Journal of Chromatography, 214 (1981) 35–46
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,945

GRADIENT ELUTION IN LIQUID CHROMATOGRAPHY

XIV. THEORY OF TERNARY GRADIENTS IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

PAVEL JANDERA* and JAROSLAV CHURÁČEK
Department of Analytical Chemistry, University of Chemical Technology, Pardubice (Czechoslovakia)
and
HENRI COLIN
Laboratoire CAP, École Polytechnique, Palaiseau (France)

(Received April 29th, 1981)

SUMMARY

The theory of ternary gradients in reversed-phase liquid chromatography, which makes it possible to calculate retention volumes, is presented. These calculations are also possible for isocratic elution using ternary solvent mixtures. The calculation procedure for retention volumes was verified experimentally and the agreement between the experimental and theoretical values was satisfactory. The results show that the calculation procedure may be suitable for the prediction of retention volumes in reversed-phase chromatography using ternary mobile phase gradients. The application of ternary gradients can improve the separation of certain solute mixtures with respect to the binary gradients. An example of the separation of phenols is discussed, where the optimal ternary gradient could be predicted theoretically. Conventional gradient elution equipment which makes possible the mixing of two liquids only can be used for work with ternary gradients also.

INTRODUCTION

Ternary mobile phases in liquid chromatography under isocratic or gradient elution conditions offer possibilities for improving the selectivity of separations[1,2]. With ternary gradients, it should be possible to adjust the retention and selectivity independently of each other to a certain extent and to change both the selectivity and retention with time more efficiently than when using binary gradients[1]. However, the phenomena in ternary solvent mixtures are, of course, more complex than in binary mobile phases. It seems that for this reason the use of ternary solvent gradients has not been studied in a systematic manner and no theoretical treatment of such gradients has been presented, as far as we know.

The theoretical treatment of gradient elution with ternary gradients can be useful in providing a better understanding of the retention in such systems and to

0021-9673/81/0000–0000/$02.50

allow the prediction of retention volumes either for better identification of sample solutes or for optimization of the separation of complex mixtures. It would be especially useful for reversed-phase systems using chemically bonded non-polar stationary phases, as these systems are at present the most frequently used in practical liquid chromatography.

In the present work, the theory of ternary gradients in reversed-phase liquid chromatography has been considered and verified. The components of the mobile phase are assumed to interact independently of each other and, under this assumption, the model presented previously for binary mobile phase gradients[3–6] is extended to describe gradient elution using ternary gradients of two different organic solvents in water.

THEORETICAL

Let us consider chromatographic behaviour in ternary mobile phases used in reversed-phase chromatography on chemically bonded non-polar materials, such as octadecyl- or octylsilica. Here, the selectivity and solvent strength of the mobile phase can be more or less conveniently adjusted by the simultaneous control of the concentrations of two different organic solvents, x and y, in aqueous solution[1]. If only a binary mobile phase containing one of each of the organic solvents in water is used, well known and widely accepted equations[4,7–12] may be written for the influence of the composition of each of the two solvent binaries on the solute capacity ratio, k':

$$\log k'_{(x)} = \log k'_{0x} - m_x c_x \tag{1}$$

and

$$\log k'_{(y)} = \log k'_{0y} - m_y c_y \tag{2}$$

where $k'_{(x)}$ and $k'_{(y)}$ are capacity ratios in the binary mobile phase containing the organic solvent x or y, at a concentration c_x or c_y (where c_x or c_y = volume $\% \cdot 10^{-2}$), respectively; k'_{0x}, k'_{0y}, m_x and m_y are experimental constants which depend on the solute and on the organic solvent but not on the concentration of the organic solvent in the mobile phase. Ideally, k'_{0x} and k'_{0y} should have the meaning of the capacity ratio in pure water ($c_x = 0$ or $c_y = 0$) and consequently

$$k'_{0x} = k'_{0y} = k'_0 \tag{3}$$

If we consider the eqn. 1–3 and assume that the two organic solvents interact with solutes virtually independently of one another, their influence on the retention is additive and we can derive an equation for k' in a ternary mobile phase containing the solvent x at a concentration c_x and the solvent y at a concentration c_y:

$$\log k' = \log k'_0 - m_x c_x - m_y c_y \tag{4}$$

This equation could theoretically be used to describe the retention in ternary mobile phases[14]. The difficulty in the practical utilization of eqn. 4 is caused by the significant differences between the values of k'_{0x} and k'_{0y} as often found by linear regressions of the experimental $\log k'_{(x)}$ *versus* c_x and $\log k'_{(y)}$ *versus* c_y plots in x–H_2O and y–$H-_2O$ binary solvent systems:

