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INFLUENCE OF THE WATER CONTENT OF THE SOLVENT ON CHROMATOGRAPHIC SEPARATIONS

LAVERNE V. BERRY and HEINZ ENGELHARDT
Angewandte Physikalische Chemie, Universität des Saarlandes, Saarbrücken (G.F.R.)
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SUMMARY

The influence of the amount of water dissolved in dichloromethane on chromatographic separations was studied. By increasing the water content of dichloromethane from 60 to 2100 ppm, the k' values decrease by over several hundred units. Peak asymmetry also decreases with increasing water content. By a continuous increase in the water content of a single solvent, an unusual "gradient elution" is achieved.

The consequences of the method described are of basic interest in conventional gradient elution in order to avoid practical problems in routine analysis.

INTRODUCTION

In liquid–solid chromatography, the water content of eluents of low or moderate polarity determines the capacity ratios (k') of the samples to be separated. It has already been pointed out[1–3] that a relationship exists between the water content in the eluent and the equilibrium amount of water on the active solid support. Recently, it has been shown[4] that by this method, for example, alumina columns can be adjusted to defined activity levels simply by changing the water content of the eluent. By increasing the amount of water (or another polar compound, *e.g.*, ethylene glycol or other alcohols) in the eluent, the amount of water (alcohol) on the adsorbent increases until all of the pores are filled with water (or with the alcohol). The chromatographic system changes continuously from an adsorption system to a more or less "pure" partition system[5]. During this period, the k' values first decrease with increase in the amount of polar liquid on the adsorbent then, after passing through a minimum, they can increase again[6]. This property can be used for an *in situ* coating procedure of pre-packed silica columns in order to achieve liquid–liquid systems[7]. It should be mentioned, however, that during this procedure the relative retentions are also changed.

This desirable but sometimes unexpected partition of a polar impurity such as water between the active solid stationary phase and an eluent that is hardly miscible with water may cause problems in different programming techniques, such as temperature programming or gradient elution. In the latter case, for example, the water partition is destroyed on passing to more polar eluents, which are miscible with water, by washing the water from the column. Returning to the initial less polar eluent, water has to be adsorbed from the now more active support in order to achieve the initial equilibrium distribution. One means of avoiding these problems is to use already pre-equilibrated solvents in the gradient device[8]. However, sometimes it is difficult to adjust the water content satisfactorily when mixtures of different solvents are used.

In a model system consisting of silica and dichloromethane as a moderately polar eluent, the influence of the water content of the eluent on the solute retention was studied. Isocratic elution with different amounts of water dissolved in the eluent was applied, as well as a "gradient elution" method with a continuously increasing water content of the mobile phase. Particular attention was paid to the study of reproducibility and column regeneration in the application of the gradient elution method.

EXPERIMENTAL

Equipment

A home-made apparatus with a pressure capability up to 250 atm, as described by Deininger and Halász[9], was used. In order to minimize the hold-up volume in the system before the column, the connections were made of 1.0-mm I.D. capillary tubing. If only one head of the pump was used, the volume could be decreased to 6.7 ml. In the gradient measurements, the flow control unit was disconnected. Pressure measurement was made outside the main flow-stream. The flow-rate was registered on the recorder trace using a siphon-type flow monitor[10]. A differential refractometer (Type R-4, Waters Ass., Milford, Mass., U.S.A., modified according to Deininger and Halász[11]) was used in series with a UV detector (Type 1205, LDC, Riviera Beach, Fla., U.S.A.). The RI detector, column and solvent reservoir were thermostatically controlled at 21.7 with a constant-temperature bath (Model NK 22, Haake, Karlsruhe, G.F.R.).

The gradient was generated on the low-pressure side by using a classical two-chamber mixing device. The second chamber was L-shaped, resulting in isocratic end elution for 150 ml with no discontinuities between the gradient and the isocratic portion of the eluent[10].

Reagents

Dichloromethane was purified and dried before use by passing it through a column (50 × 3 cm) of activated silica (Woelm, Eschwege, G.F.R.)[12]. By this method, the water content of the dichloromethane could be decreased to 19 ± 7 ppm, if the column and collecting flask were connected by glass joints and a drying tube containing silica was attached to the flask in order to exclude atmospheric moisture. Because of the hygroscopic properties of this solvent, the water content was adjusted to 60 ppm by adding an aliquot of water-saturated dichloromethane.

