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EFFECTS OF INTERMEDIATE MOBILE PHASE REMOVAL ON COLUMN PERFORMANCE IN TWO-DIMENSIONAL COLUMN CHROMATOGRAPHY

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SUMMARY

The physical removal of the first solvent between two successive developments in two-dimensional column chromatography employing two immiscible eluents is an important step which can affect adversely the performance of the overall analysis. A study of the effects of the following drying conditions on column efficiency is described: the flow-rate of the displacing gas, the duration and temperature of the purge and volume of liquid displaced. The quantitative data are in good agreement with the results obtained from the derived equation for loss of efficiency. The loss of efficiency accompanying the drying process is inversely proportional to the $1 + k'$ values of the compounds investigated, which have low but not negligible vapour pressures, and depends critically on the drying time.

INTRODUCTION

It is becoming increasingly obvious that the analysis of highly complex mixtures demands a separation power that can rarely be achieved using a single chromatographic column[1,2]. The peak capacities afforded by conventional columns usually do not exceed a few hundreds for the common range of capacity factors (k'): for $k' = 1$–10, a peak capacity of 200 requires an efficiency of 270,000 plates, which is difficult to achieve[1]. This impossibility of analysing simultaneously a sufficiently large number of compounds entails the use of selective sample extractions and/or several chromatographic separations prior to the actual analysis[3], thus resulting in a prolonged total analysis time, increased cost and additional sources of error.

In the light of these facts, it is not surprising that two-dimensional thin-layer chromatography (TDTLC) using microparticulate packing materials and automated operation is experiencing a period of intensive revival[4]. The theoretical principles of chromatography on two-dimensional columns have been discussed by Guiochon *et al.*[1]. It has been demonstrated that a careful choice of mobile and stationary phases which afford two distinctly different retention mechanisms can result in peak capacities which far exceed those commonly encountered in conventional column chromatography.

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In two-dimensional chromatography it is important to remove the first solvent prior to the second development. This is necessary in all TDTLC separations because the driving force for the solvent migration is provided by the wetting of the dry adsorbent. Thus the second development can be performed only with a dry TLC plate.

In this respect, the situation is different with two-dimensional columns. If the two solvents are compatible, the presence of a small amount of the first solvent in the second does not always markedly change the retention properties of the second chromatographic system. In these instances it is possible to operate simply a two-dimensional chromatographic column by merely pumping the second solvent in the second direction of the column after having stopped the first solvent stream along the first column direction[1].

There are cases, however, when the two solvents are not compatible[4] and the first one must be eliminated before the second can be pumped across the chromatographic bed. Two problems can arise during the drying stage of the high-performance liquid chromatographic system (HPLC)[4]. Firstly, incomplete removal of the first solvent can lead to a modified mechanism in the second dimension, compared with that obtained with the direct use of the second solvent. Secondly, improperly chosen drying conditions can lead to distortion of the spots and a concomitant loss of separation efficiency. These problems have been the topic of several studies in TLC[1,4–6]. It is difficult to extend the results of this work directly to two-dimensional column chromatography, which requires the use of a closed system to achieve constant control of the solvent flow-rate. Thus the whole surface of the chromatographic bed is not accessible, as it is in TLC, for vaporization of the solvent by gentle heating under a stream of nitrogen in an oven or a hood. One must use other methods to replace one solvent by another. This paper reports the simplest approach: the first solvent is flushed out by an inert gas stream and, subsequently, the second solvent is pumped into the column.

In an effort to evaluate the effect of drying conditions on chromatographic performance in two-dimensional column chromatography, we have conducted a series of experiments in which several operating parameters, such as the duration of purge, the temperature, inert gas flow-rate and volume of the liquid displaced, were varied.

