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Effect of the sample solvent on band profiles in preparative liquid chromatography using non-aqueous reversed-phase high-performance liquid chromatography

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ABSTRACT

If a solvent with a higher elution strength than the mobile phase is used to dissolve poorly soluble samples in preparative chromatography, significant deformation of the band profiles occurs, especially when the column is overloaded. Eventually, band splitting may take place. This behavior was observed in non-aqueous reversed-phase chromatography of cholesterol and other low-polarity sample compounds dissolved in a non-polar solvent, such as dichloromethane, at concentrations exceeding their solubilities in the mobile phase, a higher polarity solvent such as acetonitrile. In this chromatographic system, the dependence of the band deformation and of its splitting on the volume and concentration of the sample, the composition of the mobile phase and the column temperature were investigated. A model taking into account the dependence of the solubility of the sample components in the sample solvent and in the eluent and of the equilibrium isotherm of these components between the stationary and mobile phases on the composition of these two solvents (eluent and sample solvent) was worked out. The possible formation of supersaturated solutions was also considered in the model. This model permits the computer simulation of the band profiles for a single compound or for mixtures dissolved in a solvent different from the mobile phase. The results of the simulations are in qualitative agreement with the behavior observed experimentally.

INTRODUCTION

The main objective of optimization in preparative liquid chromatography is to obtain the lowest production cost. Academics, who lack reliable data for cost estimates, settle for finding the conditions permitting the maximum throughput of the purified material [1]. For this latter purpose, the sample load should be large. This may be difficult if the sample is not well soluble in the mobile phase. Two procedures are often used in an attempt to overcome this problem, the so-called "dry injection" of a solid sample [2] or the dissolution of the sample in another solvent in which it is more soluble than in the mobile phase. In the latter instance, great caution must be exercised to avoid the use of a solvent stronger than the mobile phase.

Very hydrophobic compounds, insoluble in water and poorly soluble in polar organic solvents such as methanol or acetonitrile, can be separated on alumina or silica, which are the preferred stationary phases for preparative applications involving these compounds. Mobile phases of low polarity must be used to achieve an adequate retention for a successful separation or purification. Partly water-saturated aliphatic hydrocarbons or dichloromethane are suitable for this purpose, but it is difficult to maintain good reproducibility in these chromatographic systems unless the trace concentrations of water in the solvents are carefully controlled, such as by using a "moisture control system" [3], a procedure which could be difficult and costly to use under

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preparative conditions. An aliphatic hydrocarbon containing 0.01–1% of a polar solvent such as propanol can be used as the mobile phase, but then the solvents used for the preparation of the mobile phase should be carefully dried to achieve good reproducibility [4,5].

The problems encountered with the control of the water content in normal-phase systems have led to attempts to separate strongly hydrophobic compounds by reversed-phase chromatography. In contrast to the polar adsorbents, the retention of these compounds on C_{18} or C_8 chemically bonded phases is large and non-aqueous eluents such as pure acetonitrile or its mixtures with chloroform, dichloromethane or tetrahydrofuran should be used to accomplish the elution in an acceptable time. For example, non-aqueous reversed-phase (NARP) systems [6] have been applied to the separations of fats [6], carotenoids [7] and sterols [8]. Under such conditions, homologs or isomers of the hydrocarbon groups may be much better resolved than on silica, which is another incentive to use NARP in preparative separations. The solubility of hydrophobic compounds in the mobile phase required for an adequate retention and a good resolution in non-aqueous reversed-phase chromatography is usually poor.

This problem is minor in analytical applications and NARP has become a popular approach. In preparative chromatography, however, mass overload of the column cannot be achieved without a high degree of volume overload when the sample is dissolved in the mobile phase. This does not permit a high production rate. Gradient elution in non-aqueous reversed-phase chromatography cannot be expected to resolve the solubility problem, as the solubility of hydrophobic samples is much lower in the weaker, starting eluent than in the stronger eluent used to generate the gradient or even than the eluent used for the isochronic, isocratic analysis.

Nevertheless, attempts are often made to overcome the limitation due to the low solubility of the sample in the eluent by using a stronger, low-polarity solvent to dissolve the sample. Considerable success has been achieved in the trace analysis of aqueous samples by reversed-phase chromatography by using the opposite approach, a mobile phase stronger than the sample solvent. Large volumes of the samples can be separated without a significant increase in band broadening because of the strong concentrating effect referred to as the sample focusing effect. Although the use of a sample solvent stronger than the mobile phase is not recommended, it usually causes only slight problems in analytical high-performance liquid chromatography (HPLC), where small sample amounts are injected. In preparative chromatography, on the other hand, the chromatograms obtained under these conditions exhibit often band profiles which are considerably deformed in unusual ways and sometimes split peaks appear [7,9]. This phenomenon leads to important separation difficulties and to a dramatic decrease in production rate [9].

In this paper, we report on an experimental and theoretical investigation of column overloading in non-aqueous reversed-phase chromatography, using cholesterol as a sample dissolved in mixtures of dichloromethane and acetonitrile.

THEORETICAL

From the theoretical point of view, the problem studied involves the simultaneous injection of a pulse of the component of interest (cholesterol) and of a pulse of the non-retained sample solvent (dichloromethane) in the mobile phase (acetonitrile). The dichloromethane band moves along the column at the mobile phase velocity, it broadens because of axial dispersion and of the finite rate of diffusion in the mobile phase and it dilutes. The profile of this band is easy to calculate at all times. The behavior of the cholesterol pulse is more complex because the presence of dichloromethane in the mobile phase changes greatly the local retention and solubility of cholesterol. Both the cholesterol solubility and its isotherm depend strongly on the dichloromethane concentration in the mobile phase. Thus, both are functions of the distance migrated by the solute along the column and of time. The effects of this dependence must be accounted for.

The semi-ideal model of non-linear chromatography was used for the calculation of band profiles [10–13]. This model assumes finite mass-transfer kinetics with rate constants and dispersion coefficients which are independent of the solute concentration in the mobile phase. It is expressed by a

system of partial differential equations, stating the mass balance of each sample component involved except the weak solvent, and of algebraic equations, stating the competitive isotherms of these components. This system of equations can be solved by numerical calculations.