Water-saturated dichloromethane was prepared by vigorously stirring silica-purified dichloromethane with excess of water in a thermostatted flask. Tests showed that saturation (2100 ppm of water at 21.7) was achieved in less than 15 min.

The water content of dichloromethane was determined with an automatic Karl Fischer titrator (Metrohm AG, Herisau, Switzerland) using 10–25 ml aliquots of dichloromethane in pre-titrated methanol. The reproducibility of the water determination by Karl Fischer titration varied with the water content of the dichloromethane. The error was found to be ±1.4% at a water content of 60 ppm and ±0.5% at a water content of 2000 ppm using 10-ml aliquots each time.

Column

The column (length 52 cm, I.D. 2.1 mm) was dry-packed with Merckogel SI 200 (E. Merck, Darmstadt, G.F.R.) wet sieved to 27–32 μm. The column contained 0.9 g of the silica. As the total porosity, ε_T, can change between 0.42 and 0.84, depending on the water content of the mobile phase[7], the volumes of the eluent used to equilibrate columns are given in terms of empty (unpacked) column volumes, V_c (= 1.80 ml), and not in terms of the more common elution volume, V_0, of a non-retained component.

RESULTS AND DISCUSSION

Water content and capacity ratio

In order to compare the systems from dry dichloromethane (with a water content of 20 ppm) to water-saturated dichloromethane (with a water content of 2100 ppm), it was necessary to select samples that are not inert in the wet system and are eluted in the dry system with a finite retention volume. A further requirement, especially in the dry system, was that the sample size required in order to obtain a detectable peak should not exceed the linear region of the adsorption isotherm. Three steroid compounds (cholest-4-en-3-one, progesterone and testosterone) gave satisfactory results. The maximum sample size for the most highly retained compound (testosterone) never exceeded $2 \cdot 10^{-5}$ g per gram of stationary phase. Doubling the sample size did not change the retention time significantly. Therefore, we assumed that we were still in the linear region of the isotherm, whereas the occurrence of a strongly tailing peak may give reason to suggest the contrary. Because of the long retention times (up to 8 h) in the dry system, care was taken to stabilize the system against changes in flow-rate, temperature, etc.

It can be seen from Table I that with increasing water content of the eluent, the k' values of the samples decrease significantly. The possible change in the k' values between the equilibrated dry system (20 ppm of water) and the wet system (2100 ppm of water) is over 100-fold for compounds that still have a finite retention in the

TABLE I

CHANGE IN CAPACITY RATIO WITH THE WATER CONTENT OF DICHLOROMETHANE

Water content (ppm)	*Capacity ratio, k'*		
	Δ4-Cholestenone	*Progesterone*	*Testosterone*
20	120	240	520
60	37	120	300
160	24	77	240
280	12	45	150
620	4.9	15	62
1400	1.6	3.1	13
1630	0	0.7	4.5
2100	0	0.1	0.5

wet system. Similar values have been reported for other systems[4,13–15]. A common result for all of these systems is that for water levels greater than 500 ppm, a given increase in the water content produces a minor change in capacity ratio for compounds with k' values of less than 60. However, if samples with higher k' values are used or the water content is less than 500 ppm, the k' values increase considerably even for small changes in water content. For example, if the water level is decreased from 300 to 200 ppm, the k' value of cholest-4-en-3-one changes from 12 to 30, while for testosterone it increases from 160 to 220. At a linear velocity of 1 cm/sec, the retention time increases by only 15 min for cholest-4-en-3-one but by over 1 h for testosterone.

It should be noted that the relative retentions also vary. For the pair testosterone–progesterone, the relative retention increases from 2.2 at a water content of 20 ppm to 4.2 at a water content of 1400 ppm, whereas the relative retention of the progesterone–cholestenone pair has a maximum value of 3.8 at a water content of 280 ppm and minimum value of 1.9 at 20 ppm and 1400 ppm of water in dichloromethane.

