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GRADIENT ELUTION IN LIQUID CHROMATOGRAPHY

VIII. SELECTION OF THE OPTIMAL COMPOSITION OF THE MOBILE PHASE IN LIQUID CHROMATOGRAPHY UNDER ISOCRATIC CONDITIONS

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SUMMARY

The influence of the composition of the mobile phase on resolution in liquid chromatography is considered from both the theoretical and the practical points of view. Different theoretical models for adsorption and ion-exchange chromatography are compared and an approach is suggested that permits calculations of the composition of the mobile phase that is necessary in order to achieve the separation required. The factors limiting the application of the mathematical approach presented are discussed. The theoretical conclusions are supported by several practical examples of chromatographic separations on silica and alumina.

INTRODUCTION

Since the comeback of liquid chromatography in the late 1960s, considerable effort has been devoted to the improvement of liquid chromatographic separations. A dramatic increase in resolution was achieved by the development of efficient column packing materials and rational instrumental design. Many papers devoted to the improvement of column and system efficiencies have been published, but far less attention has been paid to the possibilities of improving resolution by appropriate control of the composition of the mobile phase, which has been adjusted by trial and error methods until acceptable separations were achieved. Few attempts have been made to calculate the optimal composition of the mobile phase for the resolution required. The work of Snyder and Saunders[1], in which the conditions for gradient elution in adsorption chromatography were calculated in order to maintain a constant width of all peaks during the elution, is a rare exception.

If any calculations of this type are to be performed, the relationship between the composition of the mobile phase and some characteristic representing the retention of the solute in the system (such as the capacity ratio) must be known. In an earlier paper[2], we suggested a simple equation to describe this relationship:

$$k' = k'_0 c^{-n} \qquad (1)$$

where k' is the capacity ratio of the solute, c is the concentration of the stronger eluting component in a binary mobile phase and k'_0 and n are experimental constants. This equation has been shown to be valid in a number of practical separations by adsorption and ion-exchange chromatography[3–6].

Based on Snyder's theory of adsorption chromatography[7], an equation can be written[2] for the capacity ratio in a mixture of two solvents a and b:

$$\log k' = \log k'_a - \frac{A_s}{n_b} \cdot \log [c(10^{\bar{a}n_b(\varepsilon^\circ_b - \varepsilon^\circ_a)} - 1) + 1] \quad (2)$$

where A_s and n_b represent the effective molecular area of an adsorbed molecule of the sample solute and that of the more polar solvent b, respectively, ε°_a and ε°_b denote the solvent strength parameters of the two solvents a and b, respectively, $\bar{a}$ is the adsorbent surface activity function and k'_a refers to the capacity ratio of sample solute in the pure solvent a.

Eqn. 2 can be rearranged into the form

$$k' = (a + bc)^{-n} \quad (3)$$

where $a = k'^{-\frac{n_b}{A_s}}_a$, $b = k'^{-\frac{n_b}{A_s}}_a \cdot (10^{\bar{a}n_b(\varepsilon^\circ_b - \varepsilon^\circ_a)} - 1)$, $n = \frac{A_s}{n_b}$ and a, b and n are experimental constants which should not depend on the concentration of solvents a and b in the mobile phase. If the sample solutes are very strongly retained in the less polar solvent a (usually a hydrocarbon), k'_a is very high and the parameter a is very small and can be neglected. Then eqn. 3 is simplified to eqn. 1.

Recently, Scott[8] published another simple equation for the capacity ratio in binary solvent systems, which can be written in the form:

$$k' = (a + bc)^{-1} \quad (4)$$

Eqn. 4 is identical with eqn. 3 if $n = 1$, which holds provided that $A_s = n_b$, *i.e.*, if an adsorbed molecule of the sample solute occupies the same area of the adsorbent surface as a molecule of the more polar solvent. This simplified assumption, however, is generally not fulfilled. The differences in the number of polar functional groups in the solute molecules and/or solvation effects may cause differences in the area of the adsorbent occupied by various molecules. Consequently, the exponent n often deviates considerably from unity and eqn. 4 is no longer valid. In a previous study on the adsorption chromatography of azo compounds on silica in different binary solvent systems[5], we found that n varied in the range 0.5–2.5, depending on the nature of the solute and solvents used.

The adsorption chromatography of four steroids on alumina in *n*-propanol–*n*-heptane[9] is described here to illustrate some practical limitations of the mathematical models discussed. The experimental values of n for lumisterol, tachysterol, calciferol and ergosterol (Tables II–IV) are within the range 1.1–1.5. The compounds are only slightly retained in mobile phases that contain more than 1% of *n*-propanol. To compare the application of eqns. 1 and 4 to the above practical system, the relationship between $1/k'$ and c is plotted in Fig. 2, while the plot of log k' *versus* log c is shown in Fig. 1. The relationship between $1/k'$ and c is approximately linear for ergosterol and possibly for calciferol, but there are large deviations for lumisterol

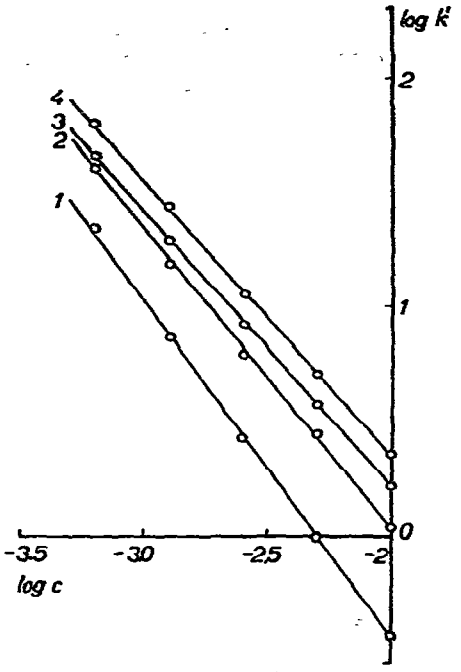

Fig. 1. Logarithmic relationships between capacity ratios (k') of lumisterol (1), tachysterol (2), calciferol (3) and ergosterol (4) and the concentration of *n*-pronapol (c, vol.-% $\times 10^{-2}$) in *n*-heptane used as the mobile phase in chromatography on LiChrosorb ALOX T. Experimental conditions are given in Table II. The relationships should be linear if eqn. 1 applies.

and tachysterol, which indicates that eqn. 4 cannot be applied to them. All of the logarithmic relationships in Fig. 2 are linear, which demonstrates the validity of eqn. 1, which is more suitable than eqn. 4 for describing the system.

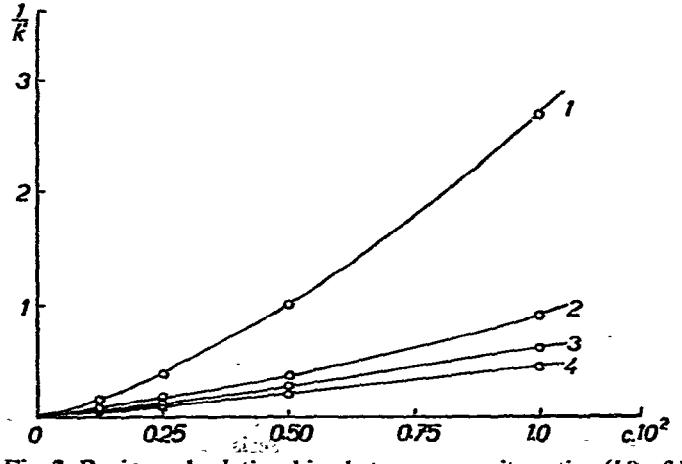

Fig. 2. Reciprocal relationships between capacity ratios (k') of lumisterol (1), tachysterol (2), calciferol (3) and ergosterol (4) and concentration of *n*-propanol (c, vol.-% $\times 10^{-2}$) in *n*-heptane used as the mobile phase in chromatography on LiChrosorb ALOX T. Experimental conditions are given in Table II. The relationships should be linear if eqn. 4 applies.

