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QUANTITATIVE ANALYTICAL ASPECTS OF REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH SLURRY-PACKED CAPILLARY COLUMNS

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SUMMARY

Conditions for reproducible packing of fused-silica liquid chromatography capillary columns were demonstrated. The plate height *vs.* velocity curves were determined for successively prepared columns. In addition, the values of separation impedance were calculated. Several liquid chromatography systems were evaluated in conjunction with the slurry-packed capillaries for the retention and peak area reproducibility.

INTRODUCTION

Microcolumn small-diameter, slurry-packed capillary columns[1–4] can effectively be employed in the analysis of very complex biochemical[5–7] and industrial[8–11] mixtures. Although their resolution capabilities have been demonstrated, considerably less attention has been given to quantitative aspects of microcolumn liquid chromatography (LC), namely the reproducibility of retention, column performance, and peak area measurements.

Analytically adequate microcolumns have been available for some time[1–4]. However, meaningful reproducibility assessments have been hindered by the generally primitive hardware. Since much improved commercial solvent delivery systems and sampling devices are becoming available for specific use in microcolumn LC, important column evaluations can now be made. This communication describes the quantitative aspects of the C_{18} reversed-phase microcolumns.

In order to evaluate the performance of successively prepared microcolumns, the reduced plate height *vs.* velocity plots were determined together with the separation impedance[12] values. The reproducibility of retention and peak area was also assessed.

EXPERIMENTAL

Evaluation procedures

The separation impedance (eqn. 1) and the reduced plate-height/velocity equation (eqn. 2), as defined by Knox and co-workers[12,13] were used to assess the micro-

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column performance:

$$E = \frac{t_0 \Delta P}{N^2 \eta} = \frac{H^2}{K^0} = h^2 \varphi \tag{1}$$

$$h = B/v + Av^{0.33} + Cv \tag{2}$$

Eqn. 1 shows that the separation impedance (E) evaluates column performance not only on the basis of total plate number (N), but it also considers the time necessary for elution of an unretained component (t_0), the pressure drop required to achieve the separation (ΔP), and the solvent viscosity (η). E is also related to the plate height, H ($H = L/N$, where L = column length) and the column permeability, K^0, or to the reduced plate height ($h = H/d_p$, where d_p = particle diameter) and the column resistance parameter ($\varphi = d_p^2/K^0$). E is dimensionless, and the lower its value the better is the performance of a column. Values as low as 2000 can be obtained for packed columns.

Eqn. 2 shows the dependence of h on the reduced velocity ($v = ud_p/D_m$, where u = mobile phase velocity and D_m = diffusion coefficient of a solute in the mobile phase). The coefficients A, B, and C are practically independent of the particle diameter and of the system used, and should only be a function of the packing method. Usually, there is a minimum in the reduced plate height between 1 and 3 for regular, homogeneous packings, which corresponds to an optimum reduced velocity of a few units.

To obtain the values of E, φ, and h, one must determine the number of theoretical plates, N, and the dead time, t_0. For a Gaussian peak

$$N = \frac{t_R^2}{\sigma_t^2} \tag{3}$$

where t_R is the retention time (first statistical moment) of the peak at the apex and σ_t^2 is the chromatographic peak variance (second central moment of the peak), expressed in time units. However, if the peak is close to the Gaussian shape, eqn. 3 can be expressed in the usual form, and the plate number is easily determined:

$$N = 5.54 \left(\frac{t_R}{w_{1/2}} \right)^2 \tag{4}$$

where $w_{1/2}$ is the peak width at one-half the peak height.

Column packing

A packing procedure similar to that previously described[4] was employed. After a porous PTFE frit[14] was fixed at the column end, 250 μm I.D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U.S.A.) were slurry-packed using a Model LC-5A pump (Shimadzu, Kyoto, Japan). The packing material, 5-μm Spherisorb-ODS (Phase Separations, Norwalk, CT, U.S.A.) was suspended in a solution containing 1.5% Nonidet P-40 (Sigma, St. Louis, MO, U.S.A.) non-ionic surfactant in acetonitrile, and then transferred in a short piece of 1/16 in. I.D. tube, serving as the

slurry reservoir. The slurry ratio was 3.8:1 (v/w), necessitating *ca.* 50–60 mg of the packing per meter of column. During the packing, the pressure was set at 50 atm and increased by 100 atm every 5 s up to 450 atm. The pump was stopped 30–60 min after the column was completely filled. When the pressure returned to zero, the solvent was changed, and water pumped through the column overnight. This step was found useful for complete compaction of the packing material.