These differences could probably be explained by possible deviations from the linear relationships between log k' and c, as given by eqns. 1 and 2, in the regions close to 0% (and to 100%) of the organic solvent in the aqueous binary mobile phase[13]. Hence, the linear plots would approximate only a part of these curved plots in the range of practically useful k' values and consequently, even though this approximation may be very good (correlation coefficient 0.9999), it would yield false values for k'_0 as k' in pure water.

To overcome this difficulty, an average value of k'_0, $\bar{k}_0$, in a given ternary mobile phase should be introduced instead of k'_0 in eqn. 4, as follows:

$$\log \bar{k}_0 = \frac{c_x \log k'_{0x} + c_y \log k'_{0y}}{c_x + c_y} \tag{5}$$

and eqn. 4 should be written as

$$\log k' = \log \bar{k}_0 - m_x c_x - m_y c_y \tag{6}$$

[It should be noted that a good validity of an empirical relationship:

$$\log k'_{(A+B)} = c_A \log k'_A + c_B \log k'_B \tag{7}$$

was found for capacity ratios $k'_{(A+B)}$ in ternary mixtures formed by mixing two binary mixtures A and B in the ratio c_A:c_B, while each of the binary mixtures contains a different organic solvent in water and k'_A and k'_B are k' in the original solvent binaries A and B, respectively[14].]

Eqn. 6 can be used for calculation of solute retentions (k') in various ternary mixtures. Its practical verification for methanol–acetonitrile–water ternary systems is given below.

Eqns. 4 and 6 can be also used as the basis for the derivation of an equation that would allow calculations of retention volumes in reversed-phase chromatography using ternary gradients. Here, both the concentrations of the solvent x and of the solvent y are changed simultaneously with time during the elution. Linear concentration gradients are very useful in reversed-phase chromatography[4,10–13] for a number of reasons: (a) they approximate well so-called "linear solvent strength" gradients, which should yield the optimal resolution of unknown solute mixtures; (b) they are simple to form, even with relatively simple instrumentation; (c) they are easy to understand, optimize and treat theoretically. Therefore, we shall limit our discussion to linear, ternary solvents gradients, where the concentrations of the two organic solvents x and y in the ternary mobile phase, c_x and c_y, respectively, are changed linearly with time, or with the volume of the eluate, V, according to the following equations:

$$c_x = A_x + B_x V \tag{8}$$

and

$$c_y = A_y + B_y V \tag{9}$$

where A_x, B_x, A_y and B_y are adjustable experimental constants that can be chosen independently of each other.

As with binary solvent gradients, the solution for the net retention volume of a solute, V'_R, in chromatography with ternary gradients should be based on the fundamental equation of gradient elution chromatography:

$$V_m = \int_0^{V'_R} \frac{\mathrm{d}V}{k'} \tag{10}$$

If we assume the validity of eqn. 4, after introducing eqns. 4, 8 and 9 into eqn. 10 we obtain the following solution for V'_R:

$$V'_R = \frac{1}{m_xB_x + m_yB_y} \cdot \log\left[2.31\, k'_0 V_m (m_xB_x + m_yB_y) + 10^{(m_xA_x + m_yA_y)}\right] - \frac{m_xA_x + m_yA_y}{m_xB_x + m_yB_y} \tag{11}$$

where V_m is the column dead volume (volume of the mobile phase in the column). Eqn. 11 is formally almost identical with an analogous equation derived previously for binary linear concentration gradients in reversed-phase chromatography[3,4], but $m_xA_x + m_yA_y$ is used here instead of mA and $m_xB_x + m_yB_y$ instead of mB.