EXPERIMENTAL

Apparatus and chemicals

Homologous *n*-alkylbenzenes were purchased from E. Merck (Darmstadt, G.F.R.), reagent-grade methanol from Carlo Erba (Milan, Italy), LiChrosorb RP-8 packing material (10 μm average particle size) from Merck and Spherisorb ODS (5 μm average particle size) from Phase Separations (Hauppage, U.S.A.). All chromatographic experiments were carried out using the following components: a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Waters Model 440 UV absorbance detector, a Rheodyne Model 7125 injection valve with a 10-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) and a Kipp & Zonen strip-chart recorder (Touzart & Matignon, Vitry sur Seine, France). The temperature of the chromatographic column was controlled using a Haake Model D3 constant-temperature circulator

(Haake, Karlsruhe, G.F.R.) and the temperature stability was ± 0.2°C. The columns were home packed at 500 atm using dibromomethane–acetonitrile (1.4:1) as the slurry solvent and pure methanol as the displacer. Two columns were used: a 15 cm $\times$ 4.6 mm I.D. column packed with 5-μm particles of Spherisorb ODS and a 25 cm $\times$ 4 mm I.D. column packed with 10-μm particles of RP-8.

Chromatographic conditions

The mobile phases were various mixtures of methanol and water, which were degassed under vacuum prior to use. The flow-rates were 2 ml/min for the Spherisorb ODS column and 1 ml/min for the LiChrosorb RP-8 column. Chromatographic solutes were detected at 254 nm. Retention times were measured manually, and peak widths with a ruled magnifying glass. A sufficiently high chart speed (0.5 mm/sec) was used in order to obtain adequate precision.

Removal of mobile phase

Prior to removal of the mobile phase from the column, a mixture of reference compounds (*n*-alkylbenzenes) was injected, the analysis started and the flow arrested at a point immediately after the volume corresponding to the maximal column porosity[7]. Next, the column inlet was connected to a nitrogen tank and the column was purged under certain conditions of time, temperature, nitrogen pressure and volume of mobile phase removed. At the end of the drying period, the column was reconnected to the pump and the analysis resumed using the same solvent. The column was weighed before and after nitrogen purging and the amounts of mobile phase displaced were calculated from the weight difference.

The displacement of the gas in the column in order to resume the analysis required approximately one column volume of the mobile phase, after which the recorder pen returned to the original baseline. Thus, under our conditions, solutes with k' values below 1.0 could not be analysed. In order to eliminate the effect of microbubbles remaining after the major portion of the gas had been displaced, a spiralled microbore tubing was attached to the detector outlet. This added backpressure (approximately 3.5 atm) was found to shorten the displacement time and helped the dissolution of small bubbles in the mobile phase. The duration and effect of this stage depends slightly upon the displacement conditions (amount of mobile phase removed, nature and speed of the second solvent).

In order to ensure that the relative loss of efficiency ($\Delta N/N$) accompanying the purge did not result from possible column degradation due to its drying or any other nitrogen impact with the packing, the test mixture was chromatographed before and after the main analysis, and the efficiencies for the respective peaks were compared. No losses of efficiency due to settling of the packing or to any other reason were encountered in the course of this work.

RESULTS AND DISCUSSION

In order to establish the optimal conditions for solvent removal in two-dimensional column chromatography, the effects of several operating parameters were studied by conventional HPLC: the duration of purging, the flow-rate of inert gas, the temperature and the volume of mobile phase removed. The experiments were

carried out using two gas flow-rates, corresponding to inlet pressures of 6 and 26 atm, respectively. In both instances the first 40% of the solvent contained in the column was flushed out mechanically in a few minutes. The remaining solvent was eliminated by vaporization only, a process whose rate is a direct function of the flow-rate, about 20 times larger at 26 atm than at 6 atm because of the compressibility of gases. The mass flow-rate of gas increases very rapidly with the pressure drop (*cf.*, Table I). At large values of the flow-rate deviations from Darcy's law appeared, as has already been observed[8]. These were due to energy losses in gas eddies at high velocities and increased with increasing gas velocities. The result is that the actual flow-rate was appreciably lower than calculated (*cf.*, Table II), where experimental data are given in parentheses, and a pressure larger than predicted may become necessary to achieve the same results. Nevertheless, extremely large gas flow-rates (up to 0.5 l/min) may be achieved through conventional LC columns, using only moderately high pressures.

