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OPTIMIZATION OF REVERSED-PHASE SEPARATIONS*

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SUMMARY

The influence of the eluent composition on the retentions of samples achieved with reversed phases (RP), including tailing, is discussed. An "eluotropic series" for RP is proposed. Analogies between the retention orders in liquid chromatography with RP and in gas chromatography with graphitized carbon black are pointed out. The separations of ten mixtures important in routine work are described.

INTRODUCTION

In a related paper[1], the preparation and properties of different reversed phases (RPs) in relation to high-performance liquid chromatography (HPLC) were discussed. The quality of the optimal organic bristle length chemically bonded on the surface of the silica and the surface properties of the support vary, depending on the most desirable features, *e.g.*, different types of RP are required if either a high speed of analysis or alternatively a high loadability (maximum sample size) is required. In this paper, the optimization of RP systems is considered.

EXPERIMENTAL AND RESULTS

The stationary phases (dichlorodibutylsilane and trichlorooctadecylsilane) were prepared as described earlier[1]. The silica had an average pore diameter of about 100 Å (Lichrosorb SI-100, Merck, Darmstadt, G.F.R.). The particle size was either about 10 μm or about 5 μm. Home built apparatus was used[3], including a device for the control of the inlet pressure[4]. A differential refractometer (Model R401, Waters Assoc., Milford, Mass., U.S.A.) or a home-built UV detector operating at 254 $\pm$ 10 nm was used. The samples were injected on the top of the column. The columns were drilled and packed by a modified slurry technique, by either a balanced-density or viscosity method[5]. All of the eluents were purified, by at least distillation, before use.

Sample structure and chromatographic retentions

The order of elution in RP systems is opposite to that in chromatography with polar stationary phases; the more polar the sample, the earlier it is eluted. Fig. 1

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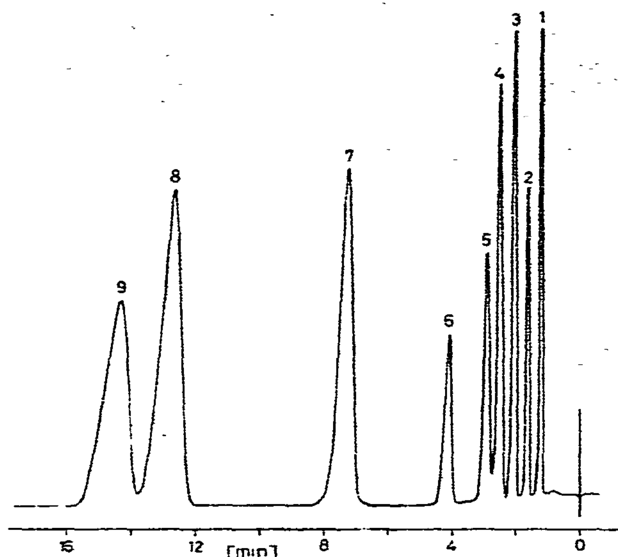

Fig. 1. Separation of phenols. Column: length, 30 cm; I.D., 4.2 mm; drilled. Stationary phase: SI-100-C_4, d_p = 10 μm. Eluent: water–methanol (7:3, v/v); u = 0.43 cm/sec; Δp = 180 atm. Samples: 1 = methanol (inert); 2 = hydroquinone (k' = 0.3); 3 = resorcinol (0.65); 4 = catechol (1.0); 5 = orcinol (1.3); 6 = phenol (2.2); 7 = *p*-cresol (4.6); 8 = *m*-xylenol (9.2); 9 = *o*-xylenol (10.5).

shows the separation of various phenolic compounds using an RP with butyl groups (SI-100-C_4) as the stationary phase. The phenols with two hydroxyl groups have the smallest retention, if the eluent is water–methanol (7:3, v/v). The addition of a methyl group to these phenols (orcinol) results in an increase in the retentions (or capacity ratios, k'). Phenol is eluted next, whereas the methylphenols (*p*-cresol) and the dimethylphenols (xylenol) are eluted much later. In this system, the addition of a methyl group almost doubles the k' value (phenol, k' = 2.24; *p*-cresol, k' = 4.6; *m*-xylenol, k' = 9.23). The more polar phenols (with three hydroxyl groups) are eluted with the inert peak, but if the water concentration in the eluent is increased they are also separated from the other phenols.

A possible explanation of retention in RP systems is the interaction of the solute with the non-polar stationary phase by dispersion or London forces. The influence of the conformation of the samples on the retention should, in a first approach, be similar to that in gas chromatography for isomeric hydrocarbons with graphitized carbon black as the stationary phase[6]. In the latter instance, the retentions increase with increasing chain length of the hydrocarbon available for interaction with the stationary phase. To illustrate these effects, the separation of isomeric aliphatic alcohols (not a real separation problem for HPLC) is shown in Fig. 2. 2-Propanol is eluted before *n*-propanol, and *tert.*-butanol before *sec.*-butanol; both of the latter are eluted before isobutanol and *n*-butanol. The relative retention of the last two components is only 1.11. After the elution of all of the butanols, a similar effect is apparent for the pentanols. The k' values increase with increasing apolar chain length of the monofunctional alcohols. In order to elute the higher alcohols, the eluting strength of the eluent must be increased. On increasing the methanol content of the eluent,