In ion-exchange chromatography, $n = 1$ is to be expected theoretically, provided that exchange between the monovalent ions takes place. If the charge of the solute ion and/or that of the counter ion differ from unity, then n will differ from unity[2] and eqn. 4 cannot be expected to be followed. Anion-exchange chromatography of guanosine 5'-mono-, di- and triphosphates on Perisorb AN in aqueous solutions of potassium dihydrogen phosphate as the mobile phase[10] represents a practical example of an ion-exchange system in which the solutes have different

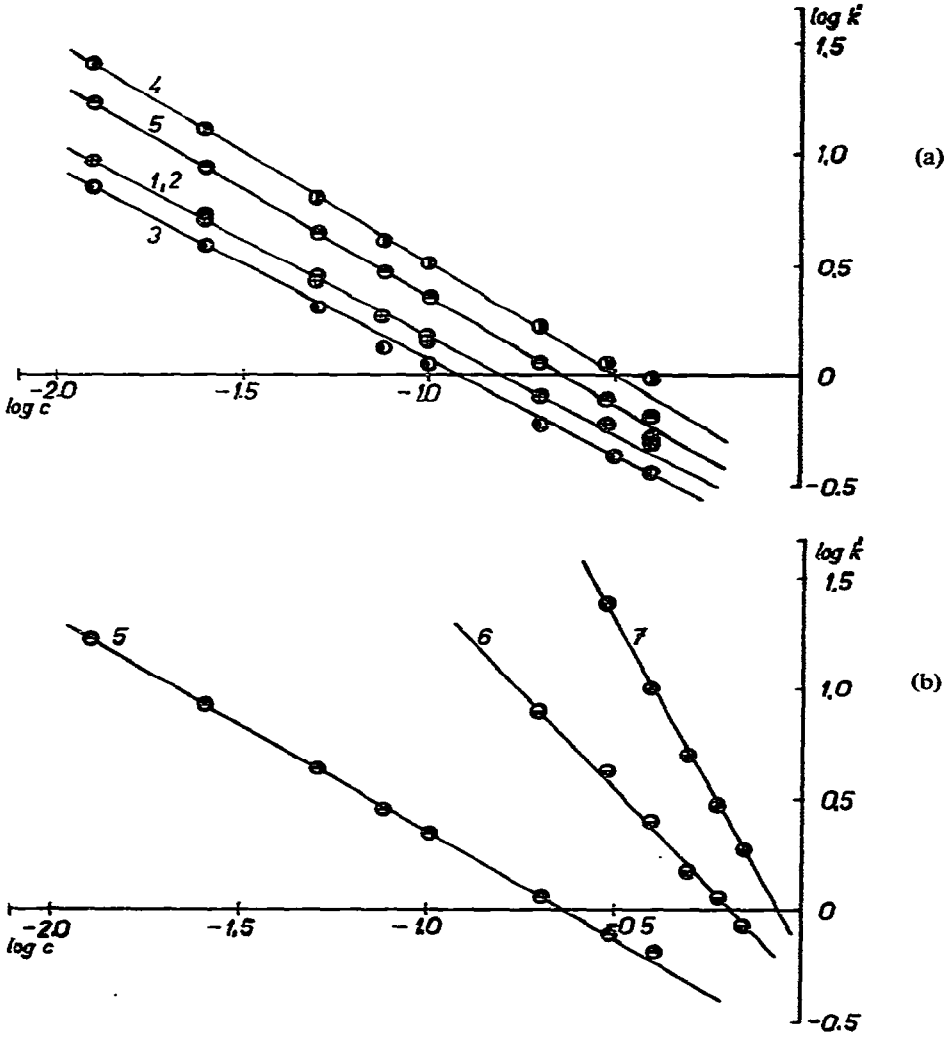

Fig. 3. Logarithmic relationships between capacity ratios (k') of nucleotides and concentration of potassium dihydrogen phosphate (c, molarity) in the mobile phase (aqueous, pH = 3.15) in anion-exchange chromatography on a column (905 × 2.3 mm) packed with Perisorb AN (30–40 μm). Numbers of compounds: (a) 1 = thymidine 5'-monophosphate; 2 = ribothymidine 5'-monophosphate; 3 = deoxyuridine 5'-monophosphate; 4 = deoxyguanosine 5'-monophosphate; 5 = guanosine 5'-monophosphate; (b) 5 = guanosine 5'-monophosphate; 6 = guanosine 5'-diphosphate; 7 = guanosine 5'-triphosphate. The relationships should be linear if eqn. 1 applies.

values of n. The experimental values in this system are very close to those predicted theoretically: 0.96 for monophosphate (theoretical value 1), 1.85 for diphosphate (theoretical value 2) and 3.03 for triphosphate (theoretical value 3). The relationship between log k' and log c (where c is the molarity of potassium dihydrogen phosphate) is plotted in Fig. 3, while Fig. 4 shows the relationship between $1/k'$ and c for the compounds studied. The graphs in Fig. 3 are linear, which shows that eqn. 1 applies well, while those for diphosphate and triphosphate in Fig. 4 are not linear. As would be expected, the plot for monophosphate is linear in the mobile phase that is less than 0.3 M in potassium dihydrogen phosphate. Fig. 4 also shows clearly that the term a in eqn. 4 is very close to zero.

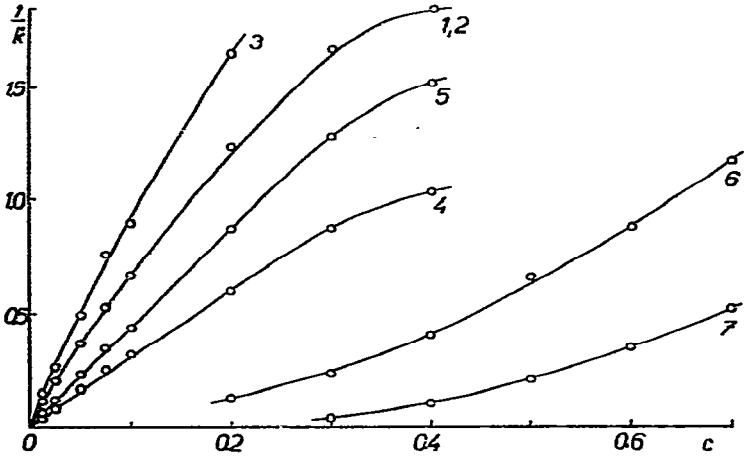

Fig. 4. Relationships between reciprocal of capacity ratios (k') of nucleotides and concentration of potassium dihydrogen phosphate (c, molarity) in the mobile phase (aqueous, pH = 3.15) in chromatography on Perisorb AN. Experimental conditions and compounds as in Fig. 3. The relationships should be linear if eqn. 4 applies.

It can be concluded that the relationship between the capacity ratio and the concentration of the binary mobile phase in adsorption and ion-exchange chromatography can generally be expressed by eqn. 3. If the molecules of the solute are placed on the surface of the column packing material in the same manner as the molecules of the stronger eluting component in the mobile phase (the same area of the adsorbent surface occupied by an adsorbed molecule; the same charge of the exchanging ions in ion-exchange chromatography), the simplified eqn. 4 can be used instead of eqn. 3. It is difficult to predict the chromatographic systems for which this assumption is fulfilled. In systems in which the solutes are strongly retained from the less efficient eluting component of the mobile phase, the term a in eqn. 3 is very close to zero and the simple eqn. 1 is valid. This applies to a number of ion-exchange systems and to the adsorption chromatography of polar compounds on silica or alumina using mobile phases that contain hydrocarbons as the less polar component.

