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HIGH-RESOLUTION REVERSED-PHASE LIQUID CHROMATOGRAPHY UTILIZING MICROBORE COLUMN CONCATENATION

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SUMMARY

It has been demonstrated previously that extremely high efficiencies can be obtained by coupling microbore columns together in series and that efficiency increases linearly with each column addition. Only recently has it become possible to prepare 50 cm × 1 mm I.D. columns packed with 8-μm reversed-phase material exhibiting an optimum plate height of about two particle diameters. A chromatographic system based on the concatenation of these columns, which allows the achievement of a high number of theoretical plates in several hours and which can be arranged with little instrument modification is reported. Experimental data presented in this paper indicate that coupling conventional larger diameter columns results in about 60% loss of column efficiency for each coupling step, whereas coupling microbore columns actually produces efficiencies of 100% for each concatenation. Thus, the design of a very high-resolution, high-efficiency high-performance liquid chromatography system makes the use of microbore columns imperative. This paper deals with both theoretical and experimental aspects of high-resolution liquid chromatography, demonstrates the feasibility of setting up the system, and shows examples of how difficult separations such as for deuterium-labeled isotopic compounds can be achieved easily.

INTRODUCTION

A number of papers have appeared in the literature recently[1–5] demonstrating the design of chromatographic instrumentation incorporating microbore columns to achieve high-speed and high-efficiency separations. It is quite clear that these 1 mm I.D. packed columns offer substantial economy of solvent, column packing material, sample and associated column hardware. Additionally, they perform a wide range of operations from high-speed analyses at advanced flow-rates (1–2 ml/min) to discrete separations at reduced flow-rates (5–20 μl/min). Wider bore columns are restricted in the range of linear velocities that can be employed and because of the expenditure of vast quantities of solvent. Because extremely low flow-rates are essential for interfacing with a mass spectrometer, microbore columns are likely suited for this purpose[6,7]

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and can be considered somewhat analogous to capillary column interfacing in gas chromatography–mass spectrometry (GC–MS)[8]. The interest in column coupling was revived recently by Snyder and coworkers[9–11] with the concept of "boxcar chromatography". It also was demonstrated by other workers[12] that 2 mm I.D. columns can be successfully coupled to achieve a high number of theoretical plates. Theoretical discussion of the preparation of high-efficiency long columns has been published by Guiochon[13].

It is the intent of this paper to study microbore column separations at nominally low flow-rates for purposes of achieving maximum efficiency and resolution. Microbore columns are then coupled together in series to improve upon those results obtained with a single column. It will be shown that, unlike conventional columns which produce a sharp reduction in performance with each coupling step, microbore columns actually give 100% theoretical plate counts for each coupling step. Theoretical treatment of column concatenation is also discussed in some detail.

THEORETICAL

Approaches to the achievement of high column efficiencies

There are basically two chromatographic systems which can be used to generate high numbers of theoretical plates: the recycling system where the solute mixture is allowed to pass through one or two columns repeatedly, and the system based on the use of long columns operated isocratically close to the optimum linear velocity. Any analytical system can be characterized in terms of three basic attributes, namely the resolution, the speed and the scope. Both high-efficiency systems differ substantially as far as the advantages and disadvantages are concerned but both must effectively trade the speed (time of the analysis) and scope (peak capacity) for an increase in resolution and the number of theoretical plates. Unfortunately, because of the limited volume capacity of the recycling loop system and increasing band spreading with each cycle number, there is a limited number of cycles which can be used. Thus, the recycling system has a further disadvantage over the long column approach in that only the solutes with a relatively narrow range of capacity factors (k') can be recycled. The column-switching technique, as suggested by Snyder *et al.*[10] in his "boxcar-recycle chromatography" system can clearly obviate some of the problems associated with recycling. Long microbore columns, however, can successfully separate complex mixtures with a wide k' difference as well as closely eluted compounds. Packing long microbore columns appears to be very difficult, and thus the only alternative is to couple short, highly efficient columns together in order to obtain a high-resolution system.

From the two possible ways to improve the chromatographic resolution of an isocratic system, namely to influence a change in selectivity or to optimize the column plate number (N), the latter is clearly more powerful and easier experimentally. Theoretical work of Guiochon and co-workers[13,14] showed that optimization of N is necessary. Although more than a million theoretical plates can be obtained from a single column, such separations would be impractically long. Also, the pressure rating of the chromatographic instrumentation would prevent indefinite column coupling. Thus, the current trend in high-resolution high-performance liquid chromatography (HPLC) is to concatenate short, highly efficient microbore columns packed with 5–8-

μm particles in order to achieve about 200,000 theoretical plates in several hours for well-retained components.