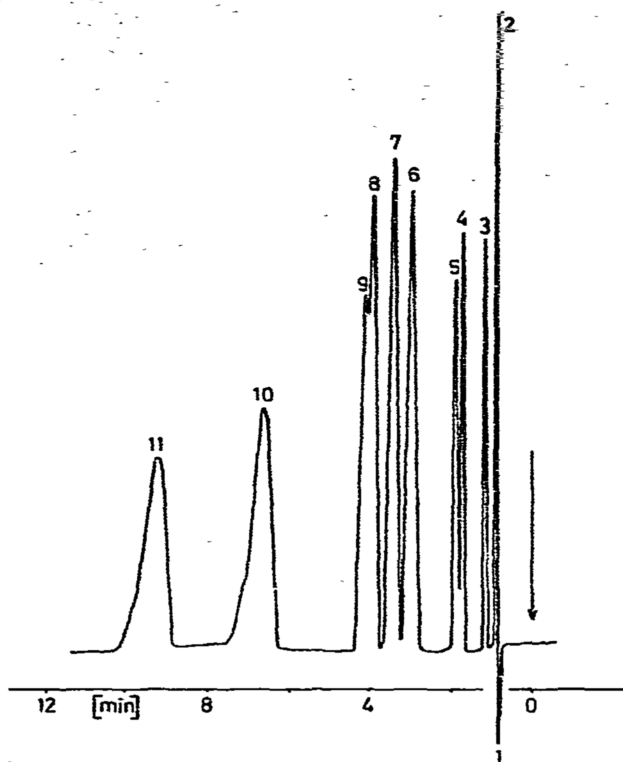

Fig. 2. Separation of aliphatic alcohols (C_1–C_5). Column and stationary phase as in Fig. 1. Eluent: water–methanol (9:1, v/v); u = 0.65 cm/sec; Δp = 180 atm. Samples: 1 = D_2O (inert); 2 = methanol (k' = 0.03); 3 = ethanol (0.5); 4 = 2-propanol (1.1); 5 = *n*-propanol (1.42); 6 = *tert.*-butanol (2.7); 7 = *sec.*-butanol (3.2); 8 = isobutanol (3.8); 9 = *n*-butanol (4.2); 10 = *tert.*-pentanol (7.3); 11 = *sec.*-pentanol (9.7).

the alcohols with longer chains are eluted. The last of each group with a given number of carbon atoms is always the *n*-alcohol. This result indicates that a similar sorption mechanism could be suggested for the retention on RP systems as in gas chromatography on graphitized carbon black.

Influence of eluent composition

With decreasing polarity of the eluent, the retention of a given compound decreases if RPs are used. The elution of solutes can be accelerated by increasing the concentration of organic solvent in water. In Fig. 3, the dependence of k' values for alcohols and phenols on the concentration of methanol in its mixtures with water is demonstrated. If the methanol concentration is less than 50% (v/v), the log k' values decrease linearly. The slope of the graphs of k' *versus* methanol concentration for these solutes seems (within experimental error) to be independent of the length of the bristle and of the quality of the sample. With pure methanol, the alcohols are almost inert ($k' < 0.02$), whereas phenol has a k' value of 0.2.

Eluotropic series are well known in chromatography with polar eluents[7–9]. The same effect can be done for RP systems. The basic eluent in this system is

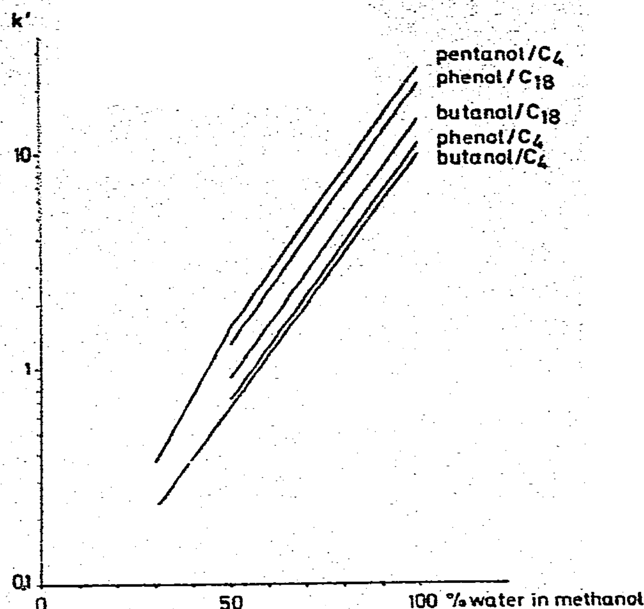

Fig. 3. Variation of k' values with eluent composition. Eluent: water–methanol mixtures. Samples: butanol, pentanol, phenol. Stationary phases: SI-100 with C_4 and C_{18} bristles.

water, because on an RP the retention of a given sample is maximal. In order to accelerate the analysis (*i.e.*, to reduce the capacity ratios, k') in water soluble and more or less apolar organic liquids have to be mixed with the water. By experience, an eluotropic series can be elucidated by measuring the retentions of the organic solvents mentioned above on an RP with water as eluent. The higher the k' value of the organic compound is in this system, the more it will accelerate the retention of a given sample if its mixture with water is used as the eluent. In Table I the retentions of some typical compounds, relative to methanol, with water as eluent using RPs with C_8 and C_{18}