As under isocratic elution conditions, eqn. 11 can hardly be used in this form in practice because of differences in the experimental values of k'_{0x} and k'_{0y} for most solutes. If we try to overcome this difficulty by introducing $\bar{k}_0$ as defined by eqn. 5 into eqn. 10 together with eqns. 6, 8 and 9, we obtain an expression that is impossible to integrate to yield an algebraic expression, and numerical integration should be used for solution. Instead, we preferred to simplify the solution by using eqn. 11 for V'_R under gradient conditions and to substitute k'_0 by an average value $\bar{k}_{0g}$, which represents an average value of $\bar{k}_0$ during the elution of the solute ($\bar{k}_0$ changes during the course of the gradient elution):

$$\log \bar{k}_{0g} = \frac{\left(A_x + B_x \cdot \frac{V'_R}{2}\right) \log k'_{0x} + \left(A_y + B_y \cdot \frac{V'_R}{2}\right) \log k'_{0y}}{A_x + A_y + (B_x + B_y) \cdot \frac{V'_R}{2}} \tag{12}$$

After introducing $\bar{k}_{0g}$ from eqn. 12 for k'_0 into eqn. 11, we obtain an implicit equation for V'_R, which should be solved by an iteration method. The calculation of V'_R, even with the use of a small programmable calculator, such as a TI 58, is very rapid and requires about 1 min for four or five iterations, which are sufficient to obtain a precision of calculation of better than 0.1 % relative.

EXPERIMENTAL

Gradient elution instrumentation

Sophisticated instrumentation making possible the mixing of three (or more) components of the mobile phase from the same number of reservoirs containing pure mobile phase components via proportioning valves or auxiliary pumping systems are, of course, most convenient and easy to use to perform gradient elution with ternary mobile phase gradients[1,2]. However, a wide variety of these gradients is possible using simple conventional devices with the possibility of mixing two different solvents only. Above all, this holds for linear concentration gradients.

Let each of the two reservoirs contain different concentrations of two organic solvents, x and y, in water, *e.g.*, solution A in the reservoir 1 contains the organic solvent x at a concentration c_{Ax} and solvent y at a concentration c_{Ay} and the concentrations of x and y in solution B in the reservoir 2 are c_{Bx} and c_{By}, respectively. Let us apply a linear concentration gradient of B in the mixed mobile phase:

$$c_B = A + BV \tag{13}$$

(A and B in eqn. 13 are constants of the gradient function used). c_B and c_A are the concentrations of B and A in the mixed final ternary mobile phase chosen in such way that

$$c_A + c_B = 1 \tag{14}$$

Then, the concentrations of x and y in the mixed mobile phase at the outlet from the gradient mixer (c_x and c_y, respectively) follow the linear relationships

$$c_x = A_x + B_x V \tag{15}$$

and

$$c_y = A_y + B_y V \tag{16}$$

where A_x, B_x, A_y and B_y are constants of the linear gradient functions for the two solvents x and y, which follow the relationships

$$A_x = c_{Ax} + (c_{Bx} - c_{Ax})\,\mathrm{A} \tag{17a}$$

$$B_x = (c_{Bx} - c_{Ax})\,B \tag{17b}$$

$$A_y = c_{Ay} + (c_{By} - c_{Ay})\,A \tag{18a}$$

$$B_y = (c_{By} - c_{Ay})\,B \tag{18b}$$

This is illustrated by a simple example in Fig. 1, where 20% aqueous acetonitrile is used as solvent A in reservoir 1 and 80% aqueous methanol as solvent B and a linear gradient of B (0–100% in 1 h) is run with a zero initial concentration of B (A = 0): straight line 1. Then, the concentration of methanol in the mixed ternary mobile phase follows the straight line 2 and that of acetonitrile is controlled by the straight line 3.