As shown in this paper, the Knox equation[15,16] well describes the reduced plate height (h)–reduced velocity (v) dependence for a microbore column packed with an average particle diameter, d_p.

$$h = (Av^{4/3} + B + Cv^2)/v \tag{1}$$

where A, B, and C are Knox coefficients. Ideally, in order to achieve the maximum number of theoretical plates ($N_{max.}$) from a column of a given length (L_1), the column should be operated at its optimum reduced linear velocity, v_{opt}, where $h = h_{opt}$ and $N_{max.} = L_1/2d_p$. However, with very small particles (typically 5–8 μm), the coefficient C, proportional to solute mass transfer in the stationary phase, appears to be very small (0.03–0.07), and thus, in most cases, it can be neglected. Under these conditions, the optimum parameters can be obtained by differentiation of eqn. 1.

$$dh/dv = -B/v^2 + Av^{-2/3}/3 = 0 \tag{2}$$

$$v_{opt} = (3B/A)^{3/4} \tag{3}$$

$$h_{opt} = \tfrac{4}{3}(3A^3B)^{1/4} \tag{4}$$

$$v_{opt} \cdot h_{opt} = 4B = 6 \tag{5}$$

Because of the very shallow slope of the HETP curve, it is not necessary to work at the optimum linear velocity when using small particle diameter packing materials. Most work in this paper has been carried out at $v = 6.8$ (equivalent to 30 μl/min or 0.07 cm/sec) and $h = 3.3$, and, in fact, no significant improvement in resolution could be obtained when operating at the optimum linear velocity.

Assuming ideal concatenation and no solvent compressibility effects, then the maximum number of columns (n) operated at a given linear velocity (u), which can be concatenated, would be limited by the pressure rating of the chromatographic system ($\Delta P_{max.}$). If reduced parameters (h, v) are substituted into Darcy's equation for column permeability (K_0), the retention time (t_R) of the retained solute could be written as follows:

$$(t_R)_n = \frac{nL_1}{u}(1 + k') = \frac{hN_{max.}\, d_p^2}{vD_m}(1 + k') = \frac{(\Delta P_{max.})\, K_0}{u^2\eta}(1 + k') \tag{6}$$

where D_m = sample diffusivity. It can be seen from eqn. 6 that column length, column efficiency, and column pressure drop are all linearly related to the solute analysis time. Thus, doubling column length ($n = 2$) would increase pressure drop, column efficiency and analysis time by a factor of 2. Resolution, however, will be increased only by a factor of $\sqrt{2}$ because peak width is inversely proportional to $\sqrt{N}$ and resolution is in turn inversely proportional to the peak width. The maximum number of theoret-

ical plates which can be achieved with coupled columns packed with a given particle diameter and operated at reduced optimal linear velocity (v_{opt}) is then given as

$$(N_{max.}) = \frac{(\Delta P_{max.})\, K_0}{\eta h_{opt}\, v_{opt}\, D_m} = \frac{(\Delta P_{max.})\, K_0}{\eta D_m\, (4B)} \tag{7}$$

and would depend only on the maximum pressure drop, column permeability, physical properties of the mobile phase such as its mobile phase viscosity (η), sample diffusivity (D_m) and Knox constant B, which is usually around 1.5 (ref. 13). Similarly, the maximum number of columns (n) which can be concatenated would depend only on d_p, L_1, $N_{max.}$ and Knox constants A and B.

$$n = \frac{4}{3} \frac{N_{max.}\, d_p}{L_1} (3A^3B)^{1/4} \tag{8}$$

It will be seen in the experimental section that theory is in good agreement with experimental data.

EXPERIMENTAL

Preparation of reversed-phase columns

Conventional 25 cm × 4.6 mm I.D. columns used here for comparison purposes in the coupling mode were prepared according to the method reported previously[17]. In all cases, DuPont Zorbax ODS material with an average particle diameter of 8 μm was employed. Microbore columns, 50 cm × 1 mm I.D., were packed with reversed-phase particles according to a modification of the previously described procedure[3], which will be reported elsewhere.

Chromatographic apparatus

Two similar systems were used to evaluate the columns singly, one of which was selected for investigations of the coupled columns of varying length. Both systems were equipped with Schoeffel SF770 UV–VIS detectors set at 254 nm containing 0.5-μl cells for microbore use and 8.0-μl cells for conventional columns. Both systems also employed a Waters Assoc. Model 6000A pump operated isocratically, a mobile phase of methanol–water (85:15) at a linear velocity of 0.07 cm/sec, and a Valco sampling valve with a fixed 0.5-μl internal loop. One system used a Hewlett-Packard Model 3311A function generator set at 7.5 Hz to achieve a flow-rate of 30 μl/min, whereas the other used a Waters Assoc. Model 660 solvent programmer set at 0.1 ml/min A + B and at 40% A to deliver the same flow-rate. The flow-rates of both systems were measured to be linear between 10 and 100 μl/min. Conventional columns were operated at the same linear velocity, equivalent to 0.5 ml/min. A Micromaster Model WP6000 microprocessor controller was used to initiate sample injection, computer acquisition, and termination of analysis for one setup, and a Valco digital valve sequence programmer 4-1 was used for the other. Data handling was done by the CIS computer system (Computer Inquiry Systems, Waldwick, NJ, U.S.A.).