TABLE I

RELATIVE RETENTIONS (RELATIVE TO METHANOL) ON RPs WITH WATER AS ELUENT ("ELUOTROPIC SERIES")

Compound	*Bristle length*	
	C_8	C_{18}
Methanol	1.0	1.0
Acetic acid	2.7	—
Ethanol	3.2	3.1
Acetonitrile	3.3	3.1
2-Propanol	8.4	8.3
Dimethylformamide	9.4	7.6
Acetone	9.3	8.8
n-Propanol	10.8	10.1
Dioxan	13.5	11.7

bristles are given. The hold-up time was always determined with D_2O using a differential refractometer as detector. If ethanol and 2-propanol with identical concentrations in water are used as eluents, the retention time of a sample will be lower with the 2-propanol system. On the other hand, it is an empirical rule, well known in column liquid chromatography, that it is better to use higher concentrations of a weak eluting liquid in water than a lower concentration of a strong eluting liquid. Using the strong liquid, the possibility of an unwanted displacement mechanism increases. Unfortunately, the change in the relative retentions is difficult to predict with the aid of the eluotropic series. Specific interactions in the RP–eluent–sample system are not unusual and can result in significant deviations from the behaviour predicted with the aid of Table I. One risk, for example, is that the acidic silanol groups of the RP (if present) can catalyse the self-condensation of acetone mixed with water, to give diacetone alcohol. If the quality of the eluent changes inside the column, the chromatographic properties are not predictable.

Acetic acid in "normal-phase" chromatography is sometimes a stronger, (here more polar) eluent than water. In RP chromatography, however, it acts in a similar manner to methanol. In the separation of acidic or basic substances, the addition of acetic acid to the eluent also reduces the tailing of the peaks. The influence of acetic acid is shown in Figs. 4 and 5, for the separation of hydroxyphenylacetic acids (some metabolics of adrenaline). In Fig. 4, 2.5% (v/v) acetic acid in water was used as the eluent. In contrast to the result with water alone as eluent, the peaks show no tailing. The analysis is completed after 14 min. In Fig. 5, the same separation is demonstrated

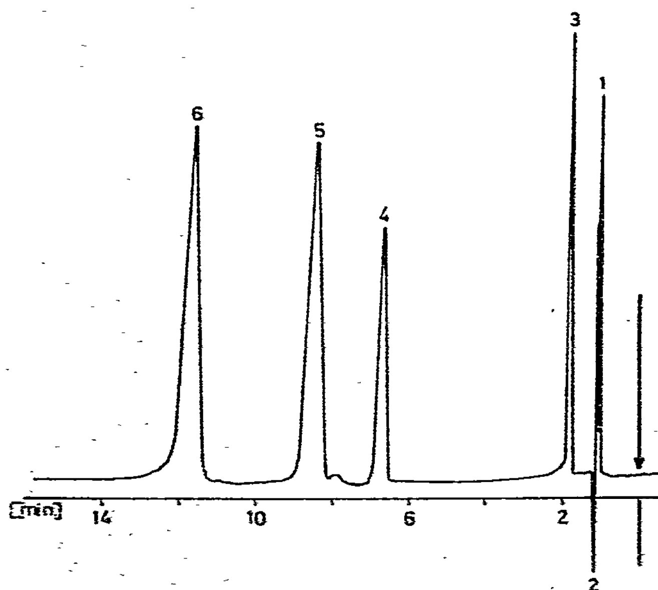

Fig. 4. Separation of aromatic hydroxy carboxylic acids. Column as in Fig. 1. Stationary phase: SI-100-C_{18}, d_p = 10 μm. Eluent: water + 2.5% (v/v) acetic acid; u = 0.8 cm/sec; Δp = 180 atm. Samples: 1 = noradrenaline (bis-tartrate) (inert); 2 = unknown; 3 = 4-hydroxy-3-methoxymandelic acid (k' = 0.7); 4 = 4-hydroxyphenylacetic acid (5.2); 5 = 3-hydroxyphenylacetic acid (6.8); 6 = homovanillic acid (9.7).