In our experiments with ternary gradients we used the equipment described previously[4,15]. A low-pressure gradient two-plunger pump (PPM-68005, Workshops of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia) served as a gradient mixer delivering the mixed mobile phase to a high-pressure reciprocating

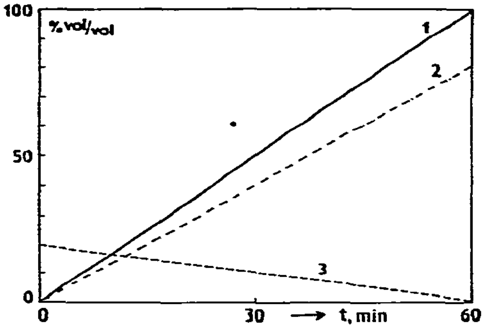


Fig. 1. Example of concentration profiles in ternary mobile phase gradients prepared from two binary solvents: (A) 20% acetonitrile in water; (B) 80% methanol in water. Lines: (1) % (v/v) of B in the mobile phase; (2) % (v/v) of methanol in the mobile phase; (3) % (v/v) of acetonitrile in the mobile phase. t = Time from the start of the gradient.

pump (M 6000, Waters, Milford, MA, U.S.A.) at a flow-rate 1.20 ml/min, the working flow-rate of the M 6000 pump being set at 1.00 ml/min. Further, a Waters U6K injector, a stainless-steel column (300 × 4.2 mm I.D.) packed with octadecylsilica prepared from LiChrosorb Si 100 (10 µm) by reaction with *n*-octadecyltrichlorosilane[16] and a Waters M 440 UV detector (at 254 nm) were used. The gradient delay of the equipment was compensated for by injecting the sample at a pre-calculated time after the start of the gradient run (198 sec at 0.97 ml/min). The solutions in the two reservoirs were either the pure organic solvent and water (in experiments with binary gradients) or pre-mixed solutions of the organic solvents in water. The two reservoirs were kept at *ca.* 60°C to degas the components of the mobile phase before their mixing.

The methanol and acetonitrile used as the components of the mobile phase were of spectroscopic grade. Deionized water was distilled with addition of potassium permanganate and sodium hydrogen carbonate, passed through a column (150 × 20 mm I.D.) packed with octadecylsilica prepared from silica of particle diameter 100–150 µm (Silasorb C18; Lachema, Brno, Czechoslovakia). The void volume of the column was determined as the retention volume of D_2O measured with a differential refractometer (Waters R-401) and was 3.08 ml.

Phenolic sample compounds were commercial reagent-grade materials.

RESULTS AND DISCUSSION

Retention in ternary mobile phases under isocratic conditions

To verify the validity of the eqn. 5 for capacity ratios in ternary solvent mixtures, the retention of phenolic compounds was measured in methanol–water and acetonitrile–water solvent binaries. Four different concentrations of the organic solvent in the mobile phase were tested for each organic solvent–water combination. From linear regression of the experimental plots of log k' of phenols *versus* concentration of the organic solvent, the parameters k'_{0x}, m_x, k'_{0y} and m_y in eqns. 1 and 2 were calculated (Table I). The correlation coefficients for the experimental plots were *ca.* 0.9990–0.9999. Then, the capacity ratios of phenols were measured in the two ternary mobile phases acetonitrile–methanol–water (20:25:55) and (10:40:50). The experi-

TABLE I

CALCULATIONS OF CAPACITY RATIOS IN TERNARY MOBILE PHASES

I, Methanol–acetonitrile–water (25:20:55); II, methanol–acetonitrile–water (40:10:50). Column: C18/LiChrosorb Si 100, 10 μm; 300 × 4.2 mm I.D.; V_m = 3.075 ml. k'_{ox}, m_x, k'_{oy}, m_y = constants in eqns. 1 and 2 (x = methanol; y = acetonitrile).