Chromatographic system

The system employed for column evaluation utilized a modified Varian Model 8500 syringe pump (Varian Instrument Division, Palo Alto, CA, U.S.A.) operated in the constant-pressure mode. A UV absorbance detector (UVIDEC-100-$\underline{\text{v}}$, Jasco, Tokyo, Japan) with an in-house modified 10-nl cell was employed to detect the solutes at 254 nm. A four-port internal-loop, electrically activated Valco injection valve (Model ECI4W, Valco Instrument, Houston, TX, U.S.A.) with a 0.2-μl rotor was used for sampling. To avoid large variances, it is also important to inject only a portion of the volume contained in the sample loop. A sample volume injected totally into the column has an exponential decay profile, because the laminar flow of a displacing mobile phase causes the sample to be dragged along the wall of the sample loop. Consequently, the moving-injection technique[15] was used. For the loop used in this work, approximately 170 nl, corresponding to a 6.6-s injection time, was the maximum injection volume without undesirable peak tailing. In addition, contribution of this sample volume to the overall theoretical plate height of this sample volume was *ca.* 10% for a compound with $k' = 1$. Since a major part of the analyzed mixtures usually corresponds to compounds with larger capacity ratios, a sample volume of 170 nl is considered to be a good compromise between the efficiency and sensitivity with the slurry-packed microcolumns. The injection time (time of the valve in the Inject position) and, consequently, the amount of injected sample were controlled by an IBM personal computer, connected to the electronic actuator. The same computer was used to acquire the data (digitization frequency of 8.0 Hz) for the peak moment calculation. The calculations were performed through an in-house developed computer program.

The column testing mixture contained *m*-cresol (4 mg/ml), nitrobenzene (0.3 mg/ml), diphenylamine (1 mg/ml), and phenanthrene (0.083 mg/ml) dissolved in acetonitrile–water (75:25, v/v). The same solvent mixture was used as the mobile phase for all column measurements. Dead-volume values were determined from the acetone peak.

RESULTS AND DISCUSSION

The first three successively prepared columns were used to evaluate the dependence of h, experimentally determined by using eqn. 4 as a function of v. The corresponding semi-log plot is shown in Fig. 1, where the solid line represents eqn. 2, in which $B = 2$, $A = 1$ and $C = 0.15$. For a well-packed column, the values are expected to be $B \approx 2$, $A \approx 1$, $C \approx 0.1$ (ref. 12), B/v reflects a contribution of the axial molecular diffusion to the plate height and is responsible for the increasing value of h at low velocities. $Av^{0.33}$ arises primarily from the flow anisotropy within the mobile phase and dominates in the intermediate region, where h shows a mini-