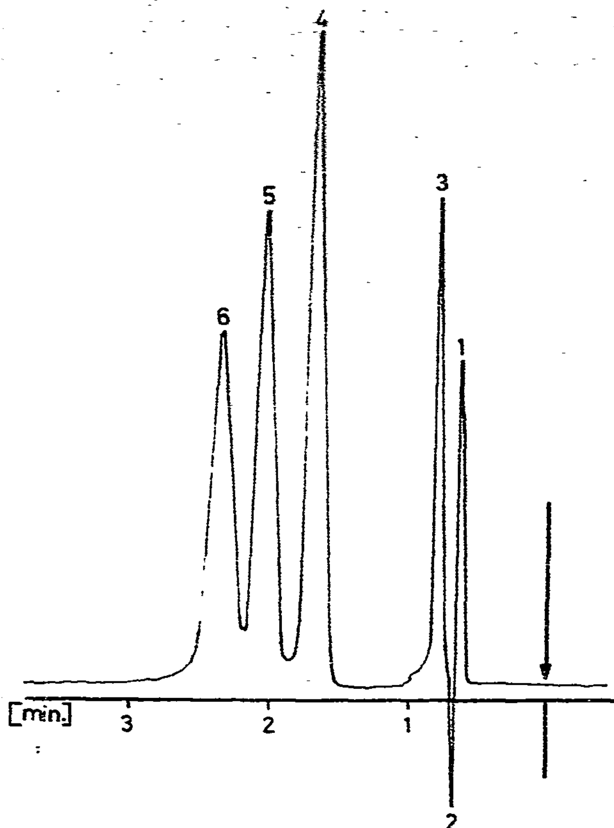

Fig. 5. Separation of aromatic hydroxy carboxylic acids. Column and stationary phase as in Fig. 4. Eluent: water + 10% (v/v) acetic acid; u = 0.8 cm/sec; Δp = 180 atm. Samples as in Fig. 4. k' values: 1 = inert; 3 = 0.3; 4 = 1.6; 5 = 2.1; 6 = 2.75.

with 10% (v/v) acetic acid in water as eluent. The separation is finished within 3 min. The k' value of homovanillinic acid decreased from 9.65 (Fig. 4) to 2.74 (Fig. 5).

It has been shown[1] that the absolute and relative retentions are higher with C_{18} than with C_4 bristles. For optimal separations, it is fortunately not necessary to have all kinds of RP stationary phases differing in their bristle lengths, as the effect of bristle length can be compensated for by changing the elution strength of the mobile phase.

In Fig. 6, the separations of carboxylic acids on a C_{18} and on a C_4 RP are shown. To obtain comparable times of analysis, the amount of acetic acid in water was doubled from 10% for the C_4 RP to 20% for the C_{18} RP. The relative retentions, however, are still higher for the C_{18} RP system.

APPLICATIONS

Although the main advantages of RP systems lie in the separation of polar samples that are usually not eluted from silica columns, it is also possible to separate hydrocarbons[1,10] and other non-polar mixtures such as pesticides. Fig. 7 shows the

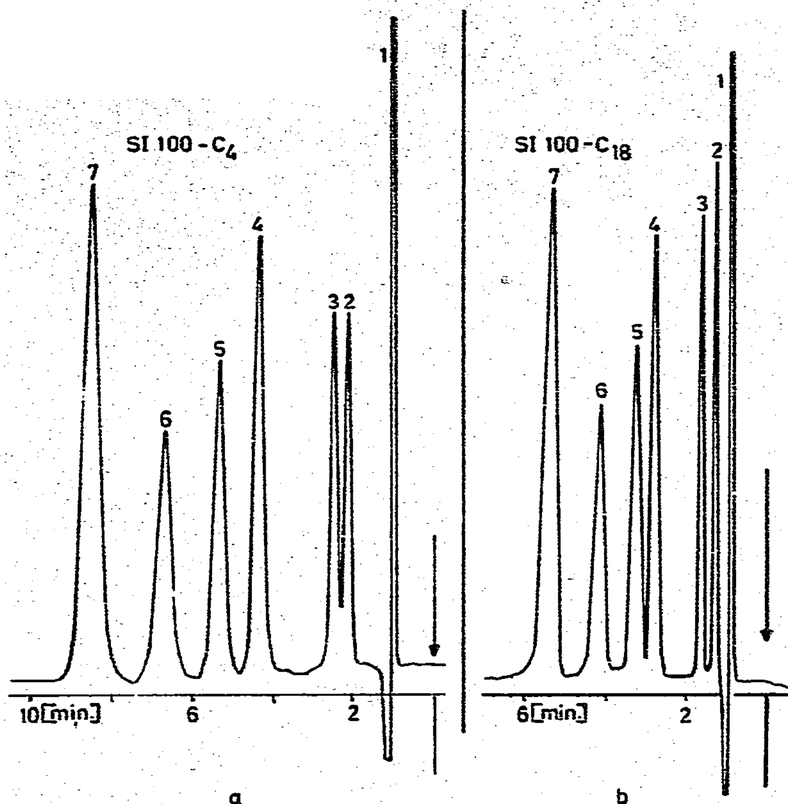

Fig. 6. Separation of caffeic acid and derivatives. Column as in Fig. 1. Stationary phases: (a) SI-100-C_4, d_p = 10 μm; (b) SI-100-C_{18}, d_p = 10 μm. Eluents: (a) water + 10% (v/v) acetic acid; u = 0.55 cm/sec; Δp = 160 atm; (b) water + 20% (v/v) acetic acid; u = 0.55 cm/sec; Δp = 160 atm. Samples: 1 = china acid (tetrahydroxycyclohexylcarboxylic acid) [inert in (a) and (b)]; 2 = chlorogenic acid [k' values: (a) 1.1; (b) 0.1]; 3 = caffeic acid [(a) 1.5; (b) 0.8]; 4 = p-coumaric acid [(a) 3.3; (b) 2.1]; 5 = m-coumaric acid [(a) 4.3; (b) 2.7]; 6 = o-coumaric acid [(a) 5.7; (b) 3.7]; 7 = coumarin [(a) 7.5; (b) 4.9].

separation of pesticides on a column packed with a C_{18} phase. The eluent is water–methanol (1:4, v/v). On increasing the water content of the eluent to 25% (v/v), the k' value of aldrin increases from 10 to 28. Even with such high k' values, symmetrical peaks are obtained.