At constant flow-rate, the time necessary for complete solvent removal from the column is, of course, a function of its composition and the vapour pressure of the different components of the mobile phase. This is illustrated in Fig. 1, which shows a plot of the amount of solvent displaced from the column *versus* composition for water–methanol mixtures. Although it is relatively easy to eliminate all methanol in about 2 h at ambient temperature, it is much more difficult to eliminate water. In fact, it is not possible to eliminate all the water without heating, even from columns packed with chemically bonded silica, rather than plain silica. This is shown in Fig. 2, for which the drying experiments were continued overnight. For mixtures with less than about 15% of methanol, approximately one third of the water is left behind. Above 20% of methanol the drying is faster (*cf.*, Fig. 1) as a large proportion of the water is carried out with the methanol.

The amount of mobile phase extracted from the column is proportional to the flow-rate, as shown by a comparison of the results obtained with 5- and 10-μm particle columns: it takes about four times longer to displace the same amount of

TABLE I

FLOW-RATES AND VELOCITIES FOR GAS PURGING OF AN LC COLUMN

$u_0 = \frac{k_0 d_p^2}{2\eta L P_0}(P_i^2 - P_0^2)$; $F = \frac{\pi d_c^2}{4} \cdot u_0$; $L = 25$ cm; $d_p = 10$ μm; $\eta = 140 \times 10^{-6}$ P (N_2); $k_0 = 1 \times 10^{-3}$.

P^* (atm)	u_0 (cm/sec)	F^{**} (cm^3/min)	ν^{***}
1	0.43	3.2	0.014
2	1.14	8.6	0.038
5	5.0	38	0.167
6	6.9	52 (39)	0.23
10	17.1	129	0.57
20	63	474	2.10
26	104	784 (411)	3.5
50	371	2800	12.4
100	1460	11000	49

* $\Delta P = P_i - P_0$; $P_0 = 1$ atm.

** $d_c = 4$ mm.

*** $D_m = 0.03$ cm²/sec, $\nu = \frac{u\, d_p}{D_m}$.

TABLE II

VOLUME OF SOLVENT PURGED FROM AN LC COLUMN

Results are % of solvent hold-up of the column. Solvent: methanol–water (80:20).

Time (h)	*Inlet pressure 6 atm*	*Inlet pressure 26 atm*
0.25	25	na
0.50	29	61
1.0	33	75
2	36	80
3	41	86

solvent from the former column than from the latter, and the flow-rates are in the same ratio. The comparison between results obtained with the same column (10-μm particles) at inlet pressures of 6 and 26 atm are also in agreement. The last layer of solvent (approximately 0.20 and 0.24 ml of pure methanol for the Spherisorb ODS and LiChrosorb RP-8 columns, respectively) is difficult to eliminate, however, and heating would help considerably.

At 6 atm nitrogen pressure at the column inlet, the entire interparticulate volume and a small fraction of intraparticulate volume could be displaced. Thus, even with a long purging time and methanol-rich mixtures, approximately 20–30 % of the total column volume remained unremoved. In order to elucidate the role of the remaining mobile phase on the extent of broadening, a series of experiments was conducted in which the column was purged at a pressure of 26 atm. The variation of