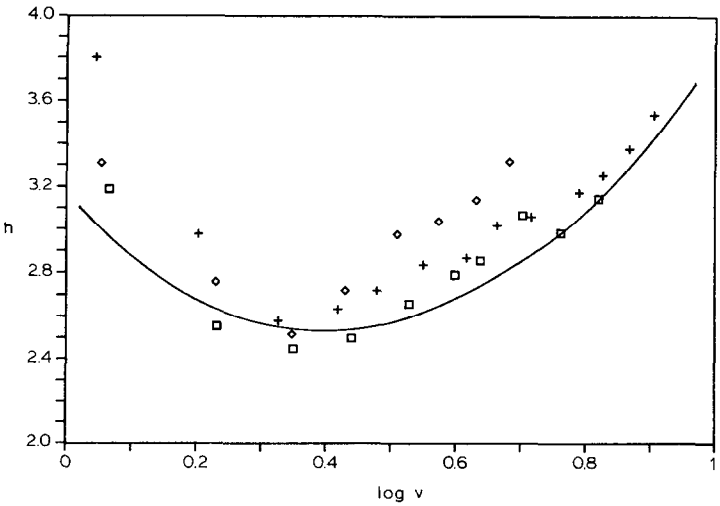

Fig. 1. Plot of h *vs.* log v for three slurry-packed capillary columns. Solute, phenanthrene (k' = 1.51, D_m = 1.41 · 10^{-5} cm^2/s). (+) Column 1, (□) column 2, (◇) column 3. Conditions as described in text. The solid line represents $h = 2/v + v^{0.33} + 0.15v$.

mum of $h \approx 2.5$ at a reduced velocity of $v \approx 2.3$. Finally, Cv is the expression of a slow mass transfer and is directly proportional to h, while its influence on the h value increases at elevated velocities. Any packing irregularities should be particularly reflected in the values of A.

As shown in Fig. 1, our packing procedure results in column efficiencies that are quite close to theoretical, which is $2d_p$. With 5-μm particles, 1-m long columns are easily prepared that yield overall efficiencies in the range of 70 000–90 000 theoretical plates.

Further data from nine additional slurry-packed capillaries are shown in Table I. These data further illustrate the point of high reproducibility, both from one run to another, and from column to column.

While the minimum plate height values are very consistent, there are some variations in the column permeability, as reflected in the values of φ and E (*c.f.*, *e.g.*, columns 5 and 6). We attribute these to minor heterogeneities in the polymeric end-frit. Although the use of this end-frit has introduced a substantial improvement over the original results with a glass-wool terminator plug[3], the average E values reported in Table I are higher than those reported earlier[14]; batch-to-batch variations in the rigid polymeric materials are suspect. For conventional and 1 mm I.D. columns, φ normally falls in the range 500–1000[16].

Decreases in the frit permeability can also be caused, in time, by an accumulation of "fines" on the frit surface, or a partially collapsed frit structure. To avoid such a collapse, the pressure was increased only gradually during the column packing procedure.

The values of h and E have been calculated from both eqns. 3 and 4. The average difference of the reduced plate height values calculated by the two different equations is only 4.4%. This reflects a good peak symmetry, a small skew and low σ/τ values, where τ represents the time constant of an exponential modifier of the

TABLE I

COLUMN CHARACTERIZATION AND REPRODUCIBILITY

Measurements for all 9 columns are based on 10 chromatographic experiments on each column. Solute, phenanthrene ($k' = 1.51$; $D_m = 1.41 \cdot 10^{-5}$ cm²/s). Flow-rate, 1.6 µl/min.

Column	φ *Mean (C.V., %)*	h_{min} *Mean (C.V., %)*		E *Mean (C.V., %)*	
		Eqn. 4	*Eqn. 3*	*Eqn. 4*	*Eqn. 3*
1	705 (1.7)	2.60 (3.6)	2.79 (3.4)	4780 (5.8)	5488 (6.0)
2	768 (1.4)	2.52 (3.6)	2.75 (2.8)	4877 (7.4)	5808 (5.9)
3	700 (2.3)	2.46 (5.2)	2.57 (5.5)	4241 (8.4)	4623 (10.5)
4	671 (1.6)	2.38 (2.1)	2.42 (5.4)	3803 (3.5)	3930 (9.8)
5	743 (1.1)	2.60 (2.3)	2.80 (5.3)	5044 (5.2)	5825 (9.1)
6	927 (2.3)	2.43 (2.0)	2.48 (6.6)	5469 (3.1)	5701 (11.9)
7	750 (1.6)	2.40 (3.1)	2.40 (4.3)	4312 (5.8)	4320 (8.6)
8	719 (2.4)	2.49 (5.2)	2.58 (6.9)	4463 (9.7)	4786 (12.4)
9	650 (1.4)	2.31 (1.7)	2.40 (2.0)	3477 (3.8)	3744 (4.2)
Average coefficient of variation	(1.8)	(3.2)	(4.7)	(5.9)	(8.7)
Average	737 (10.9)	2.47 (4.0)	2.58 (6.9)	4496 (13.9)	4914 (16.7)
Column 1 after 3 months	806 (0.9)	2.50 (5.1)	2.64 (7.0)	5038 (9.7)	5615 (13.5)

standard deviation of a Gaussian peak[17]. The higher coefficient of variation found with the moment method originates from experimental sources such as noise and baseline drift. The calculated values of h are slightly higher than those predicted by the theory. As discussed later, this difference arises, at least partially, from extracolumn band-broadening.