The asymmetry of the peaks decreases as the water content of the eluent increases. A convenient measure of peak asymmetry (As^2) is the quotient of the square of the portion of the baseline of the peak on its tailing side divided by the square of the portion on the leading side. The asymmetry of the testosterone peak decreases from 50 at a water content of 60 ppm to 22 at a water content of 280 ppm and to 11 at a water content of 620 ppm. In the chromatogram obtained with dichloromethane containing 1630 ppm of water, the peak is symmetrical ($As^2 < 1.5$). This is demonstrated with the two chromatograms shown in Fig. 1 and Fig. 2.

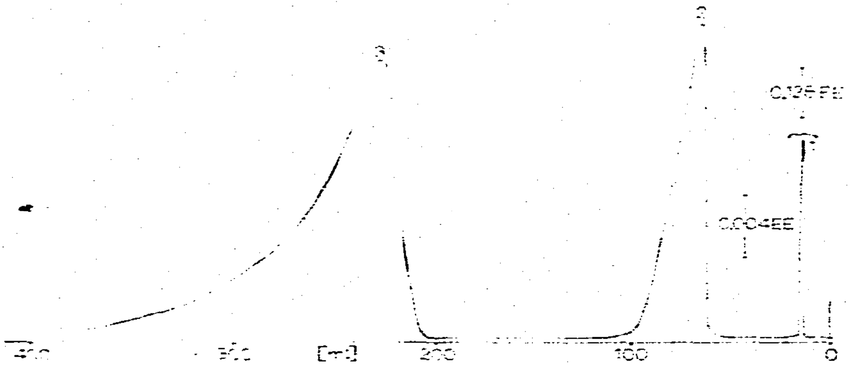

Fig. 1. Separation of steroids on silica (isocratic). Eluent: dichloromethane (80 ppm of water). Column: 52 × 0.21 cm, Merckogel SI 200, 27–32 μm. UV detector. Flow-rate: 1.6 ml/min; linear velocity: 0.9 cm/sec; Δp = 30 atm; temperature = 21.7°. 1 = Δ4-Cholestenone (80 μg); 2 = progesterone (70 μg); 3 = testosterone (170 μg). EE = Extiction units.

Problems of reproducibility

These large changes in k' values caused by small changes in the water content at low water levels makes it essential that the water content (or content of other polar contaminants) is reproduced or maintained if reproducible chromatograms are to be obtained. The reproducibility found experimentally when different batches of

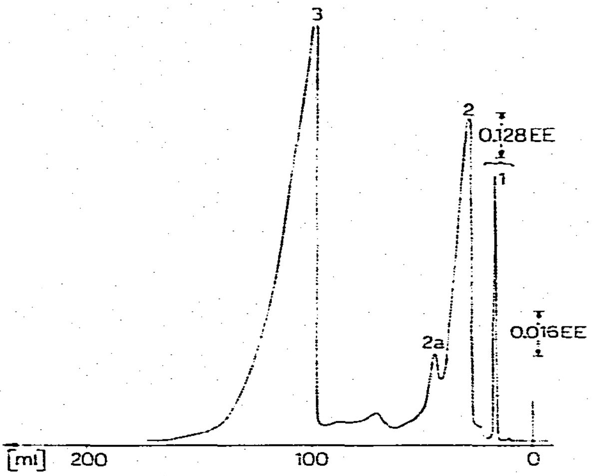

Fig. 2. Separation of steroids on silica (isocratic). Eluent: dichloromethane (620 ppm of water). Other conditions as in Fig. 1. 2a = unknown.

solvent were dried and cleaned and adjusted to a water content of 61 ± 5 ppm is shown in Table II. It can be seen that the variation in *k'* is about 10% for *k'* values between 40 and 300 with a variation in the water content of about 8%. For the "dry" system (20 ppm of water), the reproducibility of the *k'* values seems to be even better. It should be noted, however, that in instances in which atmospheric water vapour was not rigorously excluded from the dichloromethane while it was being prepared or stored in the reservoir, water was adsorbed until a water content between 30 and 40 ppm was reached.