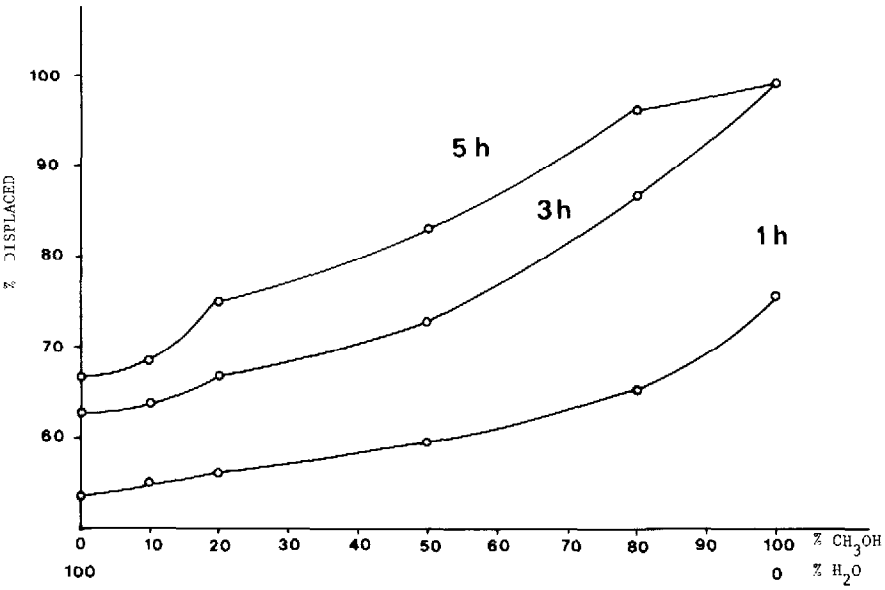

Fig. 1. Dependence of the volume of mobile phase displaced at a nitrogen inlet pressure of 26 atm as a function of mobile phase composition and purging time. Column: LiChrosorb RP-8 (250 × 4.0 mm I.D., d_p = 10 μm).

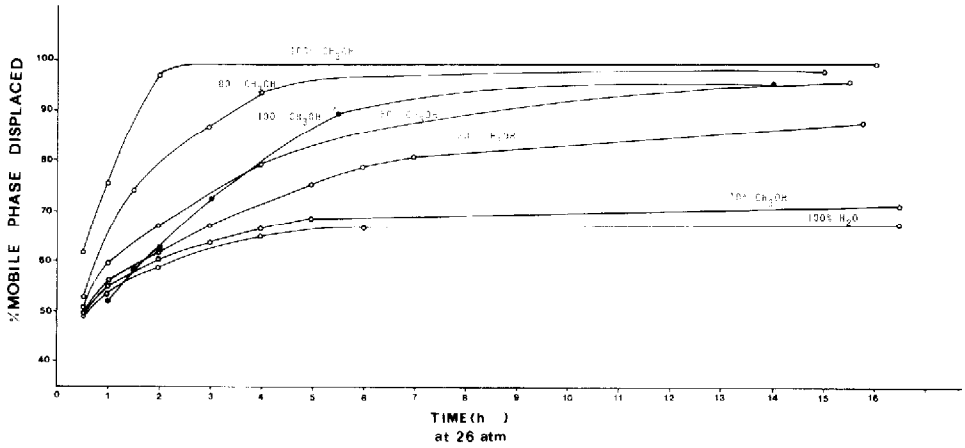

Fig. 2. Variation of the amount of mobile phase displaced as a function of purging time and solvent composition. The nitrogen pressure at the column inlet was 26 atm in all instances. ○, Data obtained for a 10-μm packing material (LiChrosorb RP-8, 250 × 4.0 mm I.D.); ●, data obtained for a 5-μm packing material (Spherisorb ODS, 200 × 4.6 mm I.D.).

the volume of liquid displaced as a function of purging time and mobile phase composition is shown in Fig. 2. The results pertaining to the 5-μm packing material indicate that, because of the lower permeability (and thus a lower nitrogen flow-rate at the same inlet pressure), the amounts of the mobile phase removed (100% methanol) are considerably lower than those for the 10-μm packing under the same conditions.

The relative loss of efficiency ($\Delta N/N$) was found to decrease markedly with increasing k' values, as illustrated in both Figs. 3 and 4. As long as a significant amount of solvent remains in the column, the loss of efficiency depends essentially on the purging time. This is shown in Fig. 3, but Fig. 5 shows that at low flow-rates, where the amount of solvent displaced varied between 23.4% and 51.0%, the loss of efficiency remains constant for a constant purging time, irrespective of the purging flow-rate; it is the same as under stopped-flow conditions (*cf.*, Fig. 3). Similarly, in Fig. 6, the loss of efficiency at a constant purging flow-rate is approximately proportional to the purging time, as long as the relative efficiency loss is small. This is consistent with a loss of efficiency resulting mainly from axial diffusion in the mobile phase while it is stagnant: as long as at least 70–80% of solvent has not been eliminated from the column, there is a significant number of large enough pools or droplets of solvent between particles, while the gas stream is also circulating around these particles, so that axial diffusion can take place in the solvent. The variance of the zone is given by

$$\sigma_a^2 = \sigma_1^2 + \sigma_p^2 + \sigma_g^2 + \sigma_f^2 + \sigma_2^2 \qquad (1)$$

where σ_a^2 is the variance leading to the observed efficiency, σ_1^2 and σ_2^2 are the variances due to the LC process prior to and after purging, so that $\sigma_1^2 + \sigma_2^2$ is the variance of the zone observed in conventional LC elution; σ_p^2 and σ_f^2 are the variance contributions due to the purging and filling processes, during which the liquid velocity is significant