INFLUENCE OF THE COMPOSITION OF THE MOBILE PHASE ON RESOLUTION

Considering the relationship between the capacity ratio, k', and the concentration, c, of the more efficient eluting component in the mobile phase (eqn. 3), we can write the following equations for the retention volume, V_R, peak width, w, and separation factor, $\alpha = V'_{R_2}/V'_{R_1}$:

$$V_R = V_m[1 + (a + bc)^{-n}] \tag{5}$$

$$w = \frac{4V_m}{\sqrt{N}}[1 + (a + bc)^{-n}] \tag{6}$$

and

$$\alpha = \frac{(a_1 + b_1c)^{n_1}}{(a_2 + b_2c)^{n_2}} \tag{7}$$

where the subscripts 1 and 2 relate to compounds 1 and 2, N is the plate number, V_m is the column void volume (the volume of the mobile phase in the column) and the coefficients a and b and the exponent n are experimental constants characteristic of the nature of the adsorbent, the components of the mobile phase and the compound being chromatographed.

The constants a, b and n are related to the capacity ratio in the pure stronger eluting component of the mobile phase, k'_0, and to the capacity ratio in the less efficient component, k'_∞:

$$k'_0 = (a + b)^{-n} \tag{8}$$

and

$$k'_\infty = a^{-n} \tag{8a}$$

Further, eqns. 5–8 a have a real physical meaning if $a \geqslant 0$. As c denotes the concentration of the stronger eluting component in the mobile phase, $k'_\infty > k'_0$ and $b \geqslant 0$; $n \geqslant 0$.

If we accept the commonly used simplified definition of the resolution of two compounds 1 and 2 as

$$R_s = \frac{V_{R_2} - V_{R_1}}{w_2} \tag{9}$$

we can derive the equation for the influence of the composition of the mobile phase on resolution:

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \frac{(a_2 + b_2c)^{-n_2} - (a_1 + b_1c)^{-n_1}}{1 + (a_2 + b_2c)^{-n_2}} \tag{10}$$

which can be expressed in a more illustrative form as

$$R_s = \underbrace{\frac{\sqrt{N_2}}{4}}_{\text{I}} \cdot \underbrace{(1 - \alpha^{-1})}_{\text{II}} \cdot \underbrace{\frac{1}{(a_2 + b_2c)^{n_2} + 1}}_{\text{III}} \tag{10a}$$

Here, three common terms for different contributions to the resolution can be distinguished: I, efficiency; II, selectivity; and III, capacity.

If the number of plates does not depend significantly on the composition of the mobile phase, the concentration of the more efficient component in the mobile phase influences the resolution by means of terms II and III. As the concentration c increases, the capacity term decreases. The selectivity term is minimal (zero) if $\alpha = 1$, and in this instance, no separation of the compounds 1 and 2 occurs. Generally, a composition of the mobile phase can be found, where $\alpha = 1$ and $R_s = 0$, from the following equation:

$$c_{(\alpha=1)} = \frac{(a_2 + b_2 c_{(\alpha=1)})^{\frac{n_2}{n_1}} - a_1}{b_1} \tag{11}$$

From this equation, the concentration c cannot be expressed in an explicit form.

The concentration $c_{(\alpha=1)}$ has a real meaning [$c_{(\alpha=1)} > 0$] if the following condition holds true:

$$a_2^{n_2} > a_1^{n_1} \tag{11a}$$

If $n_1 \approx n_2$, eqn. 11 can be simplified to the explicit form

$$c_{(\alpha=1)} = \frac{a_2 - a_1}{b_1 - b_2} \qquad (n_1 \approx n_2) \tag{11b}$$

The concentration range of a binary mobile phase can be divided into two parts, with opposite elution sequences of the two compounds. As long as $c < c_{(\alpha=1)}$, the compound with the lower value of the exponent n (if $n_1 = n_2$ and $a_1 \neq 0$ and/or $a_2 \neq 0$, the compound with the lower value of b) is eluted first, while if $c > c_{(\alpha=1)}$ the opposite occurs.

The concentration c always lies in the range between $c = 0$ and $c = c_{mp}$, where c_{mp} is a maximum possible concentration, which is either the pure more efficient eluting component of the mobile phase, or its saturated solution in the less efficient eluting agent. If $c_{(\alpha=1)} < 0$, or $c_{(\alpha=1)} > c_{mp}$, the elution sequence of the two compounds does not change over the whole concentration range. Thus, a reversal of the elution sequence with changing composition of the mobile phase can occur only if the concentration $c_{(\alpha=1)}$ is within practical limits [$0 < c_{(\alpha=1)} < c_{mp}$].

On the other hand, it can be proved that at a certain composition of the mobile phase, the resolution can reach a maximum value. The concentration of the more efficient eluting component in the mobile phase for maximum resolution, c_{max}, can be found by solving the equation

$$dR_s/dc = 0 \tag{12}$$

The equation for c_{max} can be written in the following implicit form:

$$c_{max} = \frac{1}{b_1} \cdot \left\{ \frac{1}{\frac{n_1 b_1}{n_2 b_2} \cdot \frac{(a_2 + b_2 c_{max})}{(a_1 + b_1 c_{max})^{(n_1+1)}} \cdot [1 + (a_2 + b_2 c_{max})^{n_2}] - 1} \right\}^{\frac{1}{n_1}} - \frac{a_1}{b_1} \tag{13}$$

The numbers of compounds in this equation should be chosen so that $n_1 > n_2$, otherwise eqn. 13 yields no solution.

The above conclusions hold true for concentrations $c > c_{(\alpha=1)}$, where the increasing concentration leads to an increase in the selectivity term (II) and to a simultaneous decrease in the capacity term (III). In the concentration range where $c < c_{(\alpha=1)}$, the increase in concentration is followed by a decrease in both the capacity and selectivity terms, so that maximal resolution is achieved for $c = 0$.

Thus, according to the sequence of $c = 0$, c_{max}, $c_{(\alpha=1)}$ and c_{mp}, six different situations can be distinguished. These situations are illustrated by plots of R_s *versus* c in Figs. 5 and 6 and of k' *versus* c in Figs. 7 and 8 (for clarity, the curves are approximated by lines).

(1) $0 < c_{mp} < c_{(\alpha=1)} < c_{max}$. This situation is shown in Figs. 5(1) and 7(1). Over the whole concentration range accessible, the compound with a higher value of n is eluted later than the compound with lower n. The resolution decreases with increasing concentration over the whole concentration range, so that maximal resolution is achieved at $c = 0$.

(2) $0 < c_{(\alpha=1)} < c_{mp} < c_{max}$ [Figs. 5(2) and 7(2)]. The elution sequence is the same as in (1) and the resolution decreases with increasing concentration as long as