Even aliphatic esters can be separated which such a system, as shown in Fig. 8. As the water content of the eluent is increased, the k' values increase and the peaks show tailing, because of the poor solubility of the esters in the eluent. The separations shown in Figs. 7 and 8 can also be achieved by gas chromatography. If required, the speed of analysis as a function of the viscosity of the eluent could be compared here. It should be pointed out that the sensitivity of the electron-capture detector (usual for pesticide analysis in gas chromatography) is several orders of magnitudes greater than that of the detectors used in liquid chromatography.

In Fig. 9, the separation of C_6–C_{18} aliphatic fatty acids is shown. The lower

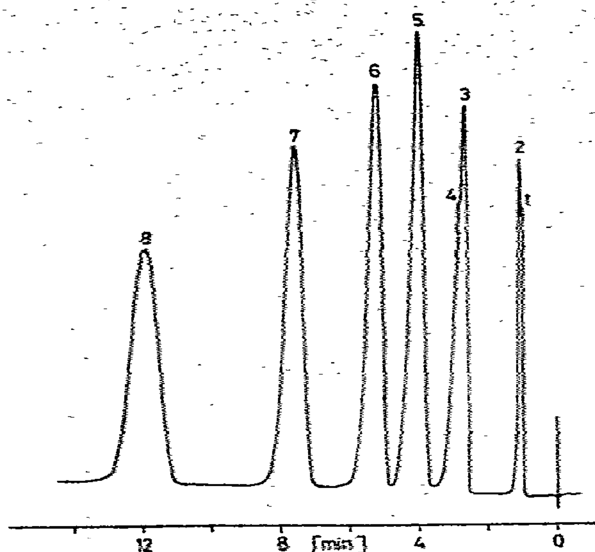

Fig. 7. Separation of pesticides. Column and stationary phase as in Fig. 4. Eluent: water–methanol (1:4, v/v); $u = 0.5$ cm/sec; $\Delta p = 170$ atm. Samples: 1 = methanol (inert); 2 = dimethoate ($k' = 0.1$); 3 = lindane (1.6); 4 = gammexane (1.7); 5 = methoxychlor (2.9); 6 = endrin (4.0); 7 = heptachlor (6.5); 8 = aldrin (10.4).

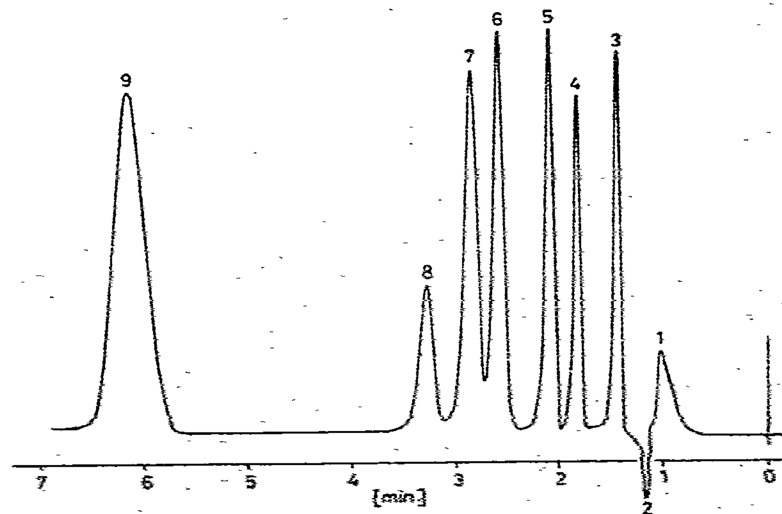

Fig. 8. Separation of carboxylic acid esters. Column and stationary phase as in Fig. 4. Eluent: water–methanol (3:7, v/v); $u = 0.5$ cm/sec; $\Delta p = 185$ atm. Samples: 1 = unknown; 2 = D_2O (inert); 3 = ethyl acetate ($k' = 0.2$); 4 = butyl acetate (0.6); 5 = ethyl valerate (0.8); 6 = butyl butanoate (1.2); 7 = heptyl acetate (1.5); 8 = ethyl phenylacetate (1.9); 9 = heptyl phenylacetate (4.3).

fatty acids can be separated at higher water contents in the eluent, but the peaks start to tail owing to dissociation. This tailing can be reduced by adding acids (compare Figs. 4–6). In contrast to gas chromatography, the fatty acids can of course be separated without derivatization.

