. Chromatogr. Sci.*, 19 (1981) 250.
15 P. Kucera and G. Manius, *J. Chromatogr.*, 216 (1981) 9.
16 P. Kucera, *J. Chromatogr.*, 198 (1980) 93.
17 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 169 (1979) 51.