TABLE I

COMPOSITION AND CAPACITY FACTORS OF TEST SOLUTES USED FOR COLUMN EVALUATIONS

Conditions: 85% methanol–water; linear velocity 0.07 cm/sec; sample volume 0.5 μl or 5 μl; 254 nm. Zorbax ODS reversed-phase column.

No.	*Component*	*Concentration (%, w/v)*	*k'*
1	Phenol	0.2	0.22
2	*o*-Ethylphenol	0.2	0.54
3	Anisole	0.1	0.62
4	Toluene	0.4	0.99
5	Ethylbenzene	0.4	1.61
6	*sec.*-Butylbenzene	0.4	2.22
7	*tert.*-Butylbenzene	0.4	3.68
8	*n*-Butylbenzene	0.4	4.10
9	*tert.*-Pentylbenzene	0.4	4.80
10	*sec.*-Hexylbenzene	0.4	5.25

Sample and standards

An alkylbenzene ten-component test mixture, covering a k' range from 0–6, was reagent-grade material. The solute concentrations and description can be seen in Table I. Deuterated benzene was 99.5% pure and was obtained from SIC (Rutherford, NJ, U.S.A.). Diazepam and D-11 diazepam were synthesized at Hoffmann-La Roche (Nutley, NJ, U.S.A.). Pine tree extract was obtained from a natural source.

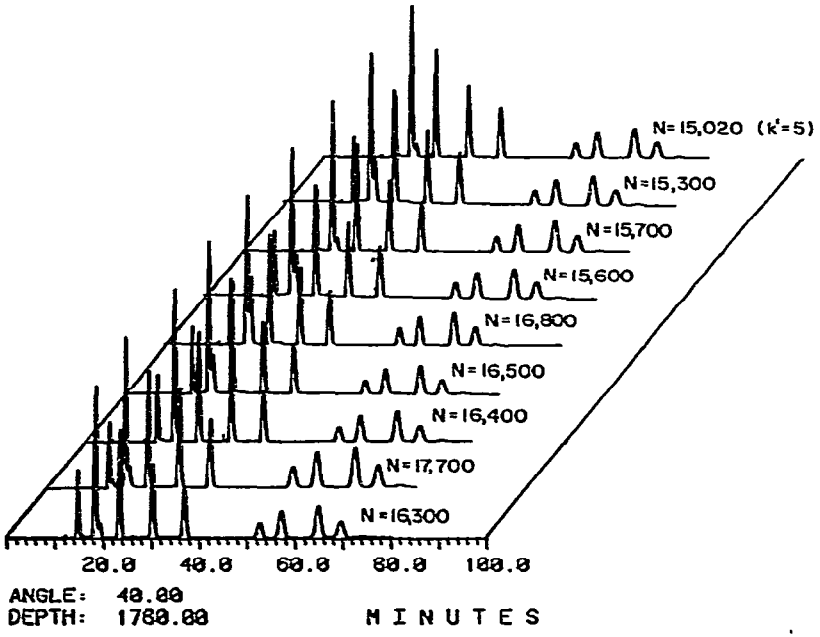

Fig. 1. Chromatograms demonstrating column packing reproducibility. Columns: 50 cm × 1 mm I.D., C_{18} reversed phase; eluent: methanol–water (85:15); flow-rate: 30 μl/min (0.07 cm/sec).

Column evaluation

The individually tested microbore columns were evaluated for reproducibility, including the ability to generate a minimum of 15,000 theoretical plates for *sec.*-hexylbenzene ($k' = 5$) at a flow-rate of 30 μl/min. Fig. 1 graphically maps the nine columns and shows the elution of the test hydrocarbon mixture on each. A few columns fell short of the minimum requirements ostensibly due to the transfer to the analytical system and were, therefore, not included. Less difficulties were experienced with conventional 4.6 mm I.D. columns, and, in this case, only columns exhibiting over 8000 theoretical plates were accepted. The necessity to impose rugged performance criteria is demonstrated in Fig. 2, which clearly shows a sharp dropoff in efficiency in the low k' region for an unacceptable microbore column compared to a linear plate count over the same k' range achieved on a satisfactory column. The flow-rate was reduced to 10 μl/min, which translates to a linear velocity of 0.03 cm/sec and which increases overall column efficiency to 23,000 theoretical plates. Fig. 3 shows the HETP *vs.* linear velocity plot for solute toluene for a typical 50 cm × 1 mm I.D.