The isotherm relates the concentrations of cholesterol in both phases at equilibrium. In the problem under investigation, the isotherm depends on the local composition of the mobile phase. The solute is introduced into the column with a pulse of the sample solvent (dichloromethane) which progressively dilutes and spreads in the mobile phase (acetonitrile); the pulse of the non-retained dichloromethane moves faster than the pulse of cholesterol. Accordingly, the isotherm changes as the environment of cholesterol is being changed from pure dichloromethane to pure acetonitrile. In contrast to the classical problem of isocratic elution, the isotherm coefficients are a function of the time and the position in the column, as during a step gradient elution. The original computer program [10,13] was adapted to take into account this dependence.

A further modification was needed to account for the fact that the solubility of the component studied decreases rapidly with decreasing dichloromethane concentration. A limiting condition was introduced in the calculation algorithm to prevent the propagation from one column space element (plate) to the next of an amount of the solute which would exceed the amount permitted by its solubility in the local mobile phase. In the last set of simulations, the calculation algorithm was further refined to take into account the possible formation of supersaturated solutions of the sample solutes, as will be discussed later.

EXPERIMENTAL

Instrumentation

An HP 1090M liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA), equipped with a 3DR solvent-delivery system, an automatic sample injector with a 250-μl sample loop, a temperature-controlled column compartment, a diode-array UV detector and a data work-station, was used both to record the band profiles of large-size samples and to acquire the data necessary for the determination of the equilibrium isotherms.

Column

We used Nucleosil 500-C_{18}, a wide-pore, spherical C_{18}-bonded silica, with an average particle size of 7 μm, available from Macherey-Nagel (Düren, Germany). The specific surface area of the support is 35 m^2/g (average pore diameter 50 nm). This material was packed in the laboratory into a stainless-steel column (250 mm × 4.6 mm I.D.) using a high-pressure slurry technique.

The column efficiency was 17 000 theoretical plates for benzene and *n*-butylbenzene as the test solutes in 100% acetonitrile at 35°C and 1 ml/min ($h = 2.1$). The column dead volume measured with acetone as the unretained compound was V_M = 3.174 ml in acetonitrile containing 0–20% of dichloromethane as the mobile phase. The total column porosity was $\varepsilon_T = 0.764$ and the phase ratio $V_S/V_M = 0.308$ (V_S is the volume of the stationary phase in the column).

Mobile phase and solutes

Spectroscopic-grade acetonitrile and dichloromethane (Burdick & Jackson, Muskegon, MI, USA) were filtered through a 0.45-μm filter (Millipore, Milford, MA, USA) and used to prepare the mobile phase by mixing in various ratios from 100:0 to 20:80. The mobile phase was de-gassed continuously in the liquid chromatograph by stripping with helium. The flow-rate was 1 ml/min in all experiments. Cholesterol (99% grade; Sigma, St. Louis, MO, USA) and tridecylbenzene (99%; Aldrich, Milwaukee, WI, USA) were used as the sample solutes.

Measurement of experimental band profiles

Samples of 5–250 μl of 32.3 and 70.8 g/l cholesterol solutions and of 91.4 g/l tridecylbenzene solution in dichloromethane were injected onto the column using the automatic sample injector. Acetonitrile and 5% and 10% solutions of dichloromethane in acetonitrile were used as the mobile phases at constant temperature (35, 40 or 45 ± 1°C).

Determination of equilibrium isotherms

The equilibrium isotherms were measured using the frontal analysis method as described previously

[12]. The mobile phase (0–80% solutions of dichloromethane in acetonitrile) was stored in one of the solvent flasks of the solvent-delivery system and a solution of cholesterol in the same mobile phase in another flask. The gradient-delivery system was used to mix and pump the solutions needed for the frontal analysis experiments. Caution was paid that the concentration of cholesterol in these solutions, C_m, was always at least 10% below the solubility limit in a given mobile phase, to avoid possible precipitation of cholesterol in the instrument lines (Table I).

The flow-rate ratio of the liquids pumped from the two flasks, which controls the concentration of cholesterol delivered continuously to the column, was changed from 0 to 100% in 10% steps. Time was allowed for the stabilization of the detector signal, recorded at 215 nm, after each concentration change. The flow-rate (1 ml/min) and the temperature (35°C) were kept constant during all the experiments.

In each experiment, the concentration of cholesterol in the stationary phase, q_i, was determined from the integral mass balance equation, using the experimental retention volume (inflection point of the breakthrough curve), corrected for the volu of the tubing between the mixing point of the liqu pumped in each channel and the top of the colu as described previously in more detail [12].

TABLE I

SOLUBILITY AND ISOTHERM PARAMETERS OF CHOLESTEROL

ϕ = Concentration (%, v/v) of dichloromethane in acetonitrile; x_s = solubility (g/l) of cholesterol; c_m = concentration (g/l) of cholesterol solutions used for frontal analysis measurements (see procedure under Experimental); a_1, a_2, b = best values of the parameters of the isotherm obtained by fitting the experimental data to eqn. 3. Column: 250 × 4.6 mm I.D. Nucleosil 500-C_{18} (7 μm); 35°C.

ϕ	x_s	c_m	a_1	a_2	b (l/g)
0	0.872	0.728	6.958	13.924	11.645
5	1.582	1.372	5.166	9.922	7.447
10	2.241	2.097	4.259	7.558	4.472
20	3.932	3.285	2.638	3.624	0.781
30	6.794	5.822	1.617	1.762	0.433
40		10.106	1.055	0.963	0.246
50		15.72	0.690	0.452	0.127
60		25.31	0.572	0.237	0.162
80		34.71	0.338	0.141	0.130
100	>160	–	0.2[a]	0.1[a]	0.13[a]

[a] Values extrapolated from eqns. 4–6.

Determination of solubility of cholesterol

An excess of cholesterol was stirred with a mixt of acetonitrile and dichloromethane in an ultrasc bath for 10 min and allowed to stand overnight 20-μl sample of the supernatant saturated solut was analyzed, using pure acetonitrile as the mo phase, at 1 ml/min and 35°C. The solubility cholesterol in the saturated solution, x_s, was e uated from the results of three repeated analy using a calibration graph based on the integra peak areas (Table I).

Computer

Computations were performed on a VAX 8 computer (Digital Equipment, Marlboro, M USA) at the Computer Center of the University Tennessee. Because the computation time needec calculate a band profile is proportional to the squ of the plate number of the column [10–12] and ta several hours for a 17 000-plate column, an e ciency of only 2000–5000 theoretical plates v considered in most of these calculations.