TABLE II

REPRODUCIBILITY OF CAPACITY RATIOS WITH DIFFERENT BATCHES OF DICHLOROMETHANE

Water content (ppm)		*Capacity ratio*		
		Δ4-Cholestenone	*Progesterone*	*Testosterone*
61 ± 5		41	133	278
		52	134	302
		44	116	297
		37	104	264
	Deviation (%)	9.7	10.0	4.9
20 ± 2		114	227	477
		105	238	537
		123	239	523
	Deviation (%)	5.3	2.1	4.9

Because of problems that arise during work with hygroscopic eluents owing to the very large influence of small changes in the water content on *k'* values at these low water levels, it is not recommended to work at water levels below 50 ppm when using dichloromethane. Additional advantages are the higher linear capacity and the reduced tailing of the peaks if water-containing eluents are used[15].

Column regeneration

With "dry" solvents, some very polar sample constituents may be irreversibly adsorbed. These polar impurities react like water and reduce the k' values of later injected less polar samples. The history of the column and the nature of samples previously loaded on to the column can considerably affect the reproducibility with these dry systems. In these instances, it may be necessary to wash periodically strongly retained components from the column with a very polar eluent, *e.g.*, methanol. Even here reproducible chromatograms can then be achieved.

It was found that washing a column with about 20 column volumes of methanol (35–40 ml) is sufficient to remove even very polar samples such as dansylated amino acids from the column. However, a much larger amount of dichloromethane is needed afterwards in order to achieve the initial equilibrium conditions (*i.e.*, removal of the methanol and introduction of the equilibrium water-content to obtain the standard k' values).

TABLE III

VOLUME OF DICHLOROMETHANE NEEDED IN ORDER TO OBTAIN EQUILIBRIUM CONDITIONS AFTER WASHING WITH METHANOL

Water content of methanol used for washing (ppm)	*Desired water content of eluent dichloromethane (ppm)*	*Volume of dichloromethane needed (ml)*
170	60	210
200	60	145
500	60	100
500	160	110
500	280	120

The results in Table III show the volume of dichloromethane required to achieve equilibrium conditions after washing with methanol. In the first column of Table III the water content of the methanol is given. After the washing procedure, dichloromethane with different water contents (polarities) was introduced, as shown in the second column of Table III, and in the third column the amount of eluent required to achieve equilibrium inside the column is given (*i.e.*, the concentrations of water in the dichloromethane at the inlet and outlet of the column must be identical). The water content was followed by Karl Fischer titration and/or with a differential refractometer ($8 \cdot 10^{-5}$ refractive index units full scale). It should be noted that as the water content of the methanol (washing agent) increased from 170 to 500 ppm, the volume of dichloromethane (with 60 ppm of water) required to achieve equilibrium conditions decreased from 207 to 100 ml. Also, as the water content of the dichloromethane (eluent) increased from 60 to 280 ppm, the volume required for equilibration increased only slightly, from 100 to 120 ml, if the methanol used for washing contained 500 ppm of water. It is conceivable that the water content of the methanol can be adjusted so that the volume necessary for equilibration with dichloromethane of a given water content is much less than the values given here. As soon as the water content at the column outlet reached the equilibrium concentration, the k' values of the standards were identical with those before the methanol wash.

Relationship between the water content of the eluent and of the silica

Table IV summarizes the volumes of new solvent required to obtain equilibrium conditions, if the water content in the column has to be changed from a low to a high level. It can be seen that in all instances the volume required is 60 V_e (108 ml) or less if the water content of the dichloromethane changes between 90 and 1630 ppm. The water content in the column effluent and the k' values of the samples remained constant during the wash between 60 V_e and 2800 V_e, indicating that the system was in equilibrium and stable for a long period of time (more than 2 days at a velocity of 1.6 ml/min). The total porosity, a measure of the pore volume occupied by a stationary liquid phase immiscible with the eluent[7-16] decreased from 0.84 to 0.81. From this value and from experimentally determined differences in the water content of the dichloromethane entering and eluting from the column, it can be calculated that at a water level of 1630 ppm about 0.06 g of water is coated per gram of silica. Similar measurements with dichloromethane at a water content of 60 ppm gave a water coating of 0.006 g per gram of silica.