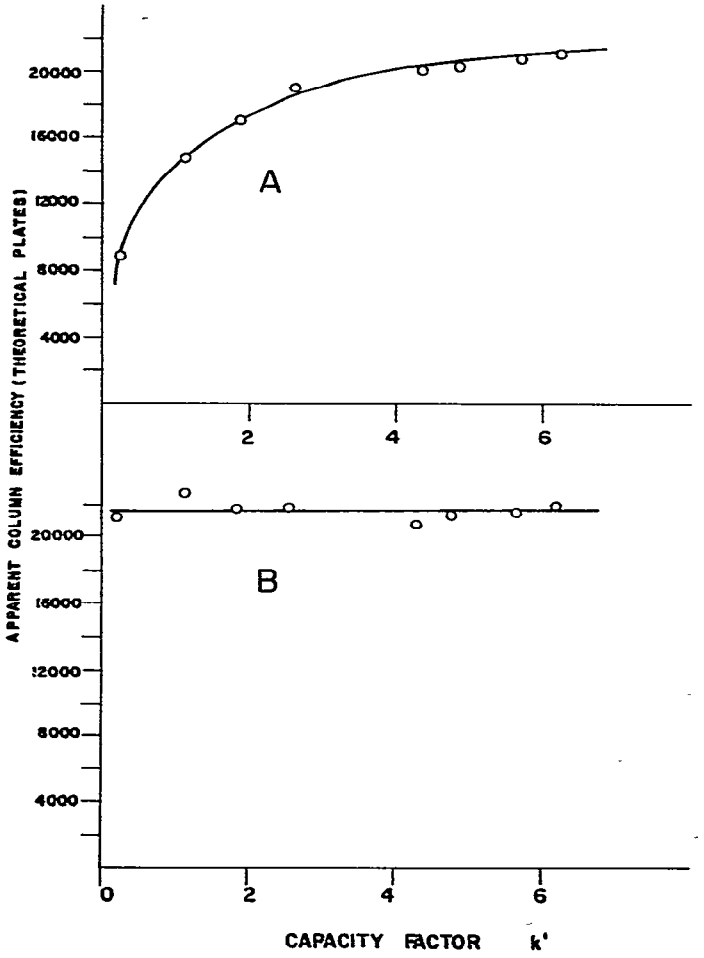

Fig. 2. Demonstration of extra column effects. Column and eluent as in Fig. 1; flow-rate: 10 μl/min.

microbore column. The optimum plate height achieved was 2.3 at a reduced velocity of 2.0 (0.02 cm/sec). The HETP curve was determined with a method previously described[1]. The column efficiencies were computer-calculated using the band width at 0.6065 of the peak height. The experimental data from 18 measurements were fitted into the Knox equation and then a series of determinants was employed to calculate average values of Knox coefficients A, B and C. The constant A was equal to 1.3, and this demonstrates a well-packed column with good homogeneity of packing material. The term B, which is partially a function of k', accounts for the axial diffusion and was found to be equal to 1.4. Mass transfer coefficient C was rather low and equal to 0.07, which indicates relatively fast mass transfer for the solute in the stationary phase and explains why the trade-off in time for improved resolution is not entirely warranted in this case. Assuming that $C = 0$ and $B = 1.4$ and using eqns. 3, 4 and 5, the constant A can be calculated to yield $A = 1.4$. This is in good agreement with the experimental data ($A = 1.3$).