RESULTS AND DISCUSSION

Experimental band profiles

If cholesterol is injected as a very dilute solutio pure acetonitrile as the mobile phase, its capac factor is 6.4. When larger samples are injected, band becomes asymmetric and its shape becor progressively unusual.

Figs. 1 and 2 show the experimental band prof observed at 35°C for 5 250-μl samples of 3 (Fig. 1) and 70.8 g/l (Fig. 2) solutions of choleste dissolved in pure dichloromethane, with 10(acetonitrile as the mobile phase. These concent tions are respectively 37 times and 81 times hig than the solubility of cholesterol in the pure ace nitrile reported in Table I.

In both figures and for a sample size of 5 (profiles 1), the cholesterol band exhibits the typi shape, with a sharp front and tailing rear end, wh correspond to column overloading with a com nent having a convex upward isotherm. For lar

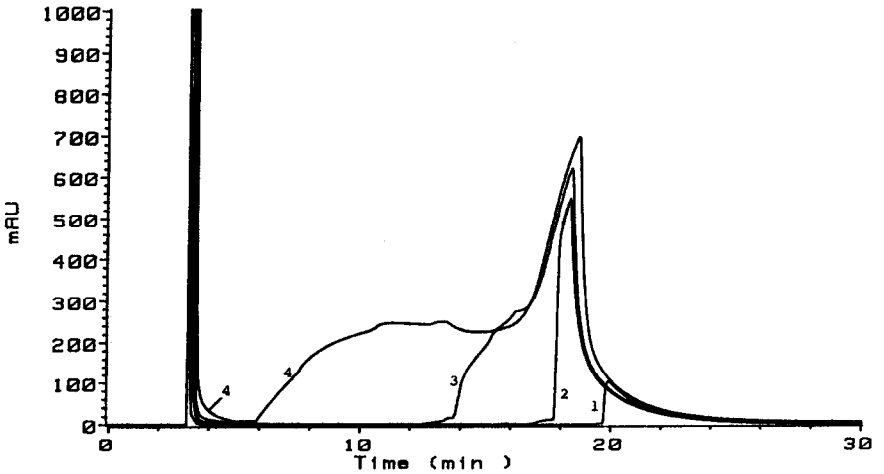

Fig. 1. Experimental band profiles of samples of cholesterol dissolved in dichloromethane. Column, 250 × 4.6 mm I.D., Nucleosil 500-C_{18} (7 μm); mobile phase, acetonitrile; temperature, 35°C; mobile phase flow-rate, 1 ml/min; detection, UV at 215 nm; sample concentration, 32.3 g/l of cholesterol. Sample volume: 1 = 5; 2 = 20; 3 = 50; 4 = 100 μl.

sample sizes, the profile becomes different. The front shock layer does not extend from the baseline to the band maximum, but the top of the band front is slanted (profiles 2 in both figures). When the sample size is increased further (profiles 3 in both figures), a hump appears, preceding the band maximum. With increasing sample sizes (profiles 4 in both figures), this hump grows and appears earlier and earlier. The retention time of the peak maximum is nearly constant in Fig. 1 (profiles 2–4); it increases slowly with increasing sample size in Fig. 2 (profiles 2–5). From the detector calibration graph we calculated that the maximum band height in Figs. 1 and 2 corresponds to *ca.* 80% of the cholesterol solubility in dichloromethane.

This behavior contrasts with that observed previ-

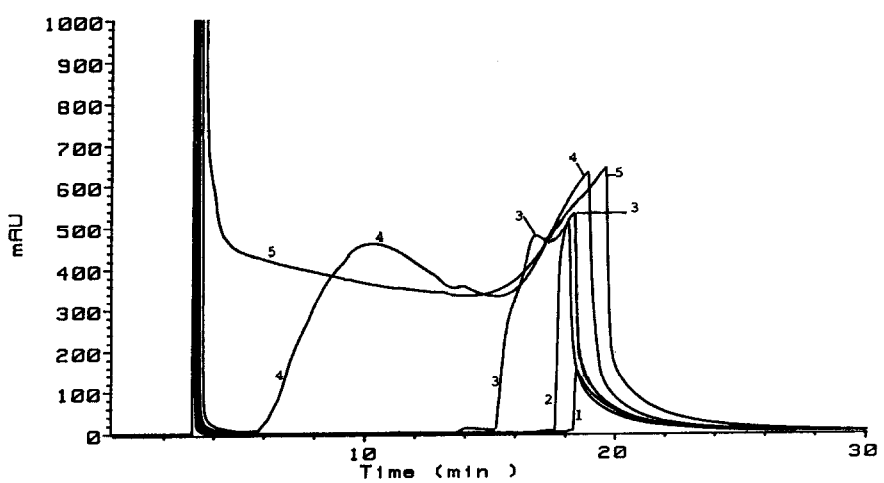

Fig. 2. Experimental band profiles of samples of cholesterol dissolved in dichloromethane. Conditions as in Fig. 1, except cholesterol concentration, 70.8 g/l, and sample volume: 1 = 5; 2 = 10; 3 = 30; 4 = 100; 5 = 250 μl.

TABLE II

HEIGHTS OF THE "DEAD VOLUME" PEAK

c_s = Concentration (g/l) of cholesterol in dichloromethane as the sample solvent; h_5–h_{100} = heights of the "dead volume" peak (in milliabsorbance units) in the chromatograms of samples with volumes of 5, 20, 30, 50 and 100 μl, respectively (see Figs. 1 and 2).

c_s	h_5	h_{20}	h_{30}	h_{50}	h_{100}
32.3	800	1700	1870	1950	2050
70.8	640	1450	1750	2050	2500

ously [13,14]. The most striking characteristic of these chromatograms, however, is their second mode, a feature which is exceedingly rare in chromatography. With profile 2 in both figures, a second peak appears at the column dead volume. Its relative importance increases with increasing sample size. The hump of the main band merges eventually with the dragging tail of the dead volume peak at large sample sizes (profile 5 in Fig. 2).

Dichloromethane is not retained and is eluted at the column dead volume. However, the first peak, eluted at the dead volume, cannot be attributed to this solvent alone. The presence of cholesterol in the "dead volume" peak is proven by the following observations: (a) the UV spectra recorded for this peak are very similar to the spectrum of cholesterol in dichloromethane; (b) the "dead volume" peak increases with increasing concentration of cholesterol in a sample of constant volume (50 or 100 μl) whereas the concentration of dichloromethane in the sample actually decreases, in contrast to lower sample volumes where only dichloromethane with no cholesterol is present in the "dead volume" peak (Table II); (c) re-injection of a fraction collected during the elution of the "dead volume" peak gives a peak at the cholesterol time.