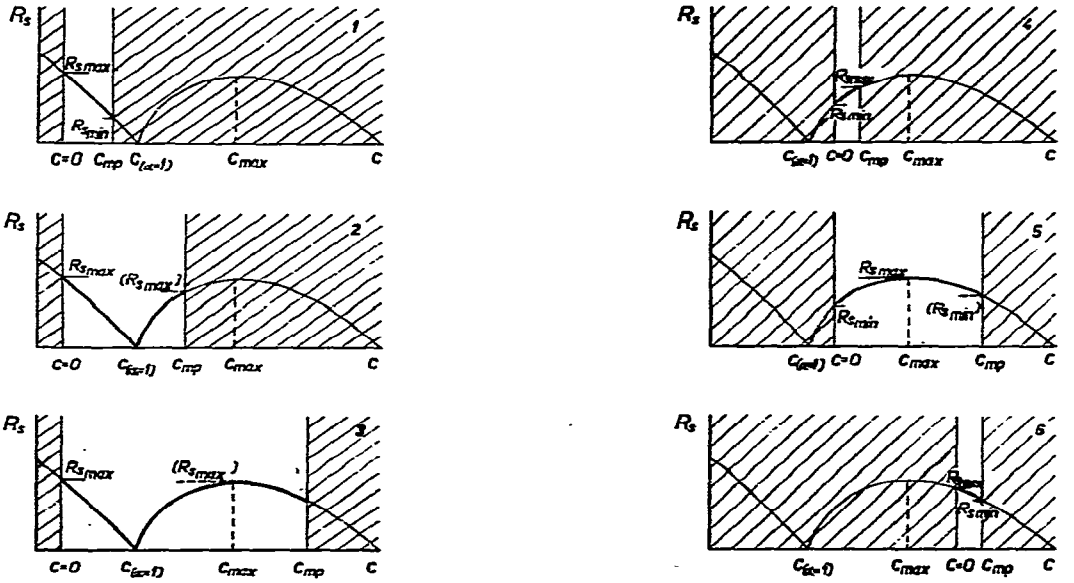

Fig. 5. Representative relationships between resolution (R_s) and concentration (c) of the more efficient eluting agent in the mobile phase. (1) Instance 1, $0 < c_{mp} < c_{(\alpha=1)} < c_{max}$; (2) instance 2, $0 < c_{(\alpha=1)} < c_{mp} < c_{max}$; (3) instance 3, $0 < c_{(\alpha=1)} < c_{max} < c_{mp}$. $c_{(\alpha=1)}$ = Concentration at which $\alpha = 1$; c_{max} = concentration corresponding to the maximum on the R_s *versus* c curve; c_{mp} = maximal practically available concentration; $R_{s\,max}$ = maximal resolution; $R_{s\,min}$ = minimal resolution.

Fig. 6. Representative relationships between resolution (R_s) and concentration (c) of the more efficient eluting agent in mobile phase. (4) Instance 4, $c_{(\alpha=1)} < 0 < c_{mp} < c_{max}$; (5) instance 5, $c_{(\alpha=1)} < 0 < c_{max} < c_{mp}$; (6) instance 6, $c_{(\alpha=1)} < c_{max} < 0 < c_{mp}$. Symbols as in Fig. 5.

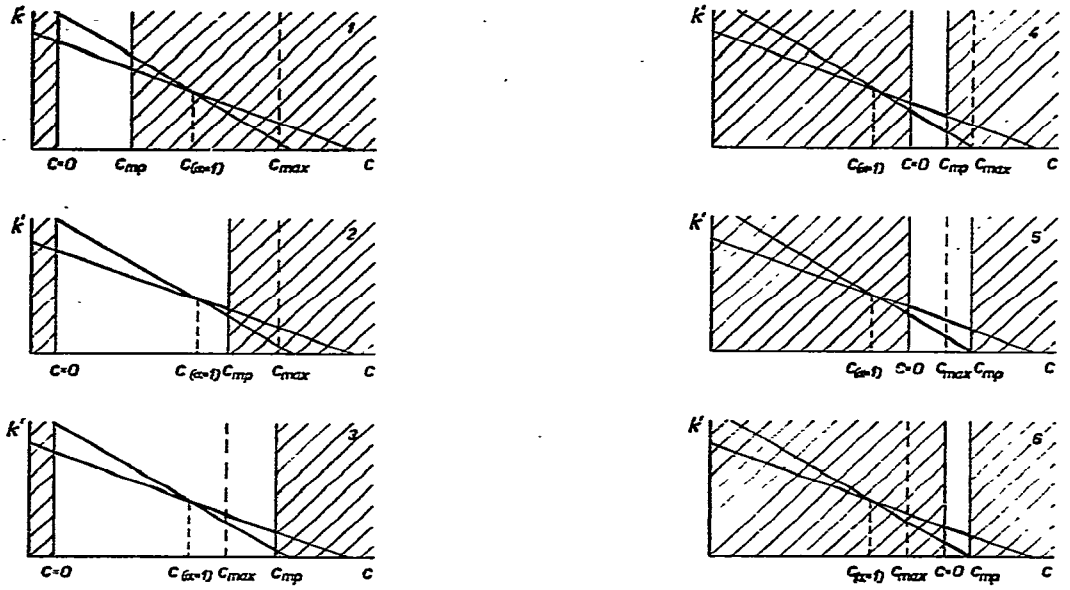

Fig. 7. Representative relationships between capacity ratios (k') and concentration (c) of the more efficient eluting agent in the mobile phase. For clarity, the curves are simplified as lines. (1) instance 1, $0 < c_{mp} < c_{(\alpha=1)} < c_{max}$; (2) instance 2, $0 < c_{(\alpha=1)} < c_{mp} < c_{max}$; (3) instance 3, $0 < c_{(\alpha=1)} < c_{max} < c_{mp}$. Symbols as in Fig. 5.

Fig. 8. Representative relationships between capacity ratios (k') and concentration (c) of the more efficient eluting agent in the mobile phase. For clarity, the curves are simplified as lines. (4) Instance 4, $c_{(\alpha=1)} < 0 < c_{mp} < c_{max}$; (5) instance 5, $c_{(\alpha=1)} < 0 < c_{max} < c_{mp}$; (6) instance 6, $c_{(\alpha=1)} < c_{max} < 0 < c_{mp}$. Symbols as in Fig. 5.

$c < c_{(\alpha=1)}$. At $c_{(\alpha=1)}$, $R_s = 0$. For $c > c_{(\alpha=1)}$, the elution sequence reverses (the compound with higher n is eluted first) and the resolution increases with rising concentration up to $c = c_{mp}$. The higher of the two values of the resolution at $c = 0$ and $c = c_{mp}$, respectively, gives the maximal resolution that can be obtained. The speed of chromatographic separation at $c = c_{mp}$ is much higher than that at $c < c_{(\alpha=1)}$.

(3) $0 < c_{(\alpha=1)} < c_{max} < c_{mp}$ [Figs. 5(3) and 7(3)]. This situation is similar to (2), but in the concentration range where $c > c_{(\alpha=1)}$ maximum on the R_s *versus* c curve is achieved. Thus, the resolution decreases from $c = 0$ to $c_{(\alpha=1)}$, where $R_s = 0$, then the elution sequence is reversed and the resolution increases to a maximal value at c_{max}. Increasing the concentration above c_{max} leads to a further decrease in resolution up to c_{mp}. The higher of the two values of the resolution at $c = 0$ and $c = c_{max}$, respectively, gives the maximal resolution that can be obtained. The chromatographic separation at c_{max} is much faster than that at $c < c_{(\alpha=1)}$.

(4) $c_{(\alpha=1)} < 0 < c_{mp} < c_{max}$ [Figs. 6(4) and 8(4)]. The compound with a higher value of n is eluted first over the whole accessible concentration range. The resolution increases with increasing concentration and acquires a maximal value at $c = c_{mp}$.

(5) $c_{(\alpha=1)} < 0 < c_{max} < c_{mp}$ [Figs. 6(5) and 8(5)]. The elution sequence is the same as in (4). The resolution increases with increasing concentration from 0 to

c_{max}, where maximal resolution is achieved. A further increase in concentration above c_{max} leads to a decrease in resolution.

(6) $c_{(\alpha=1)} < c_{max} < 0 < c_{mp}$ [Figs. 6(6) and 8(6)]. The elution sequence is the same as in (4). The resolution decreases with increasing concentration from its maximal value at $c = 0$ to the minimal value at c_{mp}. The decrease in resolution over the whole concentration range is similar to that in (1), but there the elution sequence was reversed in relation to the values of n.

A practical chromatographic system involving the separation of two compounds can easily be attributed to one of the above instances by comparing the known value of c_{mp} with calculated values of $c_{(\alpha=1)}$ (eqn. 11) and c_{max} (eqn. 13). If $c_{(\alpha=1)} < 0$ or $c_{max} < 0$, the eqns. 11 and 13 give no solution (instances 4–6).

The maximal resolution that can be obtained in a given system (or the maximal tolerable ratio $R_s/\sqrt{N}$) can be calculated from eqn. 10 by introducing $c = 0$, $c = c_{max}$ or $c = c_{mp}$. This calculated value of the ratio $R_s/\sqrt{N}$ must not be exceeded, otherwise eqn. 10 yields no solution or no solution with a practical meaning.