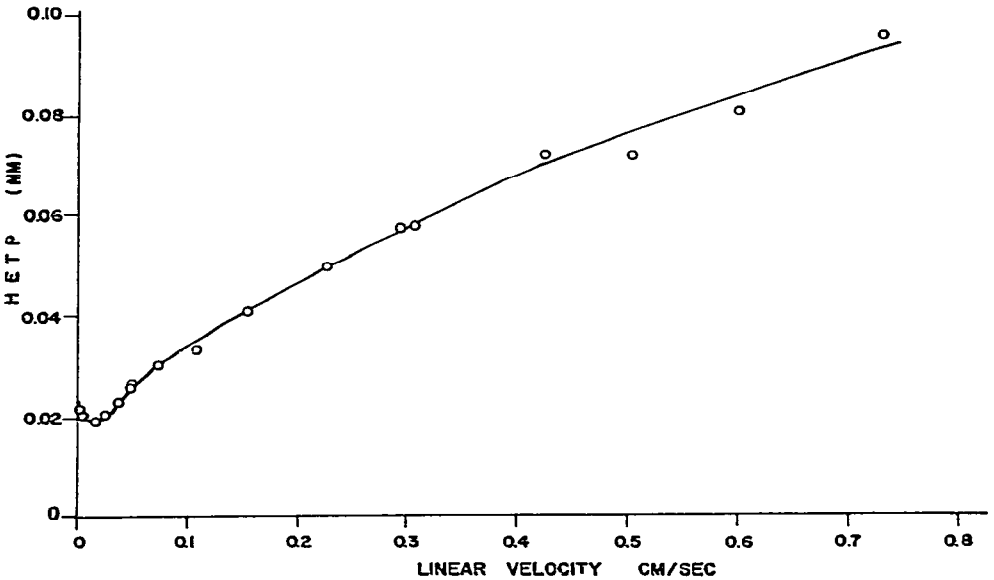

Fig. 3. Graph of height equivalent to a theoretical plate (HETP) against mobile phase velocity for reversed-phase microbore column 50 cm × 1 mm I.D. Eluent: methanol–water (85:15); solute: toluene ($k' = 1$).

Column concatenation experiments

It was noted previously that any connecting system other than direct column butt-to-butt coupling introduced significant band spreading in the coupling union[1]. Therefore, both conventional 4.6 mm I.D. columns as well as 1 mm I.D. columns were connected together using a simple modified Swagelok union as shown in Fig. 4. Utmost care was taken not to disturb the packing during the coupling operation. In all, nine microbore columns were packed and evaluated separately. The columns were then coupled in series, first in three, six and then nine column lengths (total length 4.5 m). After each coupling, the column system was evaluated. Similar experiments were carried out with 4.6 mm I.D. columns packed with the same packing material and

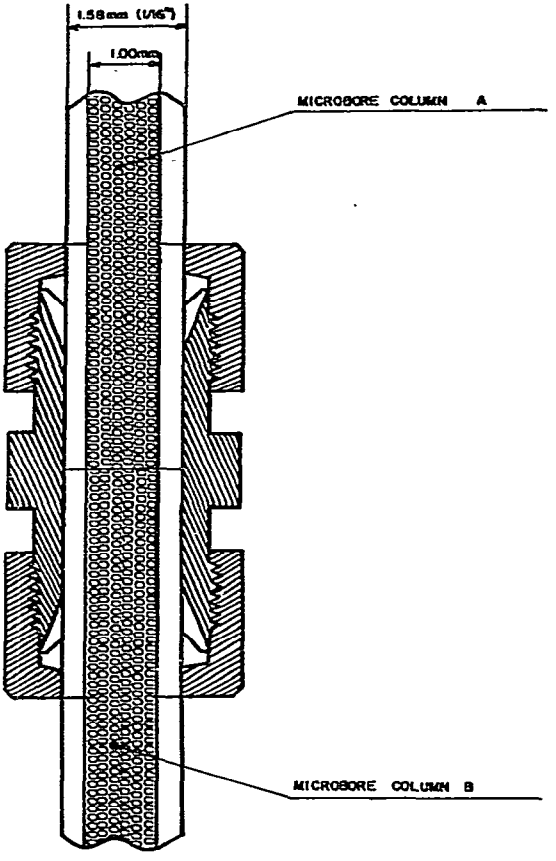

Fig. 4. Detail of microbore column coupling utilizing a modified Swagelok union.

TABLE II

COMPARISON OF COLUMN CONCATENATION EXPERIMENTS FOR COLUMNS OF DIFFERENT DIAMETERS

Conditions: 85% methanol–water; linear velocity 0.07 cm/sec; sample volume 0.5 μl or 5 μl; 254 nm, Zorbax ODS reversed-phase column.

Column	*Total column length (cm)*	*sec.-Butyl benzene, k′ = 2.22*	*n-Butyl benzene, k′ = 4.10*	*Average plate count, N**	*Column pressure drop, ΔP (p.s.i.)*
50 cm × 1 mm I.D.	50	15,523	15,735	15,400	730
	150	46,392	49,732	47,420	2260
	300	92,720	93,210	92,800	4530
	450	162,624	159,436	139,060	6800
25 cm × 4.6 mm I.D.	25	8645	8360	8350	370
	50	12,351	11,785	11,830	810
	100	16,120	16,960	16,690	1540
	150	20,030	19,895	19,500	2355

* Average plate count taken for all solutes ranging from k' 0 to 5.

operated under the same chromatographic conditions, but, in this case, columns were coupled in two, four and six column lengths (total length 1.5 m). The results from these experiments can be seen in Table II where apparent column efficiencies for *sec.*-butyl benzene and *n*-butyl benzene for different coupled column systems are shown. An average plate count for all ten solutes of the test mixture and column pressure drop were also determined for different total column lengths. The nine coupled microbore columns, giving a maximum of 250,000 theoretical plates (at optimum linear velocity, 0.02 cm/sec), were used for the high resolution separation of deuterium-labeled compounds. Fig. 5 shows the ten-solute test mixture of alkylbenzenes separated on the nine coupled microbore columns exhibiting approximately 200,000 theoretical plates at a flow-rate of 20 μl/min, or about 0.045 cm/sec.