Similar behavior was observed with samples of solutions of tridecylbenzene in dichloromethane (Fig. 3). The nearly symmetrical peak obtained for the low-volume samples (profiles 1 and 2) indicates that the sorption isotherm is almost linear in this size range and suggests that band deformation and splitting may occur even under conditions where the column is not overloaded. Most probably the tridecylbenzene contains some isomeric and homologous impurities to which the smaller peaks in the earlier part of the chromatogram can be attributed. However, as for the chromatograms in Figs. 1 and 2, when the sample size is increased, a new peak appears at the "dead volume" and a hump is observed at the front of the retained band; it grows and eventually merges with the dead volume peak, whereas the retention time of the band maximum remains nearly constant and, at very high sample sizes, begins to increase with increasing sample size.

Quantitative study of the chromatograms in Figs. 1 and 2 shows that the hump appears for a

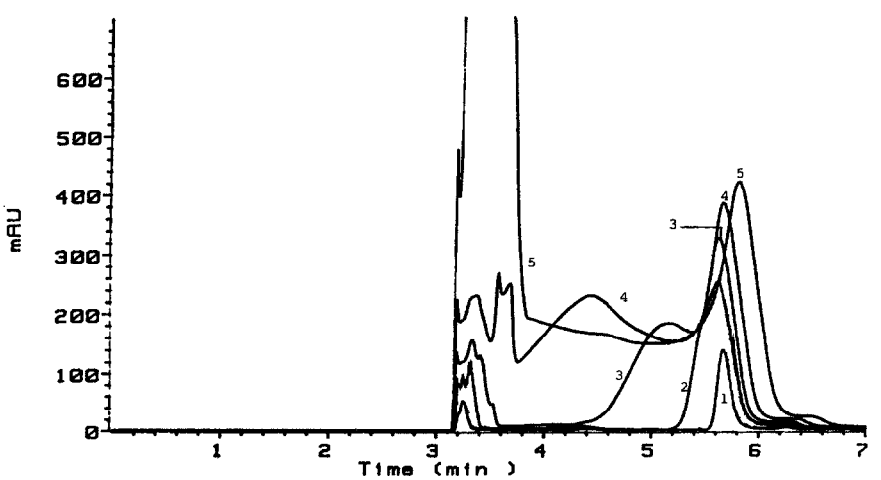

Fig. 3. Experimental band profiles of samples of tridecylbenzene dissolved in dichloromethane. Conditions as in Fig. 1, except sample concentration, 91.4 g/l, detection wavelength, 230 nm, and sample volume: 1 = 5; 2 = 20; 3 = 50; 4 = 100; 5 = 250 μl.

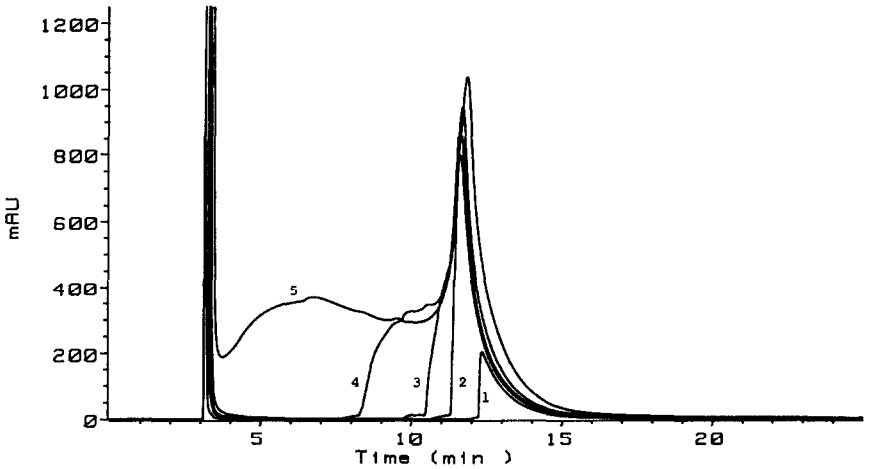

Fig. 4. Experimental band profiles of samples of cholesterol dissolved in dichloromethane. Conditions as in Fig. 1 (including sample volumes), except temperature, 45°C.

sample volume of 50 μl with the 32.3 g/l cholesterol solution (Fig. 1, profile 3) and for a sample volume of 30 μl with the 70.8 g/l solutions (Fig. 2, profile 3), corresponding to sample amounts of 1.5 and 2.1 mg, respectively. The behavior of the front of the retained band ceases to be normal when the sample exceeds *ca.* 0.7 mg. However, the amount of cholesterol injected does not control the phenomenon entirely. The amount of cholesterol for which splitting and band deformation begin to occur increases with decreasing sample concentration.

To investigate further the effect of the experimental conditions, band profiles of samples of increasing volume of a 32.3 g/l cholesterol solution in dichloromethane were recorded at 40 and 45°C (Fig. 4) with pure acetonitrile as the mobile phase, and at 35°C (Fig. 5) with 5% and 10% dichloromethane solutions in acetonitrile as the mobile

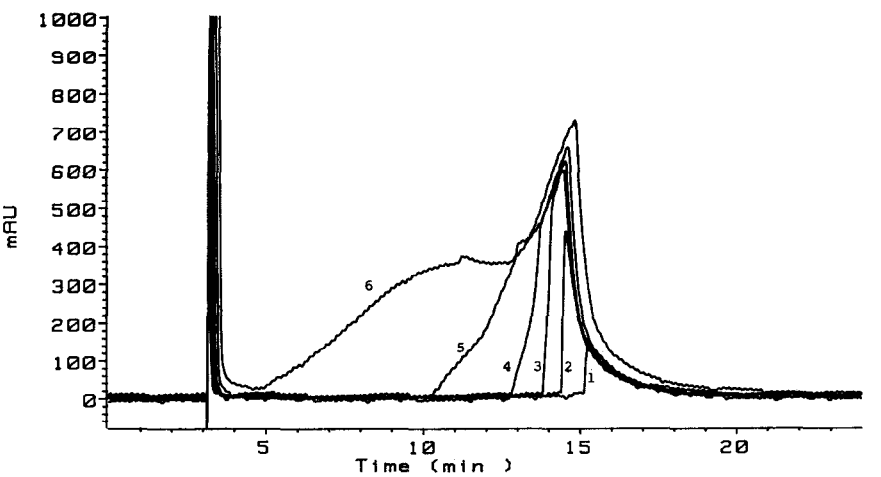

Fig. 5. Experimental band profiles of samples of cholesterol dissolved in dichloromethane. Conditions as in Fig. 1 (including sample volumes), except the mobile phase, acetonitrile–dichloromethane (95:5).