The concentration necessary in order to obtain a required resolution can be calculated after rearranging eqn. 10 into the following two forms:

$$c = \frac{1}{b_1} \cdot \left[\left(1 - \frac{4R_s}{\sqrt{N_2}}\right)(a_2 + b_2 c)^{-n_2} - \frac{4R_s}{\sqrt{N_2}} \right]^{-\frac{1}{n_1}} - \frac{a_1}{b_1} \tag{14}$$

and

$$c = \frac{1}{b_2} \cdot \left\{ \frac{\sqrt{N_2}}{4R_s} \left[1 - \frac{(a_2 + b_2 c)^{n_2}}{(a_1 + b_1 c)^{n_1}} \right] - 1 \right\}^{\frac{1}{n_2}} - \frac{a_2}{b_2} \tag{14a}$$

If the compounds are numbered so that $n_1 > n_2$, the solutions of eqns. 14 and 14a give the concentrations for the required R_s at each side from the maximum R_s ($R_{s\,max}$) on the R_s *versus* c curve. Eqns. 14 and 14a cannot be expressed in an explicit form and mathematical solution by an approximation method is required. Eqn. 14 gives the concentration for the required R_s on the lower concentration side from the maximum on the R_s *versus* c curve, while eqn. 14a gives the corresponding concentration higher than c_{max}. The values of the first approximation of concentration used when eqns. 14 and 14a are being solved must be chosen from the appropriate region in order to obtain a solution. Hence the first approximation, c_I, for eqn. 14 should be chosen so that $c_{(\alpha=1)} < c_I \leqslant c_{max}$ and c_I for eqn. 14a should not be lower than c_{max}; $c_I \geqslant c_{max}$.

If we use eqn. 14a with the inverse sequence of the parameters n, *i.e.*, $n_1 < n_2$, we obtain the solution for the left branch of the R_s *versus* c curve [for concentrations lower than $c_{(\alpha=1)}$]. Here again, the first approximation c_I should be chosen so that $0 < c_I < c_{(\alpha=1)}$.

In all calculations using eqns. 14 and 14a, care must be taken that the value of $R_s/\sqrt{N_2}$ does not fall outside the range limited by the minimal and maximal values given by concentrations $c = 0$, c_{max} and c_{mp} (this range should be considered individually in each of the instances 1–6).

In some instances, when the numbers of plates for the two compounds 1 and 2

being chromatographed differ considerably, the simplified definition of resolution according to eqn. 9 may no longer be satisfactory and a more rigorous equation for resolution should be considered:

$$R_s = 2 \cdot \frac{V_{R2} - V_{R1}}{w_2 + w_1} \tag{15}$$

Introducing eqn. 5, we can derive the relationship between resolution and the concentration of the more efficient eluting agent in the mobile phase in the following form:

$$R_s = \frac{(a_2 + b_2c)^{-n_2} - (a_1 + b_1c)^{-n_1}}{2\,[N_2^{-\frac{1}{2}} + N_2^{-\frac{1}{2}}(a_2 + b_2c)^{-n_2} + N_1^{-\frac{1}{2}} + N_1^{-\frac{1}{2}}(a_1 + b_1c)^{-n_1}]} \tag{16}$$

It can be shown that eqn. 11 for $c_{(\alpha=1)}$ and eqn. 13 for c_{max} apply in this instance as well as for the simplified definition of resolution. All of the other considerations and conclusions also remain valid, only eqns. 14 and 14a for the concentration at which a required resolution can be achieved acquire somewhat altered forms; eqn. 17 must be used instead of eqn. 14 and eqn. 17a instead of eqn. 14a:

$$c = \frac{1}{b_1}\left[\frac{1 + \frac{2R_s}{\sqrt{N_1}}}{(a_2 + b_2c)^{-n_2}\left(1 - \frac{2R_s}{\sqrt{N_2}}\right) - 2R_s\left(\frac{1}{\sqrt{N_2}} + \frac{1}{\sqrt{N_1}}\right)}\right]^{\frac{1}{n_1}} - \frac{a_1}{b_1} \tag{17}$$

and

$$c = \frac{1}{b_2}\left[\frac{1 - \frac{2R_s}{\sqrt{N_2}}}{(a_1 + b_1c)^{-n_1}\left(1 + \frac{2R_s}{\sqrt{N_1}}\right) + 2R_s\left(\frac{1}{\sqrt{N_2}} + \frac{1}{\sqrt{N_1}}\right)}\right]^{\frac{1}{n_2}} - \frac{a_2}{b_2} \tag{17a}$$

If the compounds are very strongly retained on the column in the mobile phase containing only the pure less efficient eluting agent, then a in eqn. 3 is close to zero and can be neglected. Then, eqn. 1 can be used to describe the relationship between the capacity ratio, k', and the concentration, c, of the more efficient eluting agent in the mobile phase. Taking into account the simplified definition of resolution (eqn. 9), we can write the relationship between resolution, R_s, and concentration, c, in the following form:

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \frac{k'_{02}c^{-n_2} - k'_{01}c^{-n_1}}{1 + k'_{02}c^{-n_2}} \tag{18}$$

or, after rearrangement:

$$R_s = \underset{\text{I}}{\frac{\sqrt{N_2}}{4}} \cdot \underset{\text{II}}{(1 - \alpha^{-1})} \cdot \underset{\text{III}}{\frac{k'_{02}}{k'_{02} + c^{n_2}}} \tag{18a}$$

where

$$\alpha = \frac{k'_{02}}{k'_{01}} \cdot c^{(n_1 - n_2)} = \alpha_0 c^{(n_1 - n_2)} \tag{19}$$

and terms I (efficiency), II (selectivity) and III (capacity) are as in eqn. 10a.

All of the considerations concerning the influence of concentration on resolution remain unchanged, except that the equations for $c_{(\alpha=1)}$ and c_{max} acquire the following forms:

$$c_{(\alpha=1)} = \alpha_0^{\frac{1}{n_2 - n_1}} \tag{20}$$

and

$$c_{max} = \left(\alpha_0^{-1} \cdot \frac{n_1}{n_2} \cdot c_{max}^{n_2} + k'_{01} \cdot \frac{n_1 - n_2}{n_2} \right)^{\frac{1}{n_1}} \tag{21}$$

Eqn. 21 must be solved by an approximation method.

The two compounds are very strongly retained on the column if $c = 0$, and therefore the resolution at $c = 0$ has no practical meaning. To calculate the maximal practical value of the resolution on the lower concentration side from $c_{(\alpha=1)}$ of the R_s *versus* c curve, the resolution (and the minimal practical concentration) must be calculated on the basis of the maximal value of k' ($k' = 20$, for instance) which can be allowed considering the time of analysis.

The values of k' for the two compounds become extremely large as the concentration c is decreased to zero ($k'_1 \to \infty$, $k'_2 \to \infty$). Hence any intersection of the two $\log k' = f(\log c)$ lines must be found in the region where $c \geqslant 0$. In this instance, the number of different situations is limited to the instances 1–3 (see above discussion and Figs. 5–8), as the situations shown in instances 4–6 are not possible.