No.	*Compound*	k'_{ox}	m_x	k'_{oy}	m_y	*k′ in ternary mixture*			
						I		*II*	
						Calc.	*Exptl.*	*Calc.*	*Exptl.*
1	4-Cyanophenol	36.73	3.212	13.94	2.970	0.96	0.91	0.79	0.80
2	Phenol	20.10	2.618	12.90	2.662	1.07	1.16	0.89	0.95
3	3-Methoxyphenol	40.62	3.113	15.87	2.840	1.21	1.22	0.99	0.91
4	2-Methoxyphenol	37.93	3.028	13.87	2.603	1.27	1.25	1.04	1.02
5	4-Fluorophenol	32.25	2.821	20.59	2.881	1.38	1.45	1.13	1.17
6	3-Fluorophenol	46.66	2.919	22.96	2.850	1.71	1.78	1.43	1.43
7	3-Nitrophenol	59.68	3.029	26.17	2.944	1.87	1.91	1.58	1.61
8	*m*-Cresol	81.59	3.215	26.07	2.870	2.06	2.02	1.74	1.77
9	*o*-Cresol	70.17	2.993	27.89	2.807	2.28	2.21	1.94	1.97
10	4-Chlorophenol	142.45	3.268	53.62	3.234	3.17	3.01	2.74	2.52
11	4-Iodophenol	471.81	3.738	136.35	3.666	5.84	5.55	5.06	4.58
12	2-Phenylphenol	1047.60	3.909	487.79	4.307	9.86	10.12	7.97	8.23
13	3-*tert*.-Butylphenol	1775.3	4.021	337.35	4.016	10.94	10.85	9.42	9.03

mental values are compared in Table I with capacity ratios calculated from eqns. 5 and 6. The average deviation of the calculated *k′* values from the experimental values is 3.8% relative; only four of the calculated values differ by more than 5% relative from the experimental values, and these four deviations do not exceed 10% relative. Consequently, eqns. 5 and 6 seem to be well suited for calculations of retention in ternary solvent mixtures in reversed-phase chromatography from the data obtained with binary mobile phases.

Verification of calculations of retention volumes in chromatography with ternary solvent gradients

To verify the calculation procedure for retention volumes in reversed-phase chromatography using ternary mobile phase gradients, four ternary and two binary gradients were run with selected phenols. The experimental retention volumes of the test compounds are compared in Table II with the values calculated by iteration using eqns. 11 and 12 and the values of k'_{0x}, m_x, k'_{0y} and m_y from Table I.

The four methanol–acetonitrile–water ternary mobile phase gradients investigated involved one ternary gradient using a simultaneous linear increase of the concentration of methanol from 0 to 60% (v/v) and of that of acetonitrile from 0 to 40% (v/v) in 60 min and three other gradients where the concentration of methanol always increased from 0 to 100% (v/v) in 60 min, but the concentration of acetonitrile decreased simultaneously from 10 to 0% (v/v) in 60 min, from 20 to 0% (v/v) in 60 min and from 30 to 0% (v/v). The average differences between the calculated and experimental retention volumes were *ca.* 3% relative in experiments with binary methanol–water and acetonitrile–water gradients and *ca.* 3–5% relative in gradient

TABLE II

VERIFICATION OF CALCULATION OF RETENTION VOLUMES (V_R) IN CHROMATOGRAPHY USING TERNARY GRADIENTS

Column and compounds as in Table I. Gradients, 0–100% B in 60 min. I, A = water, B = methanol; II, A = water, B = acetonitrile; III, A = water, B = methanol–acetonitrile (3:2); IV = A = acetonitrile–water (1:9), B = methanol; V, A = acetonitrile–water (1:4), B = methanol; VI, A = acetonitrile–water (3:7), B = methanol. A_x, B_x, A_y, B_y = constants in eqns. 8 and 9 (x = methanol; y = acetonitrile); concentration of the organic solvent (c_x, c_y) as % (v/v)·10^{-2}.

Parameter	*Gradient*					
	I	*II*	*III*	*IV*	*V*	*VI*
A_x	0.2	0	0	0	0	0
B_x	0.017182	0	0.010308	0.017182	0.017182	0.017182
A_y	0	0	0	0.1	0.2	0.3
B_y	0	0.017182	0.006873	−0.0017182	−0.0034364	−0.0051546