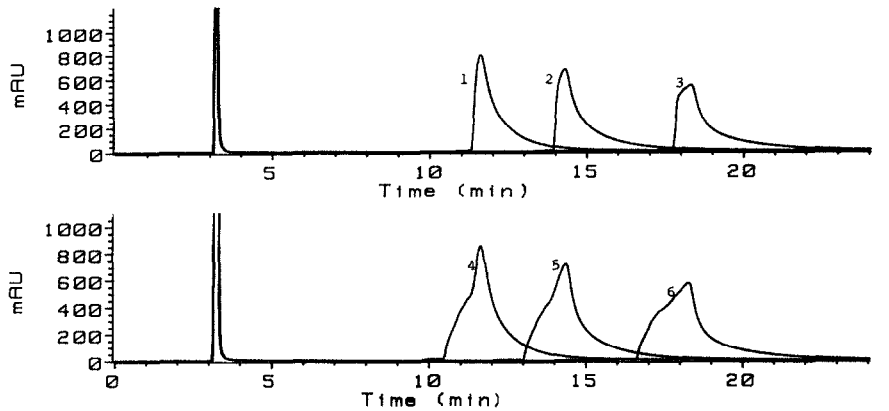

Fig. 6. Experimental band profiles of samples of cholesterol dissolved in dichloromethane. Conditions as in Fig. 1. Band profiles of 20 μl (top, 1–3) and 30 μl (bottom, 4–6) samples of a 32.3 g/l cholesterol solution, measured at (1, 4) 45°C, (2, 5) 40°C and (3, 6) 35°C.

phase. Increasing the temperature or the concentration of dichloromethane results in an increase in the cholesterol solubility and a decrease in the retention times. The aforementioned behavior of the band profiles is still observed, however. Fig. 6 shows the band profiles for the same sample sizes at 35, 40 and 45°C and Fig. 5 compares the band profiles in acetonitrile containing 0, 5 and 10% dichloromethane. The profiles in Figs. 4 and 5 show the same trends as those in Figs. 1 and 2. The profiles in Figs. 6 and 7 show that, at constant sample size, the intensity of the band deformation decreases slightly with increasing temperature and increasing dichloromethane concentration.

The phenomena observed cannot be explained by the appearance of system peaks, because pure acetonitrile was used in most experiments reported here and because dichloromethane is not retained under the chromatographic conditions used. The probable cause of these results is the variation of the solubility of cholesterol in the mobile phase during the migration of the dichloromethane band, as already suggested by Khachik *et al.* [7] to explain similar observations. In this instance, the band

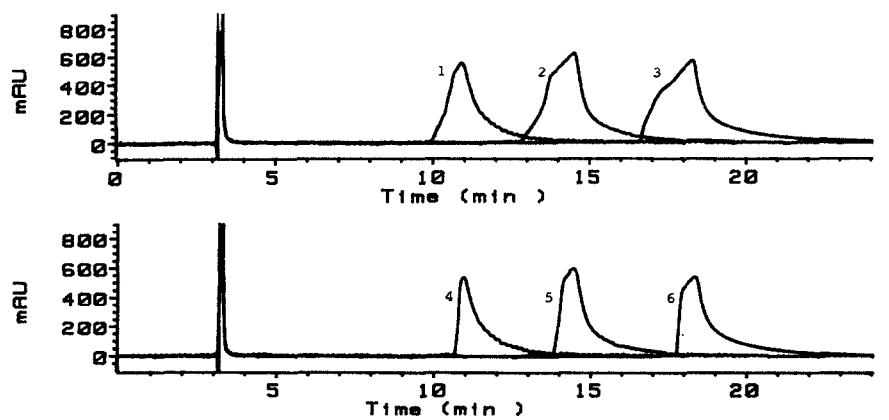

Fig. 7. Experimental band profiles of samples of cholesterol dissolved in dichloromethane. Conditions as in Fig. 1. Band profiles of 30 μl (top, 1–3) and 20 μl (bottom, 4–6) samples of a 32.3 g/l cholesterol solution, eluted with acetonitrile containing (1, 4) 10%, (2, 5) 5% and (3, 6) no dichloromethane.

profile could depend on the shape of the sorption isotherm, the solubility of the sample in the solvent mixtures, the exact injection profile of the sample and the possible formation of supersaturated solutions close to the solubility limits. The formation of supersaturated solutions of cholesterol in the solvents used was observed during the study.

These factors are taken into account in an approximate model which can be used for simulation of the behavior observed. First, an appropriate description of the experimental isotherms is needed.

Equilibrium isotherms

The equilibrium isotherms of cholesterol were measured in various mobile phases containing 0–80% dichloromethane in acetonitrile, in order to collect the data necessary to perform the calculations. As an example, the experimental data points are reported in Fig. 8 (symbols) for pure acetonitrile. These points were fitted to the Langmuir isotherm equation:

$$q_i = \frac{ac_i}{1 + bc_i} \tag{1}$$

where q_i and c_i are the stationary and mobile phase concentrations, respectively, and a and b are numerical parameters. The result was not satisfactory (Table III). An attempt to fit these data to the bi-Langmuir isotherm:

$$q_i = \frac{a_1c_i}{1 + b_1c_i} + \frac{a_2c_i}{1 + b_2c_i} \tag{2}$$

where a_1, b_1, a_2 and b_2 are numerical parameters, was also unsuccessful. Small and sometimes negative values of b_2 were obtained.