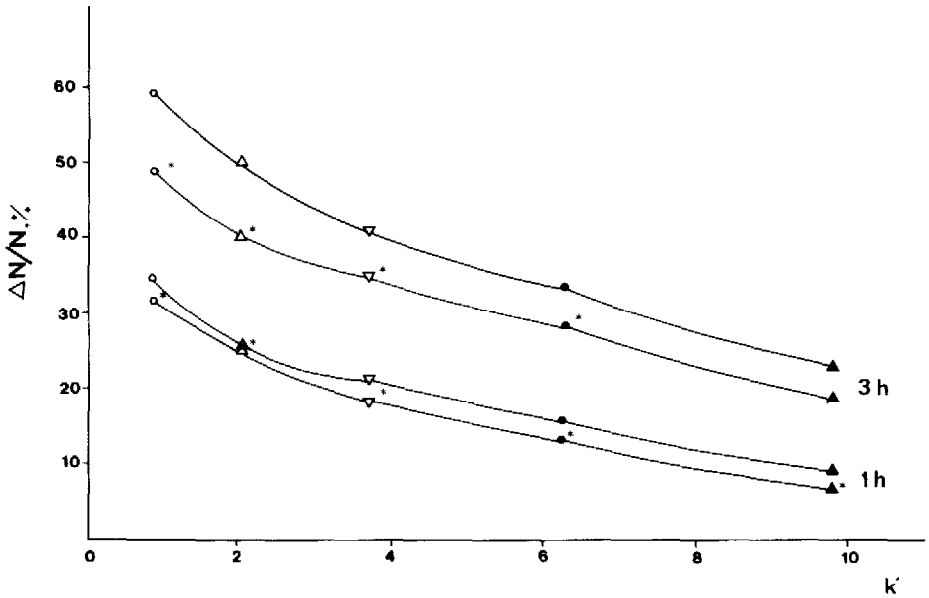

Fig. 3. Relative loss of efficiency ($\Delta N/N$) as a function of k' values of the solutes investigated. ○, Propylbenzene; △, butylbenzene; ▽, pentylbenzene; ●, hexylbenzene; ▲, heptylbenzene. Symbols with asterisks refer to results obtained under stopped-flow conditions without column purging. Column: Spherisorb ODS (150 × 4.6 mm I.D., 5 μm). Solvent: 80% methanol 20% water.