Column 1 was retested after three months of storage. During the storage period, the column was constantly flushed with acetonitrile at low pressure (approximately 10 atm). Other storage procedures may be applicable, as long as solvent evaporation is avoided. As shown with this column, no significant changes in its chromatographic characteristics were found.

The total porosity, ε_T, of a column can be dynamically determined from the following equation:

$$\varepsilon_T = \frac{F_v \, t_0}{r_c^2 \, L_c \pi} \tag{5}$$

where F_v is the volumetric flow-rate, r_c is the column radius, and L_c is the column length. Employing column 3 as an example, the total porosity was calculated to be 0.751. The total porosity is the sum of the column inter- and intraparticle porosities (ε_u and ε_i, respectively). Thus, using a modified Kozeny-Carman equation

$$\varphi = 180 \, \psi^2 \frac{(1 - \varepsilon_u)^2 \, (\varepsilon_u + \varepsilon_i)}{\varepsilon_u^3} \tag{6}$$

where ψ is a structural constant (equal to approximately 1 for porous, spherical packings), ε_u and ε_i can be calculated. The intraparticle porosity value, a characteristic of the packing, for column 3 was determined to be 0.343. This value corresponds well with that ($\varepsilon_i = 0.321$) reported previously[18] for a very similar material (Spherisorb S5W). When the interparticle porosities for the remaining columns were calculated, their values were found to be very consistent (range of 0.398–0.416), except for column 6, presumably due to the collapse problem mentioned above. These values reflect the column packing densities that are consistent with those reported for slurry-packed conventional columns[18,19].

When N is determined from a detector signal, this value reflects not only the chromatographic band-broadening, but also any extracolumn contributions. The chromatograph may reduce the apparent column efficiency mainly through the injection system, the connections between the injection port and the column, and the connections between the column and the detector. In addition, the detector cell volume and time constant may also contribute to band-broadening. The overall loss of efficiency corresponds to individual losses. As the effects are considered to be independent of each other, the total volumetric variance σ^2_{tot} can be written as

$$\sigma^2_{tot} = \sigma^2_{col} + \Sigma\sigma^2_{ext}$$

where σ^2_{col} is the column variance due to band-broadening inside the column and the σ^2_{ext} terms are various volumetric contributions from the chromatographic system. The column volumetric variance can easily be obtained from eqns. 3 and 5 as

$$\sigma^2_{col} = F_v \frac{t_R^2}{N} = (1 + k')^2 (\pi r^2 \varepsilon_T)^2 h\, d_p L$$

TABLE II

EXTRACOLUMN CONTRIBUTIONS TO CHROMATOGRAPHIC BAND-BROADENING[21]

(1) Plug injection	$\sigma^2_I = \frac{V_s^2}{K^2}$	V_s = sample volume K^2 = constant for each injection technique. Theoretically, equal to 12, experimentally as low as 4 (ref. 22).
(2) Connecting tube	$\sigma^2_T = \frac{\pi^2 r_T^6 L_T U_T}{24\, D_m}$	r_T = tube radius L_T = tube length U_T = linear velocity within the tube D_m = solute diffusion in the mobile phase
(3) Detector cell	$\sigma^2_C = \frac{V_C^2}{K^2}$	V_C = cell volume K^2 = constant equal to 12
(4) Detector time constant	$\sigma^2_{RC} = (\pi r_c^2 \varepsilon_T U_c)^2 \tau^2$	r_c = cell radius U_c = linear velocity within the cell τ = detector time constant ε_T = column total porosity