TABLE IV

VOLUME OF DICHLOROMETHANE REQUIRED IN ORDER TO ACHIEVE EQUILIBRIUM

Water content of dichloromethane (ppm)		*Column volumes required (V_e = 1.8 ml)*
Initial	*Final*	
90	1630	60
290	620	40
620	1390	28

Using water-saturated dichloromethane (2100 ppm), it took more than the above mentioned 60 V_e volumes to obtain a stable system, because more and more water was adsorbed by the silica until a heavily water-loaded column was generated. Fig. 3 shows the differences between the water content of the dichloromethane entering the column (straight broken line at 2100 ppm) and eluting from it. After a delay of 12 V_e (= 21.6 ml of eluent, at a velocity of 1.6 ml/min corresponding to 13.5 min) the water content of the effluent increased first rapidly and finally approached asymptotically the value of the water content of the eluent entering the column. After more than 9 l of water-saturated eluent had been pumped through the column, the water content of the column effluent was identical with that of the eluent entering the column. During this period, the total porosity of the column decreased from 0.84 to 0.47, which shows that the pore volume of the silica was filled with water almost completely. About 10 l of eluent had to be pumped through the column at a velocity of 1.6 ml/min in order to obtain a final water coating of about 0.83 g per gram of silica (calculated from the change in ε_T). During this coating procedure, the k' value of testosterone, for example, changed significantly. From an initial value of 240 in the equilibrated "dry" system (60 ppm of water in dichloromethane) it decreased to 4.2 after about 250 ml of eluent (140 V_e) had been pumped through the column. The k' value remained at this level for more than 40 h (1400 V_e = 2520 ml at a velocity of 1.6 ml/

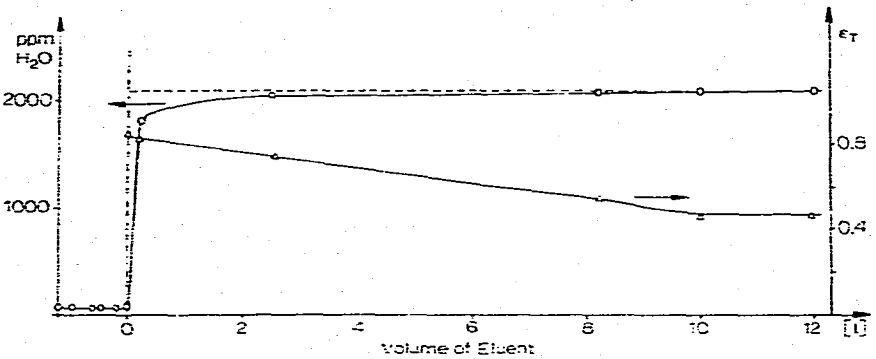

Fig. 3. Break-through curve of the "wet" eluent. Measured at column outlet by Karl Fischer titration (○) (left-hand scale). Broken line represents water content at column inlet. Right-hand scale shows the decrease in total column porosity (□) as a function of the volume flow through the column. Column and flow-rate as in Fig. 1.

min) of flow of water-saturated dichloromethane through the column. However, after a further 3–5 l of water-saturated eluent had been pumped through the column, the k' value decreased to below 0.6. At this time, more than 70% of the pore volume had been filled with water (ε_T = 0.54).

The dependence of k' values on the amount of liquid loading is similar to that measured in the system n-heptane–oxydipropionitrile–silica[6]. In the initial deactivation step, the total porosity, ε_T, decreases slowly (from 0.84 to 0.81) as the surface of the silica is covered, resulting in a large decrease in k' values. The liquid coating is about 0.1 g per gram of silica. In the following slower step, capillary condensation nearly fills the pores with stationary liquid (water), the change in the adsorptive properties is small and, with the sample used, the decrease in k' is small. With other systems, it has been observed[6] that k' can increase at higher liquid loadings for compounds that have a significant solubility in the stationary liquid phase.

The reversibility of the system is confirmed by the results shown in Fig. 4. Through a column packed with silica and heavily coated with water, "dry" dichloromethane (60 ppm of water) was passed. The changes in the water content of the column effluent and in the porosity, ε_T, were determined. It can be seen that the porosity increases linearly as the "dry" dichloromethane dissolves the adsorbed water from the silica. After a total volume of 250 V_e (450 ml), the influent and effluent water levels are indistinguishable at the precision of the Karl Fischer titration. The area under the water elution curve corresponds to the amount of water adsorbed on the silica after prolonged equilibration with water-saturated dichloromethane. A water loading of 0.78 g per gram of silica can be calculated, which compares with the amount of water adsorbed, as calculated from the change in porosity, of 0.83 g per gram of silica.