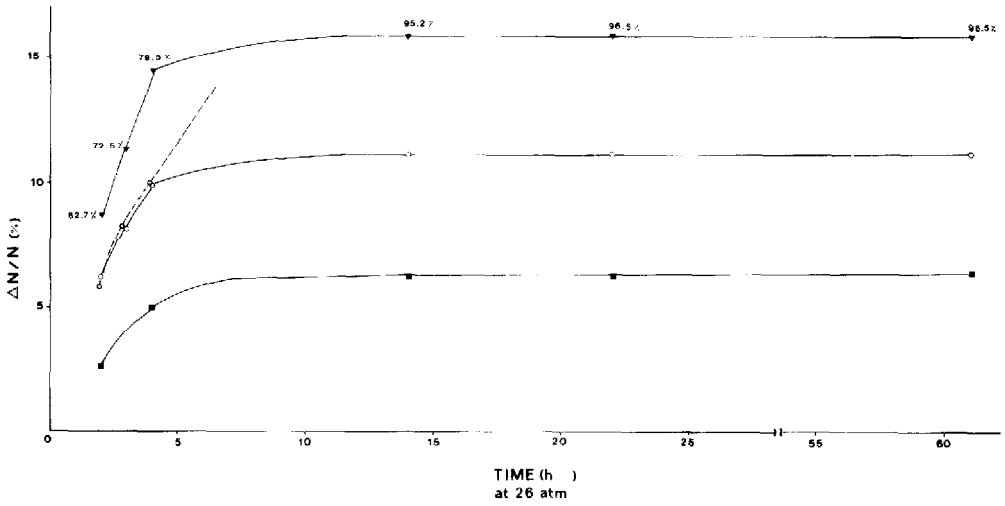

Fig. 4. Relative loss of efficiency as a function of purging time. The nitrogen pressure at the column inlet was 26 atm in all instances; the corresponding percentages of liquid displaced are indicated. Column, LiChrosorb RP-8 (250 × 4.0 mm I.D.; 10 μm), mobile phase, 100% methanol; flow-rate, 1 ml/min; temperature, ambient. Solutes: ▼, undecylbenzene; ○, dodecylbenzene, ■, octadecylbenzene. The dotted line represents the theoretical $\Delta N/N$ results for dodeculbenzene, obtained using eqn. 6. Parameters used: $\gamma = 0.80$, $D_m = 1.01 \cdot 10^{-5}$ cm^2/sec, $k' = 1.3$, $N = 4000$.

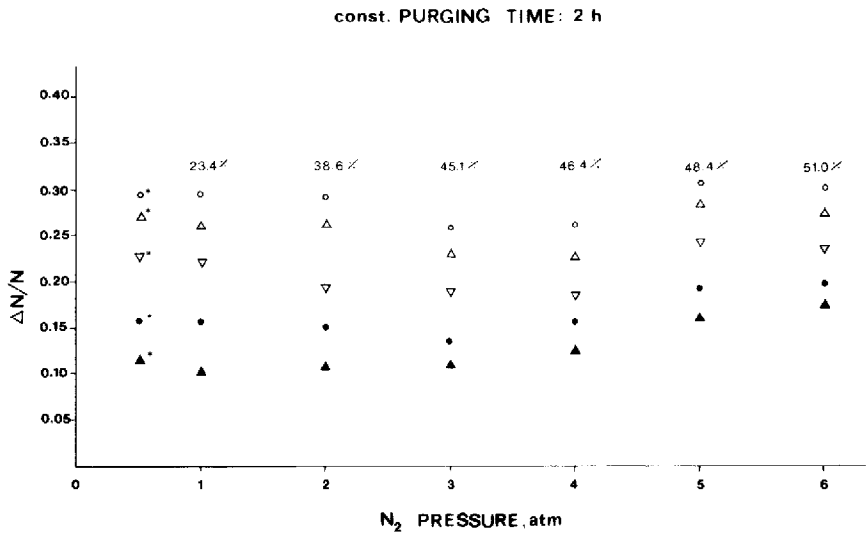

Fig. 5. Relative loss of efficiency ($\Delta N/N$) as a function of nitrogen pressure and volume of liquid displaced, at constant purging time (2 h). Symbols as in Fig. 3. The percentages refer to the volume of liquid displaced.

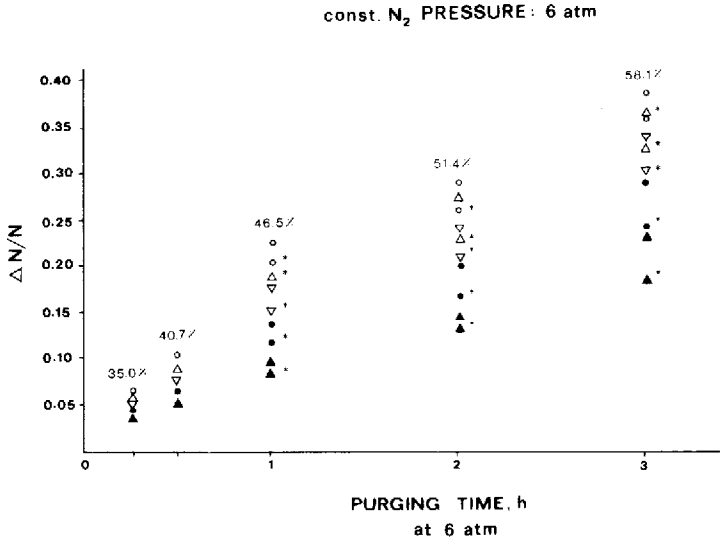

Fig. 6. Relative loss of efficiency ($\Delta N/N$) as a function of purging time at constant nitrogen flow-rate. Symbols and chromatographic conditions as in Fig. 3.