As the plots of the experimental isotherms appeared to be nearly linear in the high-concentration range, the data were fitted to the sum of a Langmuir and a linear isotherm, *i.e.*, a bi-Langmuir isotherm with the parameter $b_2 = 0$:

$$q_i = \frac{a_1c_i}{1 + bc_i} + a_2c_i \tag{3}$$

This last isotherm gives values of q_i which are in much better agreement with the experimental results

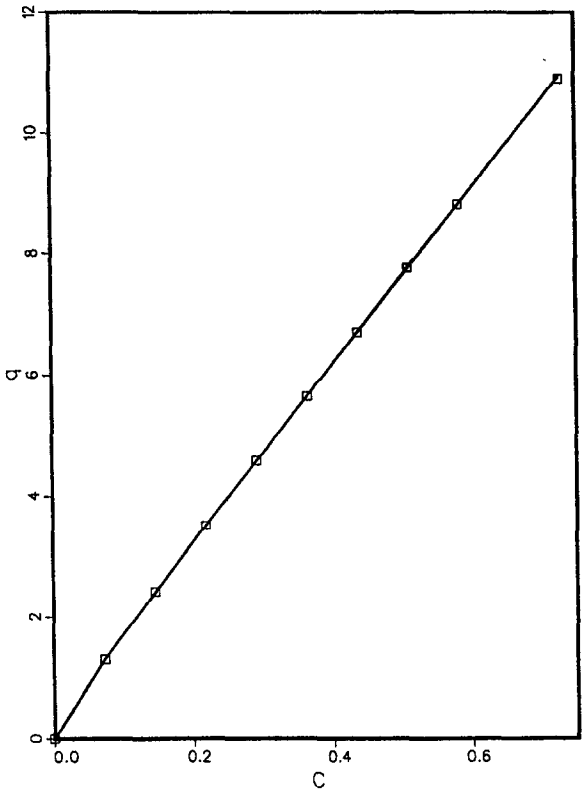

Fig. 8. Experimental isotherm of cholesterol in acetonitrile at 35°C. Symbols, data points by frontal analysis. Solid line, best fit to eqn. 3 (parameters a_1, a_2, b in Table I). c and q = concentrations (g/l) of cholesterol in the mobile and stationary phase, respectively.

TABLE III

EXPERIMENTAL AND PREDICTED CONCENTRATIONS OF CHOLESTEROL IN THE STATIONARY PHASE

c_s = Concentration (g/l) of cholesterol in acetonitrile as the mobile phase; q_i (E) = concentration (g/l) of cholesterol in the stationary phase determined experimentally by frontal analysis; q_i (C-1) = concentration (g/l) of cholesterol in the stationary phase calculated from the eqn. 1 (Langmuir isotherm); q_i (C-3) = concentration (g/l) of cholesterol in the stationary phase calculated from the eqn. 3 (combined isotherm). Column and temperature as in Table I.

c_s	q_i (E)	q_i (C-1)	q_i (C-3)
0.0728	1.296	1.234	1.298
0.1456	2.426	2.425	2.424
0.2183	3.514	3.574	3.511
0.2911	4.583	4.685	4.583
0.3639	5.645	5.759	5.648
0.4366	6.706	6.798	6.709
0.5094	7.766	7.803	7.767
0.5822	8.826	8.776	8.823
0.7278	10.898	10.633	10.932

than the first isotherm (eqn. 1), as shown in Table III. Deviations between the experimental and calculated values of the amount adsorbed at equilibrium are always less than 0.003 g/l of packing material with eqn. 3 whereas they exceed 0.1 g/l with eqn. 1. The same results were obtained for all the concentrations of dichloromethane used. The composite isotherm (eqn. 3) fitted the data very well.

The best values of a_1, a_2 and b in the equilibrium isotherms obtained for the different mobile phase compositions at which measurements were carried out are reported in Table I. The linear regression of the logarithms of the experimental parameters a_1, a_2 and b on the dichloromethane concentration, ϕ, yielded the following equations which account well for the mobile phase dependence of these parameters:

$$\log a_1 = 0.770 - 0.02056\,\phi \quad (4)$$

$$\log a_2 = 1.096 - 0.03145\,\phi \quad (5)$$

$$\log b = 0.748 - 0.04491\,\phi \quad (6)$$

Thus, a general isotherm (eqn. 3) valid over a wide range of mobile phase compositions can be formulated.

Because of too low a cholesterol retention, it was impossible to determine accurately the isotherm parameters in acetonitrile solutions with more than 80% of dichloromethane. The values of a_1, a_2 and b in pure dichloromethane necessary for the calculations discussed later were derived by extrapolation from the data measured at lower dichloromethane concentrations (see Table I).

We have at present no justification for the isotherm used, except that it fits the whole set of data extremely well. It also accounts well for the band profiles observed, which are controlled by the Langmuir term, dominant at low concentrations in spite of the little deviation observed from linear behavior [10]. The properties of the chromatographic system and of the sample solute could explain the isotherm: a bimodal surface energy distribution, a mixed retention mechanism or an accidental compensation between effects leading to the curvature of the isotherm in opposite directions (*e.g.*, surface saturation and adsorbate–adsorbate interactions). Some features of the chromatograms in Fig. 2, such as the very steep rear part of the profile, could suggest that the isotherm has an inflection point at high concentrations. For this reason, we carried out the isotherm measurements in the whole range of concentrations accessible, up to 90% of the mobile phase saturation at all compositions of this phase. Although we cannot rule out an inflection, and especially not one in the supersaturated range, we have found none. Further, measurements are not possible in the supersaturated range, even if such solutions may have a transitory existence in the column.

A calculation based on the molecular weight of cholesterol, 386.7, on the assumption of spherical molecules, on the specific surface area of the silica used (35 m^2/g), the packing density of the column (0.5 g/ml) and the volume of octadecylsilica (0.979 ml) gives an estimate of the monomolecular layer capacity of the column for cholesterol, 42 mg. Using the isotherm (eqn. 3) and the appropriate parameters from Table I, it is found that this amount would be adsorbed at equilibrium with a 3 g/l solution of cholesterol in acetonitrile, which is approximately three times the solubility of cholesterol. Again, this concentration exceeds considerably the limits within which measurements are possible.

Simulation calculations of cholesterol band profiles

Not surprisingly, calculations of the band profiles of cholesterol samples performed using the classical algorithm [9–13] and the isotherm parameters measured in pure acetonitrile (Table I) give profiles (not shown) typical of a convex upward isotherm and similar to typical Langmuirian band profiles as those reported previously [12–14]. Whatever the combination of concentration and volume used, these profiles are not like those recorded during the experiments (Figs. 1–7). The agreement with experimental profiles is not improved when the algorithm is modified to take into account the dependence of the isotherm coefficients on the local composition of the mobile phase, *i.e.*, both on the time and the position of the solute in the column, but not the limited solubility of cholesterol in acetonitrile. Thus, neither the equilibrium isotherm nor the passage of the dichloromethane band can explain the anomalous band profiles reported in Figs. 1–7.