The rate at which the water fills the pores of the silica using water-saturated dichloromethane is slower with the silica used in this work (Merckogel SI 200) than with the silica used in previous experiments[7]. This demonstrates the influence of the pore structure and the size of the surface area on the water adsorption mechanism. On the other hand, the time required to transport the water, which is soluble in the dichloromethane to only a very limited extent, into the column is not negligible. The

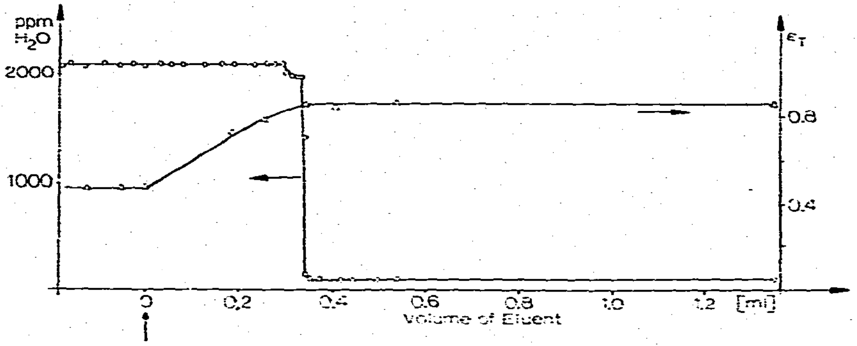

Fig. 4. Break-through curve of the "dry" eluent. Measured at column outlet ( ) by Karl Fischer titration (left-hand scale). Right-hand scale shows increase in total column porosity ( ) accompanying the removal of the water from the column. Column and flow-rate as in Fig. 1.

measured volume of 60 V_e to equilibrate columns below water saturation is comparable to a calculated volume of 40 V_e necessary to transport water into the column in order to achieve a liquid loading of 0.15 g per gram of silica, which was the maximum liquid loading obtained at equilibrium with dichloromethane just below saturation (*ca.* 2000 ppm). The rate of the water adsorption process to obtain heavily loaded columns can be improved by using a small temperature difference (*ca.* 2) between the column and the saturator which is at the higher temperature level.

Single-solvent gradient elution

Through the adsorption of water from the dichloromethane, the activity of the silica changes continuously during the elution of samples under non-equilibrium conditions, and the k' values of the samples therefore decrease during elution, resulting in a concentration of the moving zones similar to gradient elution. In an extreme case, the water will act as a displacer, resulting in elution of the strongly retained constituents of the sample in a single peak. However, this will happen only if the adsorption sites on the surface area are energetically homogeneous. If this were true, the break-through curve of the water should be steep.

In Fig. 5, an experimental break-through curve for water is shown. The lower curve was obtained with the differential refractometer by changing the water content of the dichloromethane from 60 to 2100 ppm. The dead volume of the system between the pump inlet and the RI detector was 8 ml. The point at which the RI curve and hence the water level begins to rise (break-through point) occurs after 23 ml have been eluted from the column. There is a delay in the water break-through of 15 ml (at a flow-rate of 1.6 ml/min). It can be seen that the RI curve at first rises steeply, then begins to level off at 54 ml and finally continues to rise slowly, as demonstrated in the Karl Fischer titration curve in Fig. 3. Hence it can be seen that even if the solvent is discontinuously switched from "dry" to "wet" solvent, a "gradient" of 23 ml results, corresponding to an analysis time of 15 min at the given flow-rate, if all sample constituents are eluted with this "gradient". In the upper curve in Fig. 5, the RI break-through curve of water is shown using a linear gradient (broken line) generated in a classical linear gradient device. The break-through point is considerably delayed in