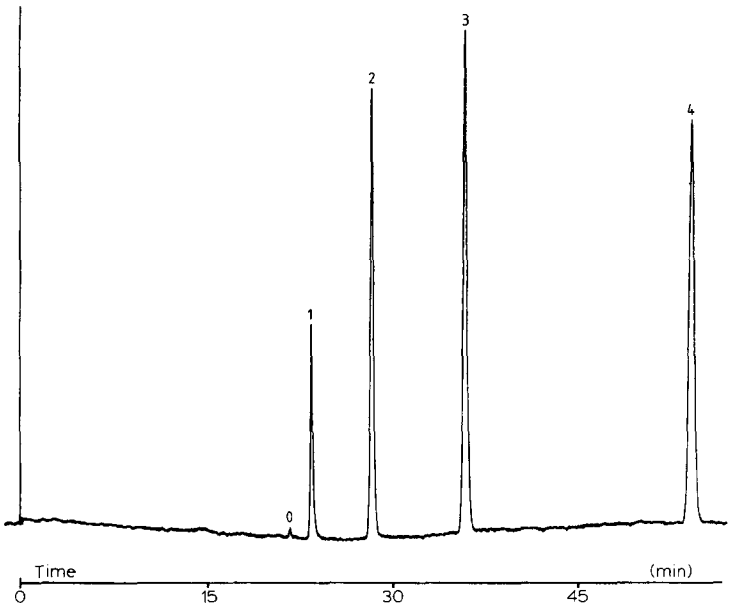

Fig. 2. A test chromatogram for the performance evaluation of slurry-packed capillary columns. Conditions as reported in text. Solutes: (0) acetone, (1) *m*-cresol (k' = 0.0867), (2) nitrobenzene (k' = 0.305), (3) diphenylamine (k' = 0.658), (4) phenanthrene (k' = 1.51).

where F_v is the volumetric flow-rate and k' is the capacity ratio of a solute. The equations for calculating the individual extracolumn contributions are given in Table II. Using the most unfavorable case of $K^2 = 4$, the injection contribution of 170 nl was calculated to be $\sigma_I^2 = 7.2 \cdot 10^{-3}\ \mu l^2$. Through a PTFE adapter, the column was attached precisely at the end of the injector hole (diameter of 254 μm), while no frit

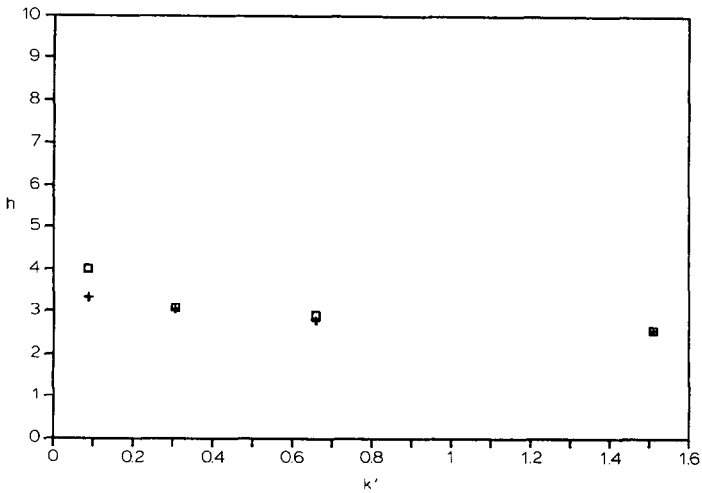

Fig. 3. Reduced plate height *versus* capacity ratio for test compounds. Column 3, (□) experimental values; (+) theoretical values for the system used, calculated assuming h_{col} = 2.38.

TABLE III

COEFFICIENT OF VARIATION IN THE PEAK AREA

Measurements for all 9 columns are based on 10 chromatographic experiments on each column with solute, phenanthrene.