The separation of quinones and substituted quinones is shown in Fig. 10, which

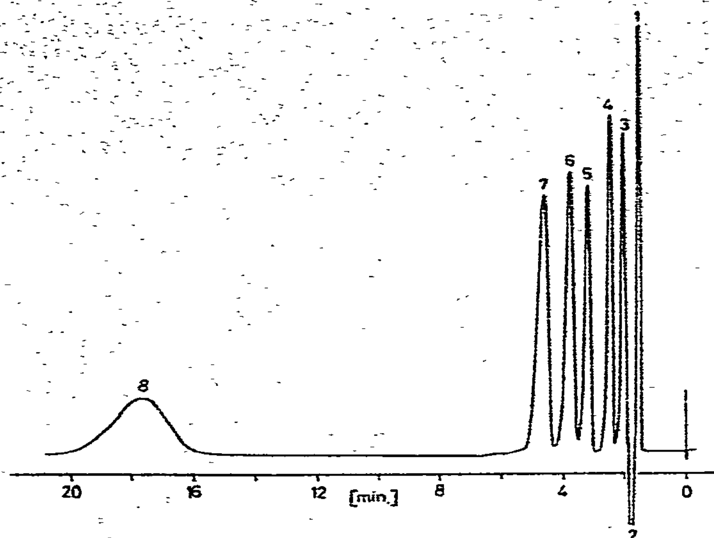

Fig. 9. Separation of aliphatic carboxylic acids. Column and stationary phase as in Fig. 4. Eluent: dioxan–water–2-propanol (4:3:2, v/v/v): $u = 0.3$ cm/sec; $\Delta p = 190$ atm. Samples: 1 = isovaleric acid (inert); 2 = unknown; 3 = caproic acid ($k' = 0.32$); 4 = caprylic acid (0.6); 5 = capric acid (1.1); 6 = undecanoic acid (1.4); 7 = lauric acid (2.0); 8 = palmitic acid (10.3).

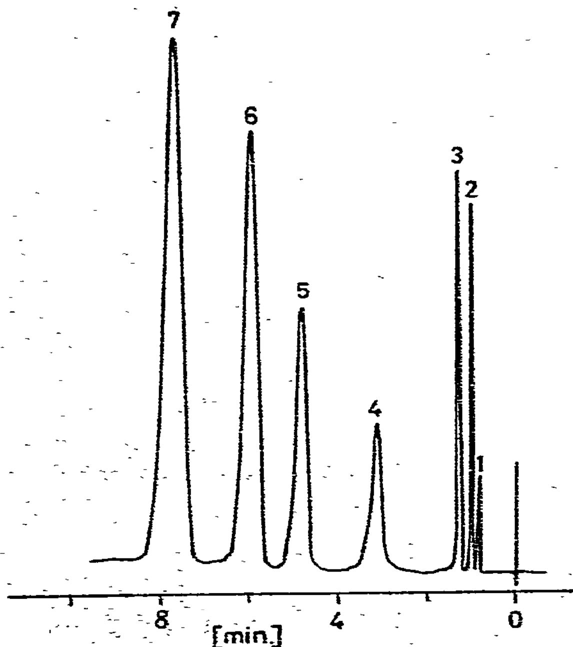

Fig. 10. Separation of quinones. Column and stationary phase as in Fig. 4. Eluent: water–methanol (1:4, v/v); $u = 0.6$ cm/sec; $\Delta p = 190$ atm. Samples: 1 = methanol (inert); 2 = *p*-quinone (k' = 0.2); 3 = naphthoquinone (0.5); 4 = anthraquinone (2.7); 5 = 2-methylanthraquinone (4.7); 6 = 2-ethylanthraquinone (6.1); 7 = 2-*tert*.-butylanthraquinone (8.1).

demonstrates the unique properties of RPs where substances that differ only in a methyl or methylene group (anthraquinone, methylanthraquinone, ethylanthraquinone) are easily separated (relative retentions > 1.3).

Fig. 11 shows the separation of tryptophan and some of its metabolites. By varying the water and methanol concentrations, the peaks of interest can be shifted to regions where they do not interfere with other components that arise from the biological material.

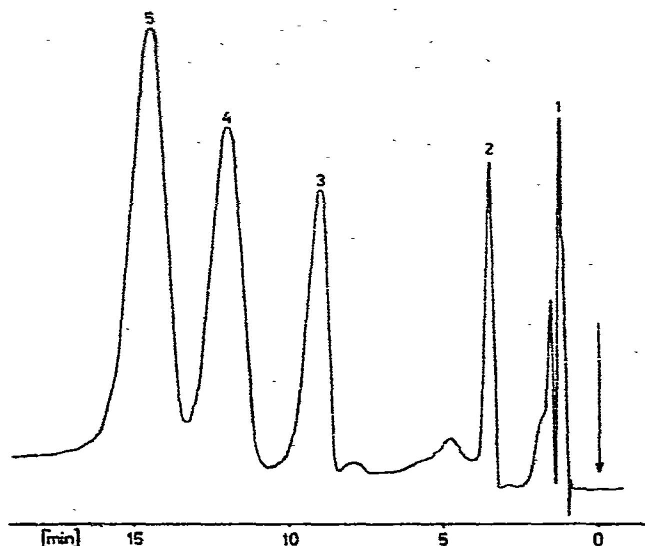

Fig. 11. Separation of tryptophan metabolites. Column and stationary phase as in Fig. 4. Eluent: water–methanol–acetic acid (200:15:5, v/v/v); $u = 0.5$ cm/sec; $\Delta p = 160$ atm. Samples: 1 = inert; 2 = 3-hydroxyanthranilic acid ($k' = 1.6$); 3 = tryptophan (5.7); 4 = xanthuric acid (7.9); 5 = kynurenic acid (9.7).