When the constraint of a limited solubility of the sample in the mobile phase was included in the calculation algorithm, a completely different picture

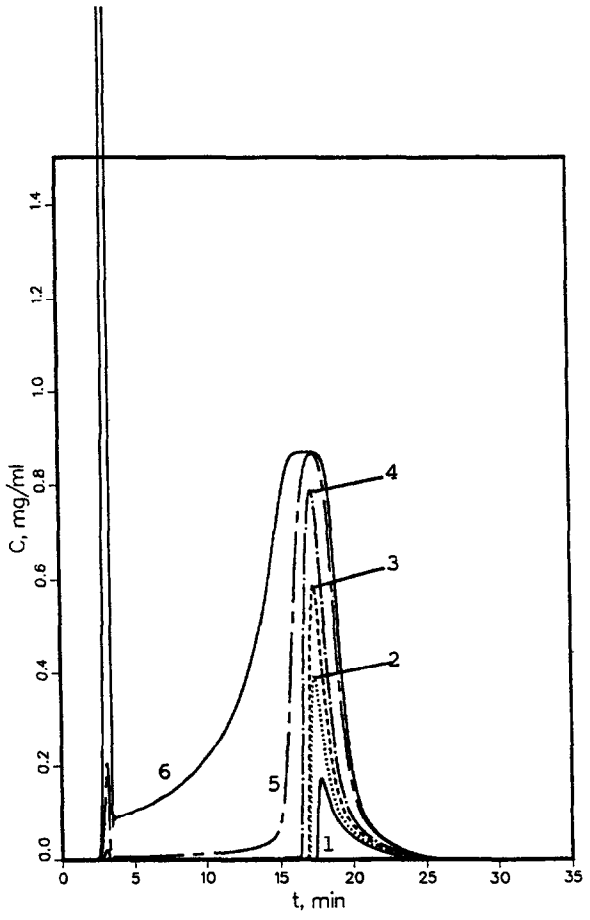

Fig. 9. Calculated band profiles of samples of cholesterol dissolved in dichloromethane. Simulated conditions as in Fig. 1; solubility and isotherm parameters as in Table I. t = Time (min); c = concentration of cholesterol (g/l). Sample volume: 1 = 10; 2 = 20; 3 = 30; 4 = 50; 5 = 100; 6 = 250 μl.

arose. Fig. 9 shows the band profiles calculated for 10–250-μl samples of a 32.3 g/l solution of cholesterol in pure dichloromethane, eluted with pure acetonitrile. These calculations were made assuming a rectangular profile of the sample plug. Some important similarities are apparent between these calculated profiles and the experimental band profiles reported in Figs. 1 and 2.

First, the calculated chromatograms corresponding to large size samples show a split peak, the second mode appearing at the column dead volume. This effect is first observed for a sample volume of 50 μl (profile 4); it increases with increasing sample volume. Second, the retention time of the band maximum remains constant. Finally, when the sample size increases, the front of the main mode band becomes increasingly skewed and eventually merges with the dragging tail of the "dead volume" peak (profiles 5 and 6). On the other hand, two major features of the calculated and experimental bands differ. The experimental bands do not exhibit a concentration plateau; the progressive formation of the hump on the band front and its profile are not correctly simulated.

Extra-column contributions affect both the retention times and the band profiles in preparative chromatography [15]. To investigate the possible influence of an actual injection profile differing markedly from a rectangular plug, a profile composed of a vertical front and an exponential rear was used in calculations. This profile describes better the actual sample pulse. However, the results of the calculations were not significantly changed, even for large sample volumes.

Finally, the formation of supersaturated solutions of cholesterol observed during the solubility measurements was taken into account as a possible contribution to the deformation of the retained band. The sample solution may become supersaturated as it starts to mix with the mobile phase before sorption equilibrium is attained. However, once the sorption equilibrium has been reached, a fresh portion of the mobile phase coming into contact with the solute in the stationary phase will not accommodate a concentration of the solute higher than the saturation. To take into account this phenomenon, we assumed in the calculation algorithm that the propagation of the sample from one column element to the next is possible up to a certain "supersaturated" solubility, higher than the experimental saturation on the band front. Below that supersaturation, we used the extrapolated isotherm. Above it, we assume precipitation in the column element. On the other hand, the solubility was set as the limiting concentration in the mobile phase for the adsorption equilibrium, on the band rear.

The supersaturated solubility cannot be measured. It was set arbitrarily at 150% of the solubility in the saturated solution. Fig. 10 shows simulated chromatograms for 20–500-μl samples of a 70 g/l solution of cholesterol in dichloromethane, with acetonitrile as the mobile phase. Only the 20-μl

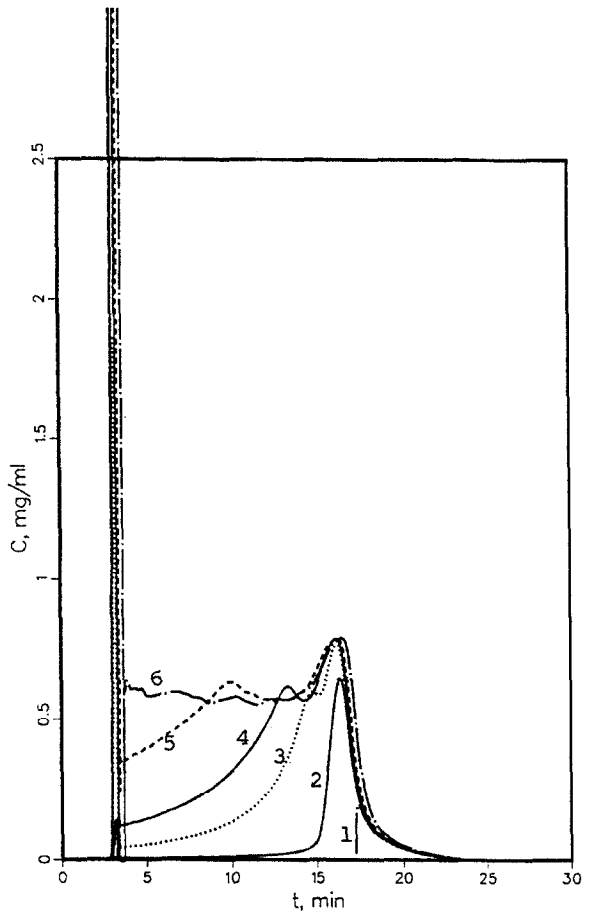

Fig. 10. Calculated band profiles of samples of cholesterol dissolved in dichloromethane. Simulated conditions as in Fig. 1; the formation of supersaturated solutions with maximum concentration equal to 150% of the experimental solubility in the mobile phase was assumed. Solubility and isotherm parameters as in Table I. t = Time (min); c = concentration of cholesterol (g/l). Sample volume: 1 = 20; 2 = 50; 3 = 100; 4 = 150; 5 = 250; 6 = 500 μl.