Column	*C.V. (%)*
1	1.5
2	3.4
3	3.0
4	1.6
5	2.1
6	2.4
7	2.0
8	2.2
9	1.9
Average coefficient of variation	2.2

was provided at the column inlet. Thus, the volume between the internal sampling loop and the column was minimal (around 50 nl, as calculated from the 1-mm-long hole; $\sigma_T^2 = 6.2 \cdot 10^{-4}$ μl^2). The lack of an inlet frit was not found troublesome, as long as sufficient care was exercised to avoid a drastic pressure decrease in the pump. The column end was connected to the detector cell with 50 μm I.D. fused-silica tubing. A segment of that tube, where the coating had been removed, served as the detection cell[20]. In total, a 10-cm capillary tube contributed to the band dispersion: $\sigma_T^2 = 9.0 \cdot 10^{-5}$ μl^2. The cell volume contribution (10 nl) was relatively small ($\sigma_C^2 = 8.3 \cdot 10^{-6}$ μl^2). At a time constant equal to 0.05 s, the contribution of this factor was $\sigma_{TC}^2 = 1.0 \cdot 10^{-6}$ μl^2. The sum of these contributions gave the value of $\sum\sigma_{ext}^2 = 8.0 \cdot 10^{-3}$ μl^2.

Considering the data in Table I and a 100-cm-long column, the average σ_{col}^2 was equal to $1.09 \cdot 10^{-1}$ μl^2. Therefore, the column contributed *ca.* 92.7% of the total band spreading, the remainder being caused by the injector (6.6%). Thus, the actual reduced plate height for the column contribution was $h_{col} = 2.38$.

In agreement with eqn. 8, the column variance has a parabolic dependence on

TABLE IV

COEFFICIENT OF VARIATION IN THE RETENTION OF ACETONE FOR FOUR DIFFERENT COMMERCIAL PUMPS

Acetone was used as a non-retained compound. Measurements for all four pumps are based on 10 chromatographic experiments with each pump.