and can vary largely during short periods of time; σ_g^2 is the variance contribution while the gas stream is actually percolating the column with essentially zero liquid-flow velocity. As a first approximation σ_p^2 and σ_f^2 are neglected. The variance during most of the purging step increases by axial diffusion:

$$\sigma_g^2 = 2\gamma D_m t \tag{2}$$

where D_m is the diffusion coefficient of the solute in the mobile phase, and t is the time spent by the solute in the mobile phase:

$$t = \frac{t_g}{1 + k'} \tag{3}$$

where t_g is the duration of the gas purging operation. Since by definition the plate number is:

$$N = \frac{L}{H} = \frac{L^2}{\sigma^2} \tag{4}$$

the relative loss in efficiency is, as a first approximation

$$\frac{\Delta N}{N} = \frac{\sigma_a^2}{\sigma_c^2} - 1 = \frac{\sigma_g^2}{\sigma_c^2} \tag{5}$$

or

$$\frac{\Delta N}{N} = \frac{2\gamma D_m t_g}{(1 + k')\, L H_c} \tag{6}$$

where H_c is the plate height of the column in conventional conditions. Eqn. 6 accounts fairly well for the experimental results. An example of the agreement between the observed $\Delta N/N$ values for dodecylbenzene ($k' = 1.30$) and those calculated using eqn. 6 is shown in Fig. 4. The dotted line which represents the theoretically calculated values parallels closely the experimental curve at drying times up to 4 h, which corresponds to approximately 80 % of mobile phase displaced. Above this point there is a significant difference between the two curves, because the equation applies only as long as there is enough mobile phase in the column to allow for axial diffusion.

The difference between the results obtained under stopped-flow conditions and during gas purging of the column is small, which validates the assumption that σ_p^2 and σ_f^2 (*cf.*, eqn. 1) are small enough to be neglected as a first approximation.

It should also be observed that D_m decreases slowly with increasing molecular weight while k' increases, which slightly amplifies the overall effect.

These low-pressure results show that it is important to eliminate rapidly a large amount of solvent to "beach" the solutes on the adsorbent surface and freeze the molecular diffusion. The results obtained at high flow-rates confirm the validity of this assumption. After 4 h the loss of efficiency becomes constant, when about 80 % of the solvent has been displaced.

Two-dimensional separations

The purpose of this study was to establish the conditions for the removal of mobile phase from the chromatographic column, and to determine the extent of peak broadening which accompanied this process. The ultimate goal was to transpose these findings to two-dimensional separations.

It is evident from the HPLC results that complete and rapid removal is possible with relatively volatile phases and high flow-rates of the purging gas. The use of low