sample (profile 1) yielded a non-split Langmuirian band profile. All the chromatograms corresponding to larger sample volumes show a "dead volume" peak in addition to the retained band. The front of the retained peak is slanted for the 50-μl sample (profile 2), which also exhibits a shallow leader as the bands for the corresponding size samples in Figs. 1 and 2. A hump appears at the front of the peak of the 100-μl sample (profile 3). The size of this hump increases and its retention decreases with increasing volume, from 100 to 250 μl (profiles 3–5). The hump eventually merges with the rear of the "dead volume" peak for the 500-μl sample (profile 6), in good qualitative agreement with the experimental behavior.

The effect of the sample volume on the deformation of the retained bands is larger in the experimental results than predicted by the calculations. This could be attributed to the arbitrary selection of the value of the "supersaturated" solubility. The qualitative agreement of the simulations with the experiments gives some support to the formation of supersaturated solutions as the origin of the "hump" on the retained sample bands.

Mechanism of band deformation

On the basis of the results, the behavior of large samples injected as solutions in a solvent which is stronger than the mobile phase can be explained principally by their interaction with the bolus of strong solvent. In this bolus, the solubility of the sample components is considerably increased. The elution strength of the sample solvent is high. When the composition of the mobile phase forming the local environment of the sample solute changes as a result of the migration and the progressive dilution of the strong solvent bolus, the sample solubility and its retention decrease rapidly. Supersaturated solutions can form when the solubility decreases and contribute to the faster migration of part of the sample. This combination of effects causes a complex band profile.

After the injection into the mobile phase of a dichloromethane solution of cholesterol, a steep gradient of decreasing elution strength is induced by the sample itself. The wave of dichloromethane propagates along the column, carrying along part of the sample. As long as the volume of the sample is small enough, dispersion dilutes both the sample and the solvent and the whole sample amount is brought into contact with a mobile phase of lower elution strength, so that it eventually elutes in much the same way as if it was injected dissolved in the mobile phase.

When large volumes of sample solution are injected, however, the bolus of dichloromethane is wider and dilutes more slowly in the mobile phase. Part of the sample is carried along with the bolus and, as its retention in presence of pure dichloromethane is negligibly small, it is eluted with it in a

"dead volume" peak. At the rear edge of the diffusing dichloromethane band, the solubility of cholesterol drops rapidly, because acetonitrile is a poor sample solvent. A supersaturated solution of cholesterol in acetonitrile is formed first. This supersaturated solution is unstable but does not precipitate instantly. It propagates along the column, and part of this cholesterol precipitates at some distance from the top of the column, so that the local cholesterol concentration in the stationary phase becomes higher than that which corresponds to the sorption isotherm. This portion of cholesterol is subsequently re-dissolved and diluted by the fresh mobile phase, in which it migrates along the remaining part of the column. Eventually it elutes and forms a hump at the front edge of the peak of the retained portion of the cholesterol sample. Because of the dilution during the re-dissolution process, the height of the hump is lower than the height of the sharp peak eluted after the hump. This sharp peak obviously can be attributed to the band of the originally saturated solution of cholesterol in acetonitrile, formed at the top of the column and moving all the distance along the column in the mobile phase.

During the re-dissolution of the precipitated cholesterol in the mobile phase, the formation of a supersaturated sample solution is most unlikely. The part of the column in which the precipitation occurs and consequently the width of the hump increases with increasing sample size. Eventually, for large enough sizes, the precipitation and re-dissolution take place in the whole column volume, resulting in a merging of the retained band and the dead volume peak.

For very large sizes (Fig. 2), the profile of the supersaturated part of the retained sample band shows a sloping front and a steep rear end, the same profile which is expected with anti-Langmuirian isotherms. Possibly the isotherm is concave in the supersaturated concentration range, but it is just impossible to measure isotherms in supersaturated solutions as the rate of precipitation is too high and uncontrollable.

Obviously, the band splitting and deformation observed here would make it very difficult to separate and purify efficiently cholesterol from other related compounds at high sample loads in a non-aqueous reversed-phase chromatographic system. This conclusion extends to many similar systems.

CONCLUSIONS

The use of a sample solvent with a higher elution strength than the mobile phase to increase the throughput in preparative liquid chromatography of poorly soluble samples may seem a good idea but leads to serious deformations and to the splitting of the sample component bands. This phenomenon limits drastically the amount of sample which can be injected, and hence the production rate. This result is of special importance in the preparative applications of non-aqueous reversed-phase HPLC of hydrophobic compounds, such as steroids.

On the basis of the results, this behavior can be explained by the abrupt and simultaneous decrease in the sample-component solubility and the change in their equilibrium isotherms which take place when the sample components are separated from the sample solvent during the early phase of their migration along the column. The possible formation of supersaturated solutions of sample solutes in the mobile phase may contribute significantly to the band deformation and the occurrence of humps on the front of the retained sample peak. The results of the simulation calculations based on this model yielded band profiles in good qualitative agreement with the experiments. Obviously, however, this agreement is not a proof of the validity of the entire model. Some features of the elution band of cholesterol, especially its sharp rear profile, are not explained satisfactorily.

The phenomena causing the band deformation interfere with the separation process and are detrimental to the production of purified material in preparative liquid chromatography, as the solutes are spread almost over the entire column at larger sample loads [9].

For these reasons, the use of sample solvents having an elution strength much stronger than the mobile phase should be avoided in preparative chromatography, especially at high sample loads. The injection of a large volume of a saturated solution of the sample in the mobile phase seems to be the best approach to column overloading. If the maximum production rate so achieved is considered to be insufficient, another chromatographic system

in which the mobile phase is a better sample solvent should be searched for. From this point of view, in spite of its own problems, preparative normal-phase chromatography, using polar adsorbents with low-polarity mobile phases containing a small concentration of a polar organic modifier, should most often be considered as a preferred alternative to non-aqueous reversed-phase chromatography for the separation or purification of hydrophobic compounds.

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