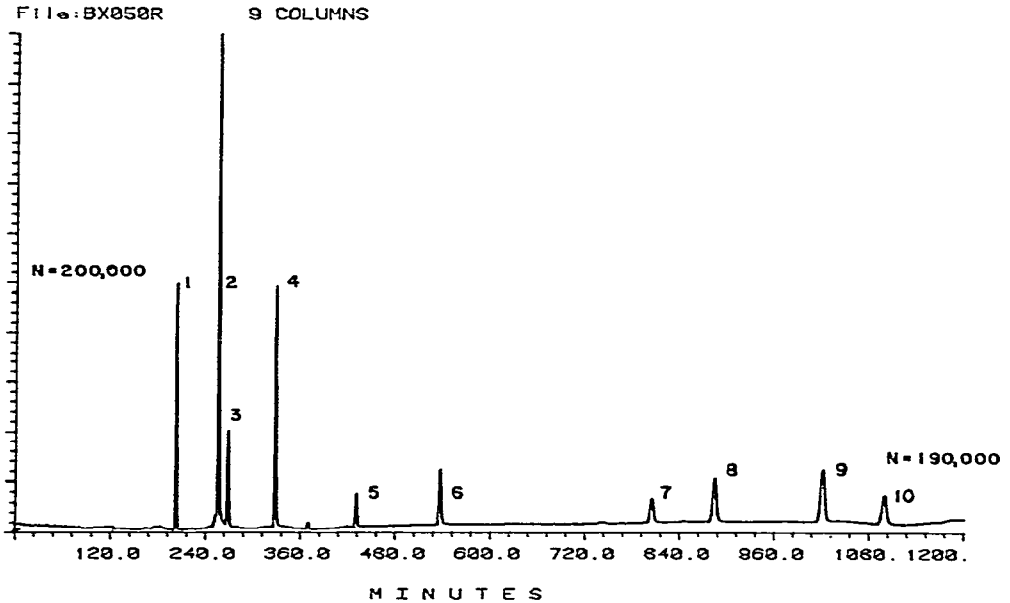

Fig. 5. High-resolution chromatogram of a test mixture of alkylbenzenes on a 4.5 m × 1 mm I.D. C_{18} reversed-phase microbore column. Eluent: methanol–water (85:15); flow-rate: 20 μl/min; detection: 254 nm.

Applications of high-resolution columns

The separation of fully deuterated D-6 benzene from H-6 benzene was obtained on a 4.5 m × 1 mm I.D. column operated at 10 μl/min with methanol–water (85:15) mobile phase and can be seen in Fig. 6. The column exhibited 230,000 theoretical plates for H-6 benzene ($k' = 0.99$) and the asymmetry factor as measured by the 60.6% of the peak height method was 1.01. The same chromatographic conditions were employed to separate D-11 diazepam from H-11 diazepam and the chromatogram can be seen in Fig. 7. Because of the lower content of deuterium (4%) and pronounced tailing in this case, only a partial separation was obtained. It is interesting to note that, in both cases, the solute containing the heavier isotope deuterium elutes first. Dispersion or London forces generally increase with solute molecular weight and, thus, it would be expected that the heavier isotope will be more "hydro-