Steroids can be separated with silica[11], alumina[12], ternary mixtures[13] and heavily loaded columns[14]. In these systems, the order of the retention is a function of the polarity of the steroids. They can also be separated on RPs, their solubility in polar eluents being greater than in apolar eluents. Optimal conditions for such separations are achieved by making slight variations to the water:methanol ratio in the eluent. In Fig. 12, the separation of some androgenic steroids is shown. The eluent is water–methanol (40:60, v/v). The corticosteroids are eluted with water–methanol (25:75, v/v), as shown in Fig. 13, while for optimal separation of the progesterones an even higher proportion of methanol is required, and in Fig. 14 a separation with water–methanol (20:80, v/v) is shown.

Heart glycosides such as digitoxin and related compounds can also be separated on RP systems, as shown in Fig. 15. The more hydroxyl groups there are in the steroid molecule, the earlier it is eluted. Acetylated derivatives are, of course, more strongly retained than the non-acetylated compounds. Increasing the methanol content from 70% to 75% (v/v) decreases the k' value of acetyldigitoxin from 13.9 to 3.1. Consequently, the reproducibility of such separations will be acceptable only if the composition of the eluent is controlled carefully.

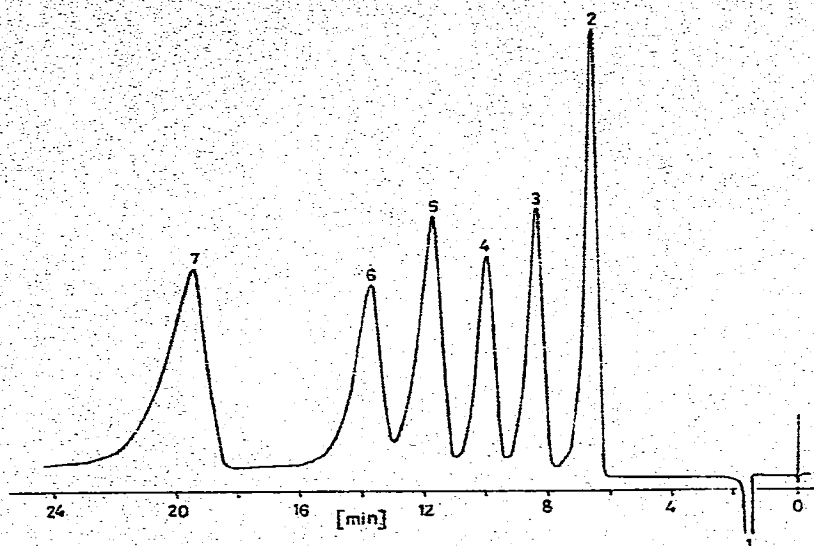

Fig. 12. Separation of androgenic hormones. Column and stationary phase as in Fig. 4. Eluent: water–methanol (20:30, v/v); u = 0.3 cm/sec; Δp = 195 atm. Samples: 1 = methanol (inert); 2 = 4-androstene-3,17-dione (k' = 3.1); 3 = testosterone (4.2); 4 = androstene-3β,17β-diol (5.1); 5 = 5α-androstane-3,17-dione (6.4); 6 = 17α-methyl-5-androstene-3β,17β-diol (7.7); 7 = androsterone (11.2).

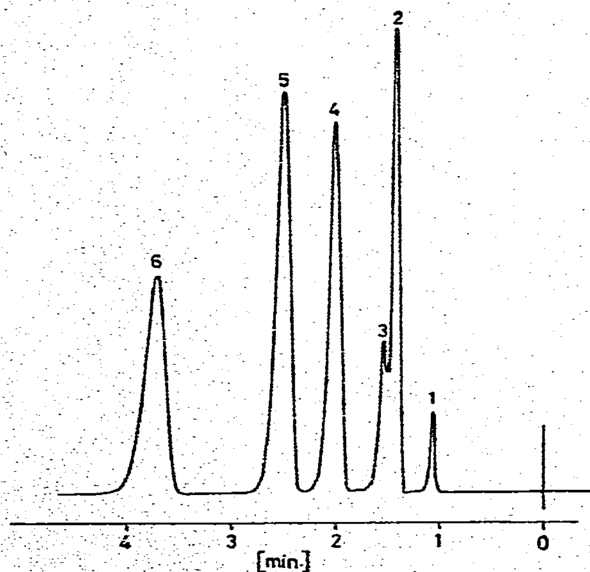

Fig. 13. Separation of corticosteroids. Column and stationary phase as in Fig. 4. Eluent: water–methanol (25:75, v/v); u = 0.5 cm/sec; Δp = 175 atm. Samples: 1 = methanol (inert); 2 = cortisone (k' = 0.3); 3 = hydrocortisone (0.45); 4 = tetrahydrocortisone (0.9); 5 = 11-desoxycorticosterone (1.2); 6 = 11-desoxycorticosterone acetate (2.5).