Pump	*C.V. (%)*
A (used during all other experiments)	2.9
B	0.55
C	0.25
D	0.15

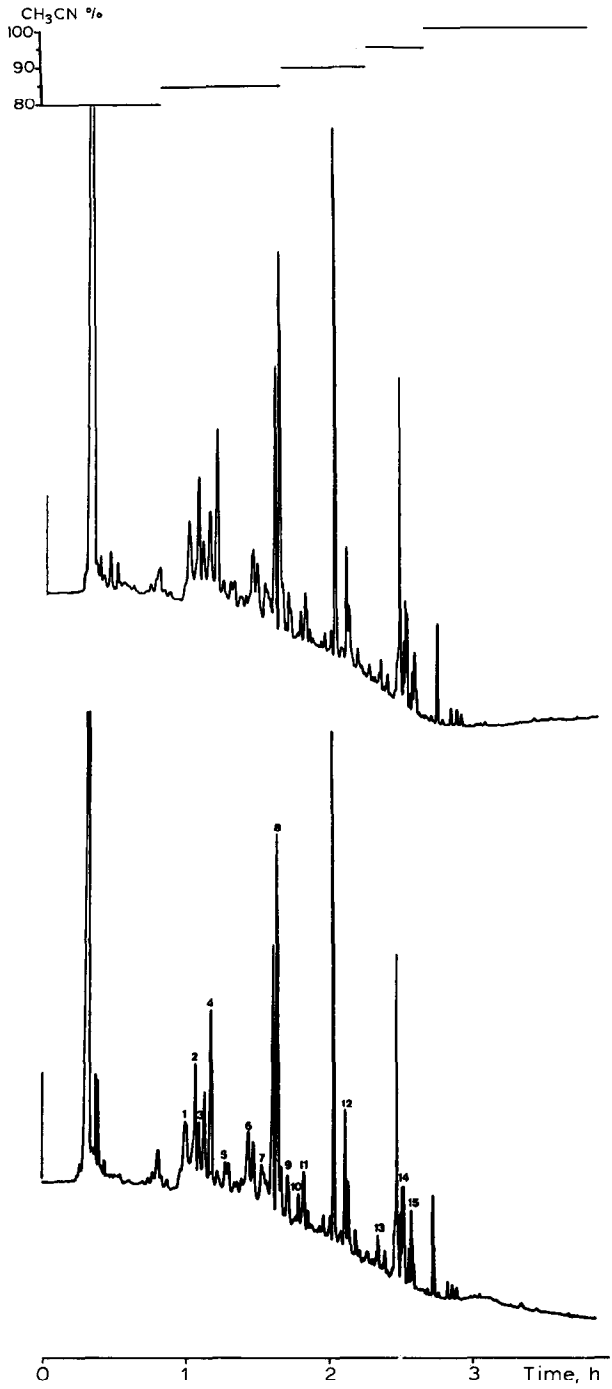

Fig. 4. Reproducibility of a complex mixture analysis. Two consecutive injections of benzoylated derivatives of hydroxysteroids isolated from human urine. Mobile phase: step gradient water–acetonitrile (top). Flow-rate: 1.5 μl/min. UV detection at 230 nm. Column: 1.1 m × 250 μm I.D. The injected amount of each steroid was *ca.* 20–100 ng. Tentative identification obtained by mass spectrometry of the isolated peaks; stereochemistry and position of functional groups are unknown. (1) a pregnen-tetrol-one; (2) an androstan-diol-one; (3) an androstan-diol-one; (4) an androstan-diol-one; (5) a pregnan-ol-one; (6) an androstan-diol-one; (7) a pregnan-tetrol; (8) a pregnan-pentol; (9) an androstan-diol-one; (10) a pregnan-pentol; (11) an androstan-diol-one; (12) a pregnen-diol; (13) a pregnan-triol; (14) an androstan-triol; (15) an androstan-diol.

the solute capacity ratio. On the other hand, the extracolumn contributions (Table II) are thought to be independent of the chromatographic characteristics of the compound injected. Therefore, the extracolumn variances have a greater influence on peak variance for substances with a low capacity ratio. A typical chromatogram of our test mixture is shown in Fig. 2. In our system (column 3), the average capacity ratios were: *m*-cresol, 0.0867 (C.V. = 1.2%); nitrobenzene, 0.305 (C.V. = 0.8%); diphenylamine, 0.658 (C.V. = 0.8%); phenanthrene, 1.51 (C.V. = 1.2%). Fig. 3 illustrates the reduced plate-height values for experimental and theoretical ratios (assuming that $h_{col} = 2.38$, and is independent of k'). Although reduced plate-height values for benzene and diphenylamine correlate well with theory, there is a discrepancy for *m*-cresol. This could be explained by an undesirable interaction with the silica packing material.

To analyze the solutes with low retention values ($k' < 1$), extracolumn variances must be minimized. A reduction in the injection volume becomes necessary, although some decrease in sensitivity will result. In sensitivity-limited applications, dissolving samples in a solution with an elution strength lower than that of the mobile phase will focus the solute at the column inlet[2], permitting a larger injection volume to be applied without a significant increase in variance.

Precision and accuracy of the injected sample volume depend on stability of the flow-rate delivered by the pump and stability of the device that controls the motion of the injection valve. Table III shows the precision data for phenanthrene. It is reasonable to assume that the flow-rate imprecision is mainly responsible for those variations. Indeed, Table IV (pump A) illustrates that the coefficient of variation for the retention time of an unretained peak well reflects the peak area accuracy. Table IV also indicates that better flow reproducibility, and subsequently, more precise injections can be obtained by using the pumps developed more recently (B, C, and D) for microcolumn technology.

When dealing with complex samples, highly repeatable analyses have to be performed in conjunction with high-efficiency separations, such as those yielded by slurry-packed capillary columns. The two chromatograms in Fig. 4, which show two consecutive injections of a hydroxysteroid sample extracted from human urine[5], exemplify the high reproducibility in terms of retention times and peak areas; a 3-h, high-resolution run still produces identical chromatograms.

CONCLUSIONS

The continued success of microcolumn LC as a powerful analytical technique will depend on precision and whether it can match the quantitation of conventional LC. Five years ago, the demands of microcolumn technology were clearly ahead of the existing instrumentation. Recent developments in both areas have made chromatography practical, using the slurry-packing technique for capillary columns for numerous applications. The readily packed reversed-phase columns reported in this work demonstrated a high degree of reproducibility. Further investigations are needed to extend the same capabilities to other column types.

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