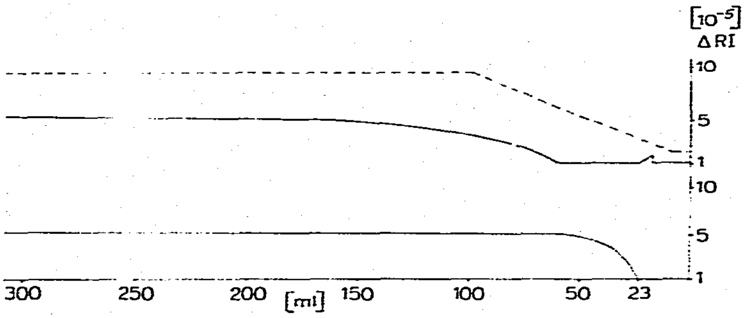

Fig. 5. Break-through curves of water measured with a differential refractometer ($8\cdot10^{-5}$ RI units full scale). Lower curve: trace obtained by discontinuously switching from "dry" dichloromethane (60 ppm of water) to "wet" dichloromethane (2100 ppm of water). Upper curve: trace obtained running a linear gradient (generated before the pump) of 46 ml of "dry" dichloromethane (60 ppm of water) and 46 ml of "wet" dichloromethane (2100 ppm of water). The broken line is the actual progress of the linear gradient as seen at the outlet of the gradient-generating device. Column and flow-rate as in Fig. 1.

the gradient system compared with the discontinuous system. Further, the rise in the water level in the gradient system is spread over a much greater volume than in the discontinuous system. This spread in the curve of 100 ml clearly shows that the gradient generated in the device (92 ml) produces a gradient in the amount of water eluted from the column, thus indicating that a continuous deactivation of the silica occurs within the column.

Fig. 6 shows the chromatogram of the same mixture used in Figs. 1 and 2 but now separated in a gradient system. The elution is finished almost at the same time

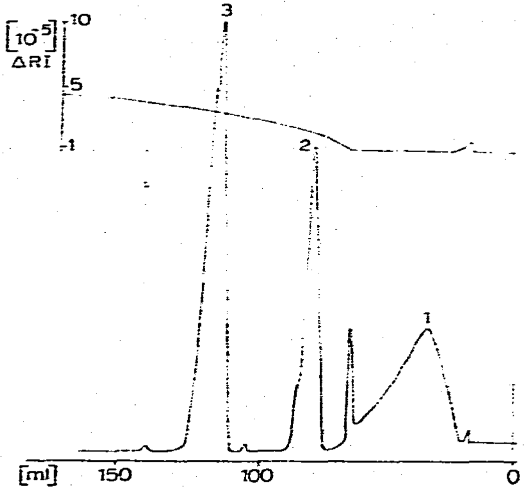

Fig. 6. "Gradient elution" of the mixture of Figs. 1 and 2. Linear "gradient" generated from 46 ml of "dry" dichloromethane (60 ppm of water) and 46 ml of "wet" dichloromethane (2100 ppm of water). Upper curve: refractometer trace.

as in Fig. 2, where a partially "wet" system (620 ppm) was used. However, the asymmetry of the peaks is less than under isocratic conditions. It should be mentioned that the small peak on the tailing edge of the cholestenone peak may be caused by a displacement of the uneluted sample because it appears exactly at the break-through point of the water. This band-splitting effect associated with solvent demixing has been described already in classical chromatography[17]. This is an unfortunate complication, because in this peak many compounds can be contained, which are pushed down the column by the advancing deactivation front on the silica. These problems are identical with those that arise in displacement chromatography[15,18].

CONCLUSION

An unusual "gradient system" has been described in which the eluent is an apolar organic liquid, and its water content changes as a function of time (retention volume). Unexpectedly, it is possible with this system to elute compounds with widely differing k' values (up to $\Delta k' = 500$). However, the k' range of the samples is much less than with the conventional gradient-elution method. An advantage of this water gradient elution is that no column regeneration step is necessary, only excess of water has to be swept out of the column. However, owing to the large influence of small differences in the water content of the eluent, great care has to be taken in adjusting exactly and reproducibly this water content. Another disadvantage of the system is the probable occurrence of band-splitting due to displacement effects arising from the water zone moving down the column.

Therefore, this unusual "gradient-elution" will have only limited practical value. However, the problems discussed here are of interest for all kinds of gradient-elution chromatography in which a polar solid stationary phase is used and the polarity of the eluent increases gradually. During this process, more or less heavily loaded columns are produced, the amount of liquid loading decreased or increased, and the composition of the liquid loading varied. Additionally, the linear velocity changes simultaneously with the changes in total porosity if the flow-rate is kept constant. All of these effects have to be kept in mind if conventional gradient-elution chromatography is performed.

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