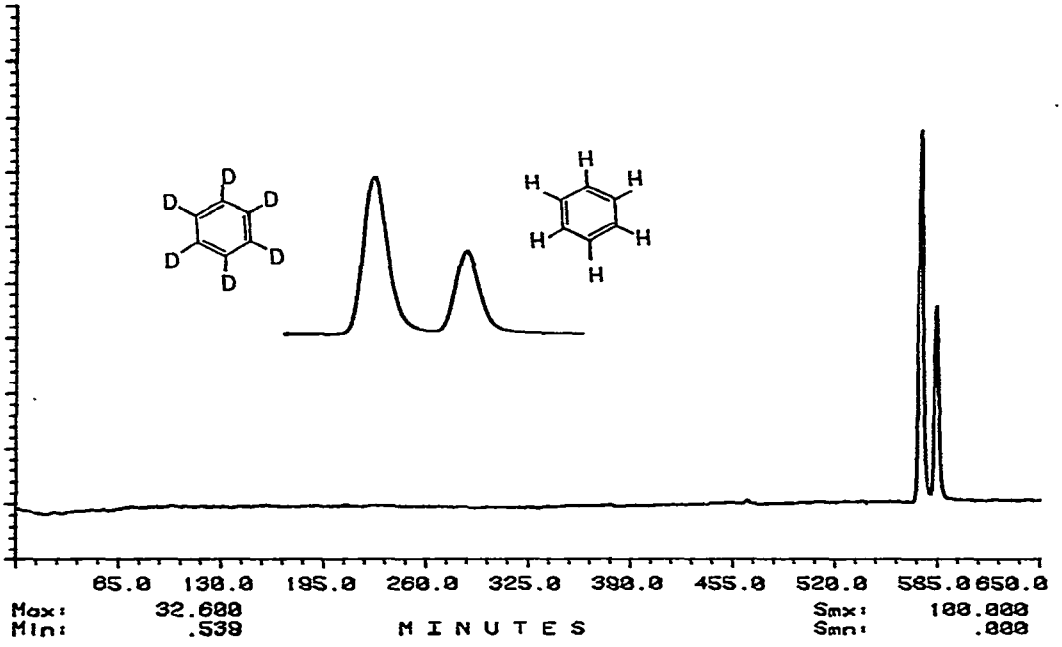

Fig. 6. Chromatogram of a mixture of D-6 + H-6 benzene obtained on a 4.5 m × 1 mm I.D. ODS reversed-phase microbore column. Eluent: methanol–water (85:15); flow-rate: 10 μl/min; sample volume: 0.5 μl; detection: 254 nm.

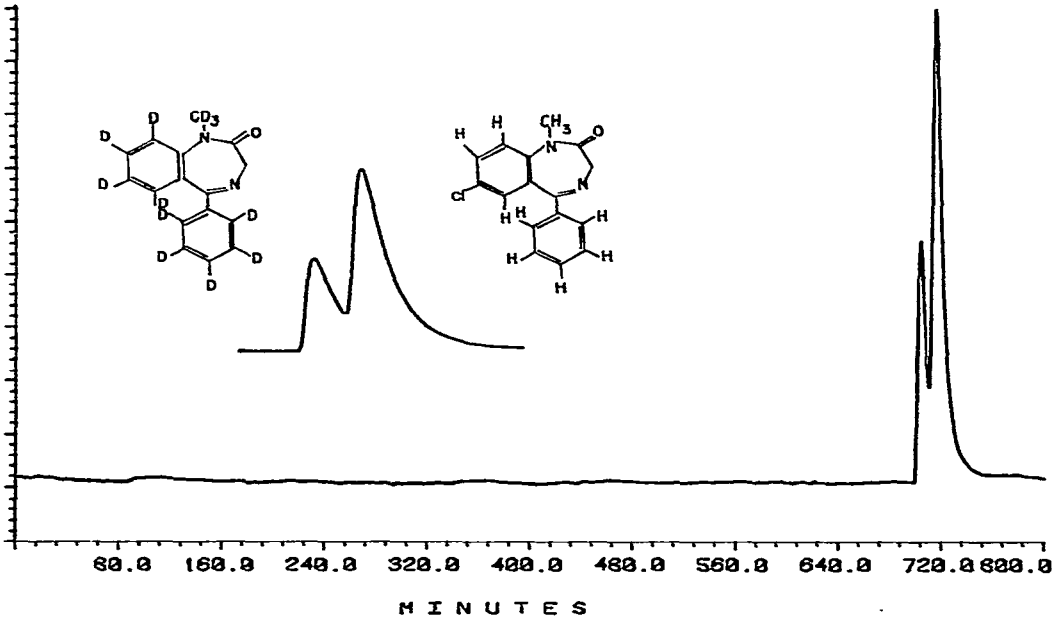

Fig. 7. Chromatogram of a mixture of D-11 + H-11 diazepam obtained on a 4.5 m × 1 mm I.D. ODS reversed-phase column. Conditions as in Fig. 6.

phobic" and elute on a C_{18} reversed-phase column after the H isotope. However, due to the relatively high concentration of methanol in the mobile phase and on the surface of the reversed phase, and due to the orientation of methanol molecules on the surface, the reversed-phase functions now as a "normal-phase" system and this explains the order of elution observed for these substances. In Fig. 8, a chromatogram of oil of dwarf pine needles can be seen. The column employed, 4.5 m × 1 mm I.D., was operated at 30 μl/min. Totally, 100 major peaks and impurities were observed on the chromatogram and this demonstrates the power of high-resolution reversed-phase microbore columns to separate complex mixtures.