The concentration necessary in order to obtain a required resolution can be calculated from eqns. 22 [$c_{(\alpha=1)} < c < c_{max}$] and 22a ($c > c_{max}$) if $n_1 > n_2$, and from eqn. 22a [$c < c_{(\alpha=1)}$] if $n_2 > n_1$:

$$c = \left[\left(1 - \frac{4R_s}{\sqrt{N_2}} \cdot \frac{k'_{02} + c^{n_2}}{k'_{02}} \right) \alpha_0 \right]^{\frac{1}{n_2 - n_1}} \tag{22}$$

$$c = \left\{ \left[\frac{\sqrt{N_2}}{4R_s} (1 - \alpha_0^{-1} c^{(n_2 - n_1)}) - 1 \right] k'_{02} \right\}^{\frac{1}{n_2}} \tag{22a}$$

If the more correct definition equation for R_s is considered (eqn. 15), then eqns. 22 and 22a acquire the following forms:

$$c = \left[\frac{k'_{01}\left(1 + \frac{2R_s}{\sqrt{N_1}}\right)}{c^{-n_2} k'_{02}\left(1 - \frac{2R_s}{\sqrt{N_2}}\right) - 2R_s\left(\frac{1}{\sqrt{N_2}} + \frac{1}{\sqrt{N_1}}\right)} \right]^{\frac{1}{n_1}} \tag{23}$$

and

$$c = \left[\frac{k'_{02}\left(1 - \frac{2R_s}{\sqrt{N_2}}\right)}{c^{-n_1}k'_{01}\left(1 + \frac{2R_s}{\sqrt{N_2}}\right) + 2R_s\left(\frac{1}{\sqrt{N_2}} + \frac{1}{\sqrt{N_1}}\right)} \right]^{\frac{1}{n_2}} \tag{23a}$$

Assuming that eqn. 1 applies and the two parameters n are close one to another ($n_1 \approx n_2 \approx n$), the separation factor, α, does not depend on concentration:

$$\alpha = \frac{k'_{02}}{k'_{01}} = \alpha_0 \tag{24}$$

and the equation for resolution is further simplified:

$$R_s = \underset{\text{I}}{\frac{\sqrt{N_2}}{4}} \cdot \underset{\text{II}}{(1 - \alpha_0^{-1})} \cdot \underset{\text{III}}{\frac{k'_{02}}{k'_{02} + c^{n_2}}} \tag{25}$$

Here, the selectivity term II is constant and a change in concentration can influence the resolution by means of the capacity term III only. In this situation, no intersection of the two lines $\log k'_2 = \mathrm{f}(\log c)$ and $\log k'_1 = \mathrm{f}(\log c)$ can be found in the whole concentration range and the elution sequence is given by the sequence of the values of k'_0. Hence, the compound with a larger k'_0 value is eluted later and should have the subscript 2 ($k'_{02} > k'_{01}$). The resolution decreases with increasing concentration over the whole concentration range from the maximal value at $c = 0$ to the minimal value at c_{mp}, as in instance 1 (Fig. 5); the two parallel lines $\log k' = \mathrm{f}(\log c)$ also decrease with increasing concentration. The concentration necessary in order to obtain a required resolution can be calculated by solving the following equation, which is expressed in an explicit form:

$$c = \left\{ \frac{\sqrt{N_2}}{4R_s}\left[k'_{02}\left(1 - \frac{4R_s}{\sqrt{N_2}}\right) - k'_{01}\right] \right\}^{\frac{1}{n}} \tag{26}$$

[the maximal value of $R_s/\sqrt{N_2}$ must not exceed $(k'_{02} - k'_{01})/4\,k'_{02}$].

Considering the more correct definition of R_s (eqn. 15), eqn. 26 acquires the form:

$$c = \left[\frac{k'_{02} - k'_{01} - 2R_s\left(\frac{k'_{01}}{\sqrt{N_1}} + \frac{k'_{02}}{\sqrt{N_2}}\right)}{2R_s\left(\frac{1}{\sqrt{N_1}} + \frac{1}{\sqrt{N_2}}\right)} \right]^{\frac{1}{n}}. \tag{27}$$

In all of the above relationships and derivations, the number of theoretical plates, N, is assumed not to depend on the composition of the mobile phase. This condition may not be strictly true and the number of plates can depend to some

extent on the capacity ratios of sample compounds. If the form and the constants of the relationship between N and k' are known, this relationship can be introduced into the above equations instead of a constant value of N. Thus, if a linear relationship between the number of plates and capacity ratio of compounds to be separated can be accepted[5], such as

$$N = C + Dk' = C + D(a + bc)^{-n} \tag{28}$$

eqn. 28 can be used for substituting N. This approach leads to more complex forms of the above derived equations; however, this is not a serious drawback if we consider that the solution is to be made by some approximation method in any case.

PRACTICAL EXAMPLES

A few practical examples will be given in order to demonstrate the applicability of the above theoretical considerations.

The first example[11] is the separation of 3-chloro-5-nitro-4-hydroxydiphenyl (compound A) and 2-fluorophenol (compound B) on a 904 × 2.3 mm column packed with Porasil A (37–75 μm), using mixtures of *n*-propanol and *n*-heptane as the mobile phase. The conditions of separation are given in Table I, together with experimentally found values of k'_0 and n. The two compounds are so strongly retained in pure *n*-heptane that $a_1 \approx a_2 \approx 0$ and eqn. 1 applies. The number of theoretical plates for the two compounds does not change significantly with concentration. In this system, $c_{mp} = 1$ (pure *n*-propanol), $c_{(a=1)} = 0.0278$ (calculated from eqn. 20) and $c_{max} = 1.7415$ (calculated from eqn. 21). Hence $0 < c_{(a=1)} < c_{mp} < c_{max}$, which corresponds to instance 2. In mobile phases that contain less than 2.78 vol.-% of *n*-propanol, compound A is eluted first, while the elution sequence is reversed in mobile phases that contain higher amounts of *n*-propanol. The resolution $R_s = 1.0$ can be obtained at two different concentrations, $c_1 = 0.0197$ and $c_2 = 0.0413$, as calculated from eqns. 22 and 22a, respectively. The experimental and calculated

TABLE I

EXPERIMENTAL PARAMETERS n, k'_0 AND N, CALCULATED VALUES OF IMPORTANT CONCENTRATIONS OF *n*-PROPANOL IN *n*-HEPTANE AND CORRESPONDING RESOLUTION AND OTHER RETENTION CHARACTERISTICS IN CHROMATOGRAPHY OF 3-CHLORO-5-NITRO-4-HYDROXYDIPHENYL (COMPOUND A) AND 2-FLUOROPHENOL (COMPOUND B) ON A COLUMN PACKED WITH PORASIL A, 37–75 μm (904 × 2.3 mm; $V_m = 3.05$ ml)

Conditions: flow-rate of mobile phase, 1.07 ml/min; pressure, 2.5 MPa; instrument, Waters ALC-100; detection, UV (254 nm). $n_A = 0.045$; $k'_{0A} = 1.206$; $N_A \approx 500$; $n_B = 0.968$; $k'_{0B} = 0.0441$; $N_B \approx 500$. Instance 2; $0 < c_{(a=1)} < c_{mp} < c_{max}$.

c (vol. % × 10^{-2})	$R_{s_{A,B}}$	V_{R_A} (ml)	V_{R_B} (ml)	w_A (ml)	w_B (ml)
0.0197	1.000	7.43	9.05	1.33	1.62
0.0278 [$c_{(a=1)}$]	0.000	7.37	7.37	1.32	1.32
0.0413	1.000	7.29	5.99	1.30	1.07
1.0000 (c_{mp})	2.94	6.72	3.18	1.20	0.57
1.7415 (c_{max})	2.955	—	—	—	—