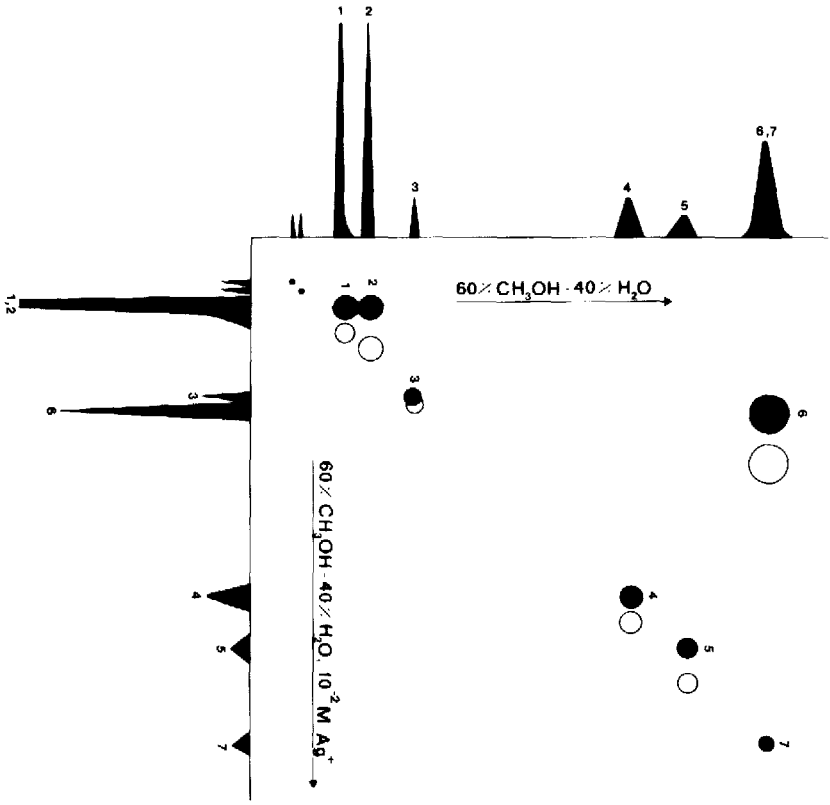

Fig. 7. Two-dimensional separation of a synthetic mixture. Solutes: 1, phenazine; 2, acridine; 3, naphthalene; 4, dibenzothiophene; 5, anthracene; 6, thianthrene; 7, *n*-butylbenzene. Column: LiChrosorb RP-8 (250 × 4.0 mm I.D., 10 μm). Solvents: (A) *x*-axis, 60% methanol–40% water, 10^{-2} *M* silver nitrate; (B) *y*-axis, 60% methanol–40% water. Flow rate: 1 ml/min; temperature: ambiant. ●, Solute positions when the column is completely equilibrated with solvent A or B and the two analyses are carried out independently of each other; ○, actual solute position when two column volumes of solvent B are passed through the column, followed by a 2 h purge with nitrogen (45% of column mobile phase displaced), and second elution with solvent A.

nitrogen flow-rates is impractical as, with short purging times, an appreciable amount of mobile phase will be left behind (which may cause problems particularly if the solvents employed in the two directions are immiscible, owing to the formation of droplets); with long purging times, the extent of peak broadening may degrade the column performance.

An example of a two-dimensional separation of a synthetic mixture of polyaromatic hydrocarbons and some sulphur- and nitrogen-containing aromatics is shown in Fig. 7. The black chromatograms on the axes refer to the conditions of complete equilibration of the column with the respective solvents. The black circles represent the solute positions which would be obtained if the column equilibration were instantaneous when switching from one solvent to the other. The open circles, however, show the actual spot position obtained by passing two column volumes of solvent (B), followed by 2-h purging with nitrogen at 6 atm (45% mobile phase

removed), and subsequent elution with solvent A. It is evident that the column was not completely equilibrated with Ag^+ ions, as the open circles are displaced with respect to the black circles. It should also be noted that, because of the complete miscibility of the two solvents used in this instance, a separation analogous to that indicated by the open circles could be obtained by sequential elutions with the two solvents, without the intermediate solvent removal. However, this is just one example, and the situation will not be analogous when partially or completely immiscible phases are used. In these instances it is critically important that the first solvent be removed as efficiently as possible with minimal band broadening.

It is also interesting that a separation of polynuclear aromatic hydrocarbons was attempted using a silica column and methanol and hexane as the solvents in the first and second directions, respectively. Although the elimination of methanol could be achieved rapidly and efficiently and hexane could be used in the second direction without interferences, it was observed that the displacement of the first solvent activated the support. Thus, the retention times increased in proportion to the purging time. This means that in TDTLC using two different phases (one of which is silica), the separation in the first direction must be carried out on silica in order to avoid activation of this support on drying.

CONCLUSIONS

Two-dimensional liquid chromatography offers attractive possibilities for difficult separations. If the solutes of interest can be separated by two different mechanisms, great control of selectivities can be achieved. This usually entails the use of partially miscible or immiscible mobile phases, which necessitates the removal of the first solvent from the column prior to the second development. This can be achieved by purging the column with an inert gas, such as nitrogen. This process gives rise to band broadening due to axial diffusion in the remaining mobile phase. For a given column, the magnitude of the loss of efficiency is directly proportional to the purging time and the diffusion coefficients of the solutes, and inversely proportional to $1 + k'$. Thus, it is desirable to displace the liquid rapidly and completely. On the basis of results obtained at 6 and 26 atm, one can predict that a nitrogen pressure of approximately 100 atm would permit very rapid solvent elimination, particularly if the more volatile solvent is used in the first direction. This does not require any particular modifications of the purging system described. In view of the considerably lower mass flow of gases compared with liquids, the column performance should not be affected by the nitrogen impact.

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