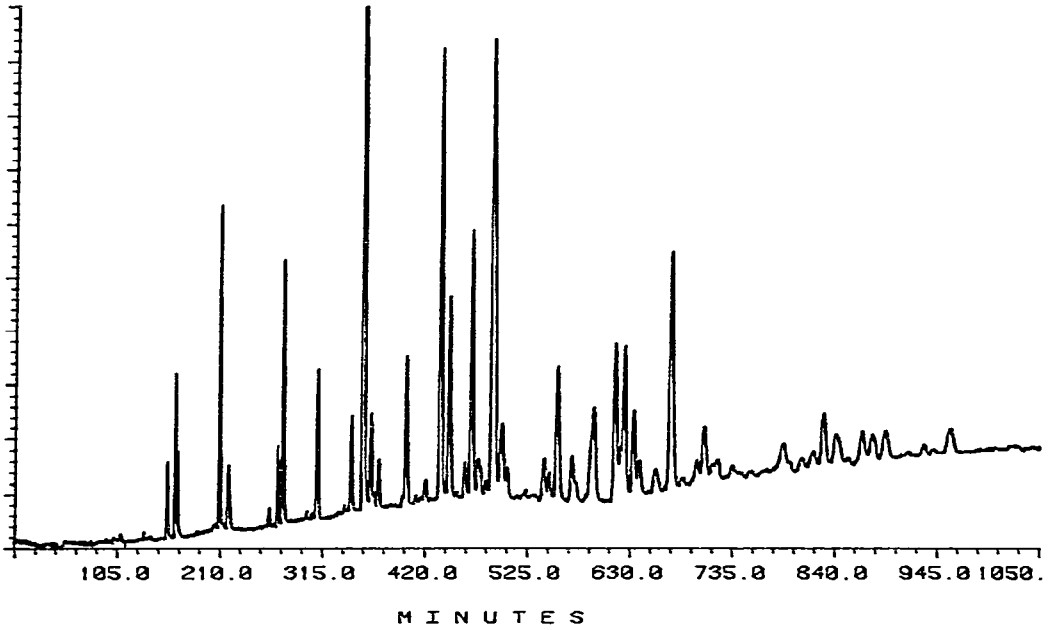

Fig. 8. Chromatogram of dwarf pine essential oil. Column and conditions as in Fig. 6, except for flow-rate: 30 μl/min.

DISCUSSION AND CONCLUSIONS

It has been assumed in the past that, when coupling columns together, some efficiency must always be lost in the connecting fitting. It can be seen now that this argument cannot be extended to columns of all diameters. The experimental data reported in this paper clearly show that excellent linearity between the average plate count and column length can be obtained when coupling 1 mm I.D. columns. The linear function gave the best fit with an index of determination of 0.999, whereas the same dependence for 4.6 mm I.D. columns could be best described by a power function with an index of determination of 0.998. Thus, there appears to be about 60–70% efficiency loss in coupling together larger bore columns. Since the same direct coupling system was used in both cases, it can be concluded that trans column band broadening effects due to the higher volumetric flow-rates are much more predominant with larger, 4.6 mm I.D. columns than with 1 mm I.D. columns. These effects are

not very well described in the conventional Van Deemter equation for plate height and should be investigated in greater detail. Relatively little data are available on the Knox coefficient C for various chromatographic systems. The results from our work indicate that the C term is usually very low, and thus the necessity to operate very close to the optimum h is not substantiated. There is a possibility that the C term may be dependent on k', d_p and column diameter. It is shown that, in a well-designed 50 cm × 1 mm I.D. column system, the extra column band broadening contribution is minimal, as evidenced by the N *versus* k' plot and by the fact that a reduced plate height of 2.3 can be achieved. As pointed out recently by Huber[18], the concept of the optimum plate height of $\times 2d_p$ does not take into account the heat transfer generated by the solvent friction and solvent viscosity and should be reconsidered.

It follows from the results reported in this paper that the road to high efficiency systems has to go through narrow-bore packed columns. It was also thought in the past that virtually indefinite numbers of theoretical plates could be obtained by coupling columns together. With the current state-of-the-art instrumentation, this view is clearly not correct since there is a maximum limiting number of theoretical plates which can be achieved within a given experimental system. The pressure rating of the instrumentation can be considered as one practical limiting factor. The stability of porous silica based packings operated at high pressures over 6000 p.s.i. is another. A very limited amount of work has been done on the effects of pressure on k' and N, but, in general, the results reported are negative. It appears that, from the previously derived equations (6, 7 and 8), and from the extrapolation from our own experimental data, this efficiency limit is about $1 \cdot 10^6$ theoretical plates with 8-μm particles and an 18-m long column operated at its optimum reduced velocity of 2 at 7000 p.s.i.

This paper describes a simple and inexpensive way to build a high-resolution system. Since microbore columns are now available from several manufacturers, it is possible for a practicing chromatographer to assemble easily such a system and to apply it to the solution of his problems. High efficiencies are clearly necessary for the separation of closely eluting compounds, such as deuterium-labeled isotopes and optical isomers, as well as for multicomponent mixtures. Besides the economy of operation of microbore columns mentioned previously, the possibility to generate high numbers of theoretical plates and the feasibility of direct LC–MS coupling are the greatest advantages which can be offered. Thus, microbore columns and, more specifically, the concatenation of them, definitely have a place in today's chromatographic work.

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