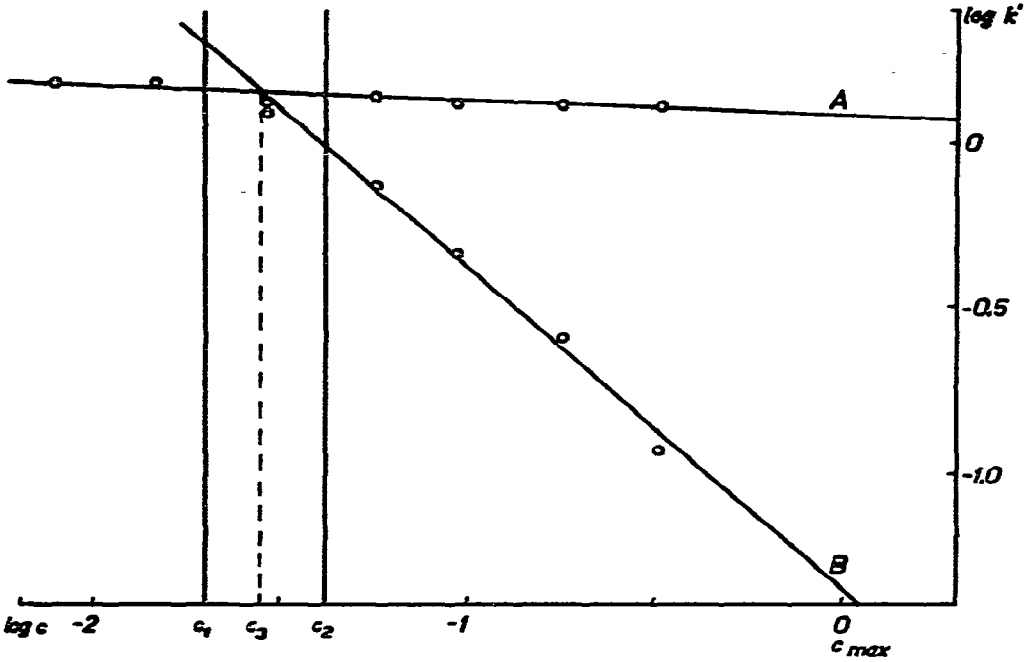

Fig. 9. Relationship between capacity ratios (k') of 3-chloro-5-nitro-4-hydroxydiphenyl (A) and 2-fluorophenol (B) and concentration (c, vol.-% $\times 10^{-2}$) of n-propanol in n-heptane used as the mobile phase. The points represent experimental values; the lines were evaluated using linear regression analysis. Operating conditions as in Table I.

plots of log k' *versus* log c for compounds A and B are shown in Fig. 9, together with concentrations c_1, c_2 and $c_3 = c_{(\alpha=1)}$.

The second example concerns the chromatography of the steroids compounds[9] lumisterol (compound A) and tachysterol (compound B) on a column packed with LiChrosorb ALOX T alumina (20 μm), also in a mobile phase composed of n-heptane and n-propanol, with a resolution $R_s = 1.5$. Here $a_1 \approx a_2 \approx 0$ and eqn. 1 again applies. The number of theoretical plates does not change significantly with variation in the composition of the mobile phase. The conditions of separation are given in Table II, together with experimentally found values of n and k'_0 for compounds A and B. Here, $c_{mp} = 1$ (pure n-propanol), $c_{(\alpha=1)} = 0.00002$ (calculated from eqn. 20) and $c_{max} = 0.00206$ (calculated from eqn. 21). Hence $0 < c_{(\alpha=1)} < c_{max} < c_{mp}$, which corresponds to instance 3. The resolution $R_s = 1.5$ cannot be achieved for $c < c_{(\alpha=1)}$ and the elution sequence where tachysterol is eluted ahead of lumisterol is not practical owing to extremely large retention volumes. $R_s = 1.5$ can be achieved at two concentrations (one on each side of the maximum on the R_s *versus* c curve), which are calculated from eqns. 22 and 22a: $c_1 = 0.00078$ and $c_2 = 0.00206$. Other retention characteristics under these conditions are given in Table II. The R_s *versus* c curve is plotted in Fig. 10 as the curve for $R_{s1,2}$ (the curve is calculated on basis of the values of n_A, n_B, k'_{0A}, k'_{0B} and N_B; the experimental values of R_s are plotted as points).

In the third example, tachysterol and calciferol are to be separated with a

TABLE II

EXPERIMENTAL PARAMETERS n, k' AND N, CALCULATED VALUES OF IMPORTANT CONCENTRATIONS OF n-PROPANOL IN n-HEPTANE AND CORRESPONDING RESOLUTION AND OTHER RETENTION CHARACTERISTICS IN CHROMATOGRAPHY OF LUMISTEROL (COMPOUND A) AND TACHYSTEROL (COMPOUND B) ON A COLUMN PACKED WITH LICHROSORB ALOX T, 30 μm (596 × 2.3 mm; V_m = 2.30 ml).

Conditions: flow-rate of mobile phase, 1.93 ml/min; pressure, 5.0 MPa; instrument, Waters ALC-100; detection, UV (254 nm). $n_A = 1.463$; $k'_{0A} = 0.000428$; $N_A = 131$; $n_B = 1.284$; $k'_{0B} = 0.00298$; $N_B = 165$. Instance 3; $0 < c_{(\alpha=1)} < c_{max} < c_{mp}$.

c (vol. % × 10^{-2})	$R_{s_{A,B}}$	V_{R_A} (ml)	V_{R_B} (ml)	w_A (ml)	w_B (ml)
0.0000195 [$c_{(\alpha=1)}$]	0.000	7653	7653	2675	2386
0.000783	1.500	36.78	69.09	12.85	21.54
0.002064 (c_{max})	1.622	10.65	21.54	3.72	6.72
0.004596	1.500	4.89	9.18	1.71	2.86
1.0000 (c_{mp})	0.008	2.30	2.31	0.80	0.72

resolution $R_s = 1.0$ under the same conditions (column, mobile phase, flow-rate) as in the second example. Here again, $a_1 \approx a_2 \approx 0$ and eqn. 1 applies. The conditions of separation and the parameters necessary for calculations are given in Table III. The calculated values are $c_{(\alpha=1)} = 0.00013$ (eqn. 20) and $c_{max} = 0.0047$ (eqn. 21); $c_{mp} = 1$ and $0 < c_{(\alpha=1)} < c_{max} < c_{mp}$, which is again the situation referred to in instance 3, as in the second example. The maximal resolution that can be obtained at c_{max} is 0.769 [operation at $c < c_{(\alpha=1)}$ involves extremely long retention times and is not practical] and therefore the required resolution cannot be achieved with the plate number given in the conditions of the experiments. If a resolution $R_s = 0.75$ is accepted, this resolution is achieved at $c_1 = 0.00308$ and $c_2 = 0.00701$ (eqns. 22

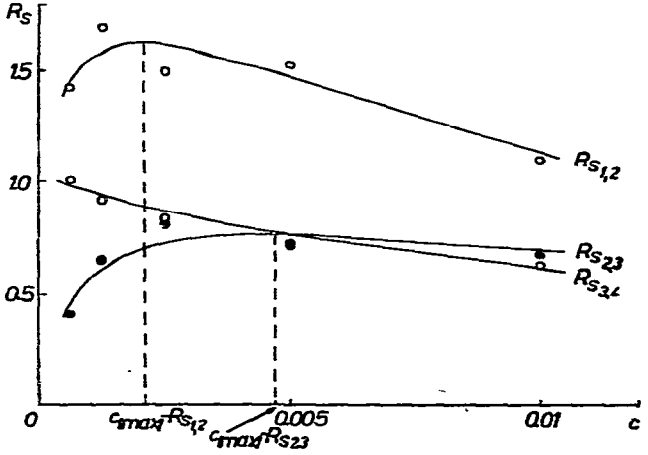

Fig. 10. Relationships between resolution (R_s; defined by eqn. 9) of steroids and concentration (c, vol.-% × 10^{-2}) of n-propanol in n-heptane used as the mobile phase. $R_{s1,2}$ = resolution of lumisterol and tachysterol; $R_{s2,3}$ = resolution of tachysterol and calciferol; $R_{s3,4}$ = resolution of calciferol and ergosterol; c_{max} = concentration corresponding to the maximum on the R_s *versus* c curve. The points represent experimental values; the curves were calculated from the parameters n_A, n_B, k'_{0A}, k'_{0B}, N_A and N_B (eqn. 18a). Operating conditions as in Table II.

and 22a). Hence in the concentration range from 0.3 to 0.7% of *n*-propanol, the resolution does not change significantly and is near to the maximal value. The corresponding R_s *versus* c curve is plotted in Fig. 10 as the curve for $R_{s2,3}$ and the calculated retention characteristics are given in Table III.