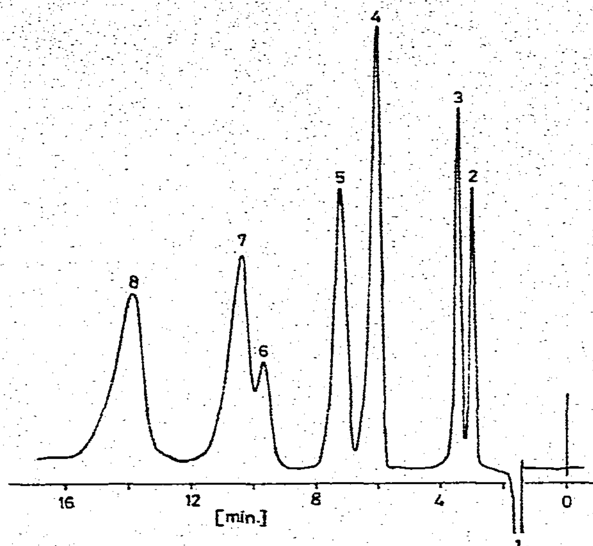

Fig. 14. Separation of progesterones. Column and stationary phase as in Fig. 4. Eluent: water–methanol (10:40, v/v); u = 0.35 cm/sec; Δp = 195 atm. Samples: 1 = methanol (inert); 2 = pregnanetriolone (k' = 1.0); 3 = 17α-hydroxyprogesterone (1.2); 4 = pregnanetriol (3.0); 5 = progesterone (4.7); 6 = pregnanediol (5.4); 7 = pregnenolone (5.9); 8 = 16α-methylpregnenolone (8.2).

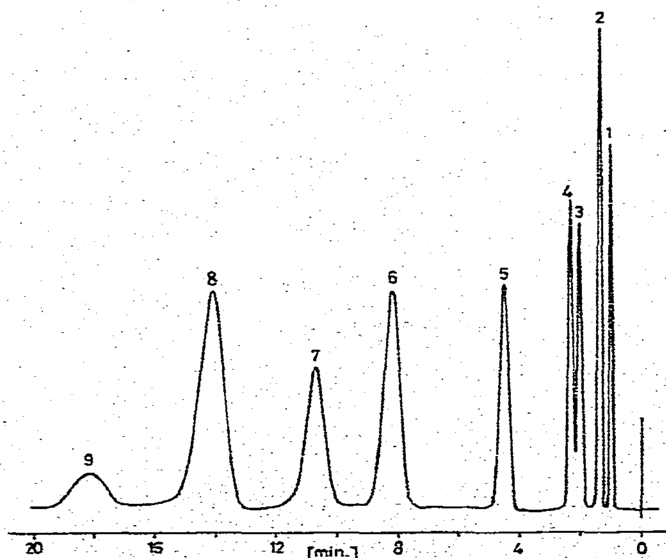

Fig. 15. Separation of heart glycosides. Column and stationary phase as in Fig. 4. Eluent: water–methanol (30:70, v/v); u = 0.4 cm/sec; Δp = 175 atm. Samples: 1 = methanol (inert); 2 = strophantin (k' = 0.4); 3 = lanatosid C (1.1); 4 = digoxin (1.4); 5 = lanatosid B (3.7); 6 = lanatosid A (7.6); 7 = digitoxin (10.3); 8 = acetyldigitoxin (13.9); 9 = unknown.

DISCUSSION

With RP systems, many types of organic compounds can be analyzed, and it is possible to separate hydrocarbons, acids, alcohols and also very polar substances. It is, of course, untrue that alkyl groups bonded chemically on the surface of silica are "the best stationary phases". Often optimal separations are achieved with silica, alumina or liquid stationary phases because of specific interactions between the sample and the stationary phase. The great advantage of the RP systems over active solids is that the water–solid adsorption equilibrium is much faster with RPs and the retentions are less influenced by the water content of the eluent. The less polar the eluent, the greater is the influence of water on the retentions. Because it is virtually impossible to ensure that the components of the eluents have corresponding water contents, a rapid water–stationary phase equilibrium is extremely important in gradient elution.

A further advantage (or disadvantage) of RPs is that small changes in the composition of the eluent (for example, methanol–water mixtures) alter the absolute and relative retentions. The methanol–water system is non-ideal: the heat of mixing is high, the viscosity and the interdiffusion coefficient are not a linear function of the concentration, and moreover they pass through a maximum or minimum. The consequence of the high heat of mixing is sometimes degassing of the eluent in the mixing chamber and other problems, for example in the solvent delivery system. The viscosity of methanol–water (40:60, v/v) is 1.6 cP at room temperature compared with 0.54 cP and 0.9 cP for methanol and water alone, respectively. The diffusion coefficients of the samples change inversely. For these reasons (among others), the height equivalent to a theoretical plate is not defined and has no physical meaning in gradient elution.

The conformation of the sample can be very important if RPs are used, because of the interaction of the apolar groups of molecules and the apolar bristles. For the same reason, the introduction of small polar or apolar groups into a large sample molecule influences the retentions significantly (Figs. 10 and 12–14). Perhaps it is possible to compare this sorption mechanism and the general rules given here with those applicable in the gas chromatographic separation of hydrocarbons on graphitized carbon black[6].

The log k' values of a homologous series change linearly with the composition of the eluent if RP systems are used, and linear eluent programming results in equidistant retentions of these mixtures.

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