TABLE III

EXPERIMENTAL PARAMETERS n, k' AND N, CALCULATED VALUES OF IMPORTANT CONCENTRATIONS OF *n*-PROPANOL IN *n*-HEPTANE AND CORRESPONDING RESOLUTION AND OTHER RETENTION CHARACTERISTICS IN CHROMATOGRAPHY OF TACHYSTEROL (COMPOUND A) AND CALCIFEROL (COMPOUND B) ON A COLUMN PACKED WITH LICHROSORB ALOX T.

Operating conditions as in Table II. $n_A = 1.284$; $k'_{0A} = 0.00298$; $N_A = 165$; $n_B = 1.189$; $k'_{0B} = 0.00698$; $N_B = 175$. Instance 3, $0 < c_{(\alpha=1)} < c_{max} < c_{mp}$.

c (vol. % $\times 10^{-2}$)	$R_{s_{A,B}}$	V_{R_A} (ml)	V_{R_B} (ml)	w_A (ml)	w_B (ml)
0.0001289 ($c_{(\alpha=1)}$)	0.000	679.4	679.4	211.8	205.4
0.003082	0.750	13.80	17.85	4.30	5.40
0.004697 (c_{max})	0.769	8.99	11.72	2.80	3.54
0.007007	0.750	6.30	8.15	1.96	2.46
1.0000 (c_{mp})	0.013	2.31	2.32	0.72	0.70

The last example concerns the separation of calciferol and ergosterol under the same conditions as in the two preceding examples. The parameters $a_1 \approx a_2 \approx 0$ and calculations based on eqn. 1 were used. The conditions of separation and the parameters necessary for the calculations are given in Table IV. Eqn. 20 yielded the concentration $c_{(\alpha=1)} = 1{,}070{,}600$ and thus it was not necessary to calculate c_{max}. Here, $0 < c_{mp} < c_{(\alpha=1)} < c_{max}$, which is the situation in the instance 1. The resolution decreases with increasing concentration over the whole concentration range, as is shown in Fig. 10 (curve for $R_{s3,4}$). Thus, $R_s = 0.75$ can be achieved at $c = 0.00536$.

If the four compounds lumisterol, tachysterol, calciferol and ergosterol are to be separated on the same column packed with LiChrosorb ALOX T, the optimal composition of the mobile phase can be chosen on basis of the above calculated values. The resolution of lumisterol and tachysterol is higher than that of tachysterol and calciferol and that of calciferol and ergosterol over the whole practical concentration range and, as the first two compounds are eluted first, their separation does not need

TABLE IV

EXPERIMENTAL PARAMETERS n, k'_0 AND N, CALCULATED VALUES OF IMPORTANT CONCENTRATIONS OF *n*-PROPANOL IN *n*-HEPTANE AND CORRESPONDING RESOLUTION AND OTHER RETENTION CHARACTERISTICS IN CHROMATOGRAPHY OF CALCIFEROL (COMPOUND A) AND ERGOSTEROL (COMPOUND B) ON A COLUMN PACKED WITH LICHROSORB ALOX T

Operating conditions as in Table II. $n_A = 1.189$; $k'_{0A} = 0.00698$; $N_A = 175$; $n_B = 1.205$; $k'_{0B} = 0.00872$; $N_B = 190$. Instance 1, $0 < c_{mp} < c_{(\alpha=1)} < c_{max}$.

c (vol. % $\times 10^{-2}$)	$R_{s_{A,B}}$	V_{R_A} (ml)	V_{R_B} (ml)	w_A (ml)	w_B (ml)
0.005362	0.750	10.34	13.22	3.13	3.84
1.0000 (c_{mp})	0.006	2.32	2.32	0.70	0.67
1,070 600 [$c_{(\alpha=1)}$]	0.000	—	—	—	—

to be considered. The resolution of tachysterol and calciferol is in the range 0.75–0.77 for concentrations of 0.3–0.7 vol.-%. The same resolution of calciferol and ergosterol can be achieved at $c = 0.005$. This concentration, *i.e.*, 0.5% *n*-propanol in *n*-heptane, is acceptable for separation, as the same resolution is achieved for the last three eluted compounds. To increase the resolution, a longer column or a lower flow-rate of the mobile phase should be used. Fig. 11 shows the separation of a mixture containing lumisterol (3), tachysterol (5) and calciferol (6) in 0.5% (A), 0.25% (B) and 0.125% *n*-propanol (C) in *n*-heptane. The improved resolution of compounds 5 and 6 and the impaired resolution of compounds 3 and 5 with increasing concentration of *n*-propanol in the mobile phase is clearly demonstrated (for comparison, see Fig. 10).

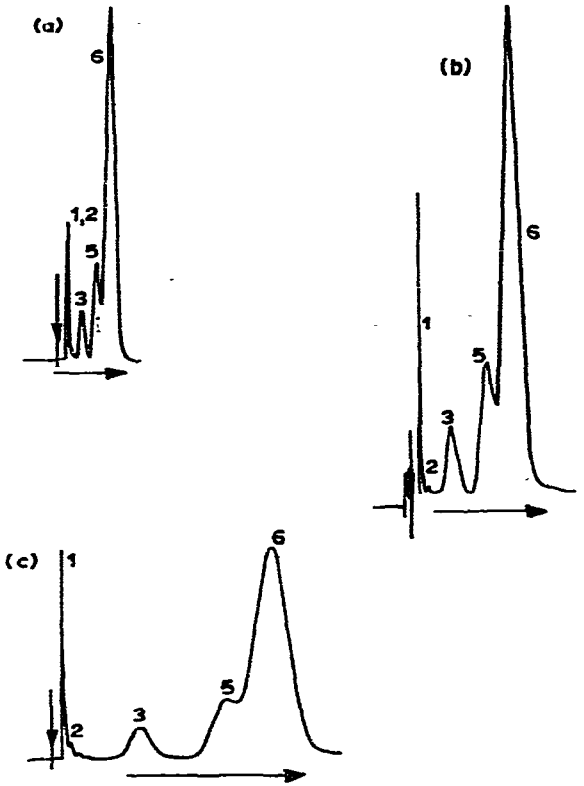

Fig. 11. Chromatographic separation of a mixture containing lumisterol (3; *ca.* 5 μg), tachysterol (5; *ca.* 8 μg) and calciferol (6; *ca.* 30 μg) on LiChrosorb ALOX T in a mobile phase containing different concentrations of *n*-propanol in *n*-heptane (A, 0.5 vol.-%; B, 0.25 vol.-%; C, 0.125 vol.-%). Compounds 1 and 2 are impurities. Flow-rate of mobile phase, 1.93 ml/min; chart speed, 2.5 mm/min; detector sensitivity, 0.64 a.u.f.s. (A) and 0.32 a.u.f.s. (B and C). Other conditions as in Table II.

CONCLUSIONS

It has been demonstrated that the experimentally found dependence of the resolution of two compounds on the concentration of the more efficient eluting agent in the mobile phase can acquire various shapes in the practically available concentration

range. Depending on the values of the parameters a, b (or k_0') and n, different sequences of four important concentrations occur, according to which the R_s *versus* c curve may be descending or ascending or may have a maximum or a minimum. These four important concentrations are as follows: $c = 0$; $c_{(\alpha=1)}$, the concentration at which $\alpha = 1$; c_{max}, the concentration at which the maximum on the R_s *versus* c curve is reached; and c_{mp}, the highest practically available concentration. These concentrations and the corresponding resolution should be calculated prior to any further theoretical predictions of the influence of c on R_s. The methods of calculation differ, depending on whether $a = 0$ for $a \neq 0$ and $n_1 \neq n_2$ or $n_1 = n_2$. If the required resolution is within the practically allowed limits ($R_{s\,min} < R_s < R_{s\,max}$), it is possible to calculate the concentration (or concentrations) at which it can be reached. In the chromatography of a multicomponent mixture, comparison of the concentration regions in which the resolution of two neighbouring compounds is achieved can lead to a rational choice of the composition of the mobile phase, which permits good separations in a reasonable time.

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