Compound No.	V_R *(ml)*											
	Calc	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*
1	14.5	12.4	18.4	17.8	22.1	20.8	18.5	16.6	12.6	11.6	8.4	7.7
4	15.6	15.3	19.3	21.1	23.2	23.5	19.9	21.1	14.1	13.2	9.7	8.9
5	15.6	15.9	21.6	21.9	23.8	24.2	20.2	22.0	14.8	14.7	10.3	9.9
6	17.6	17.6	22.6	23.4	25.8	26.1	22.5	24.1	16.6	16.8	11.5	11.2
8	19.7	19.5	23.5	24.7	27.8	28.0	25.1	26.3	18.8	18.3	12.9	12.2
10	23.3	22.8	27.3	27.6	31.6	31.7	29.5	30.8	23.3	23.2	16.4	16.2
11	27.9	27.3	31.5	31.3	36.3	36.1	35.3	36.0	29.7	29.2	22.5	22.2
12	30.5	30.6	35.5	35.0*	39.4	40.0	38.6	40.0	33.8	34.1	27.3	28.8*
13	31.5	31.2	35.0	35.0*	40.1	40.1	39.7	40.4	35.1	34.9	28.6	28.8*

* Evaluated from the maximum of a single peak for two compounds: no or poor resolution only.

elution using ternary gradients. This is also in agreement with the errors in the calculation of k' in ternary mobile phases under isocratic conditions. Therefore, the calculation procedure presented here seems to be suitable for the prediction of retention volumes in reversed-phase chromatography using ternary mobile phase gradients.

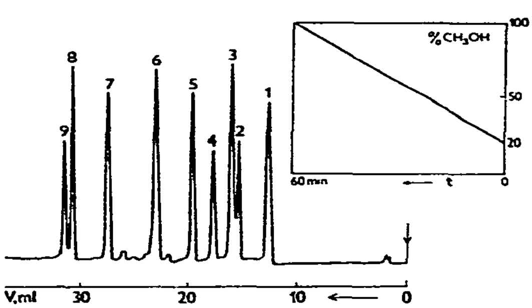
Selection of an adequate ternary gradient for a given separation problem

Ternary gradients can provide greater possibilities for the separate control of selectivity and retention (capacity term) during gradient elution with respect to binary mobile phase gradients. Bakalyar *et al.*[1] argued that it could be possible to adjust the selectivity by control of the concentration of one organic solvent in the mobile phase, while the elution strength is changed by the concentration gradient of another solvent during the elution. However, the changes in selectivity and capacity (retention) are always interconnected when the composition of the mobile phase is changed. Therefore, the influence of the ternary gradients on chromatographic behaviour may be much more complicated than with binary mobile phase gradients. For this reason, the content of one component of the mobile phase (one organic solvent) is kept constant during gradient elution, while the concentration of the other solvent in the mobile phase is increased (and that of water decreased) with time[1,2]. Such gradients can be essentially considered as binary gradients and it is often possible to achieve a similar resolution by using binary gradients only (it is almost always possible when the separation of only one solute pair presents difficulties).

Ternary gradients are useful for the separation of complex samples where the separation of a certain pair of solutes is difficult in one binary solvent system, while the separation of another solute pair (differing significantly in retention from the first solute pair) cannot be achieved easily in other binary solvent systems. Here, ternary gradients may lead to a considerable simultaneous improvement of selectivity and resolution in different parts of the chromatogram.

From eqn. 11, it follows that a change in selectivity in a ternary gradient with respect to binary gradients can be expected for pairs of solutes where the parameters m_x and m_y in eqns. 1 and 2 in two organic solvents differ from one another at least for one compound of this pair, otherwise the selectivity does not change significantly. Thus, if we inspect the values of m_x and m_y for phenols in Table I, we find that for unsubstituted phenol, halogenophenols, 3-nitrophenol and *tert.*-butylphenol $m_x \approx m_y$, whereas there are large differences between m_x and m_y for 2-methoxyphenol, *m*-cresol and 2-phenylphenol. It follows that we can use the parameters m_x and m_y to compare the selectivity in different solvent systems at least to some extent, and the data from Table I suggest differences in selective interactions of the solvent and the last phenols for methanol and acetonitrile (to a lesser extent this applies also for 4-cyanophenol, 3-methoxyphenol and *o*-cresol).

As a practical consequence, we find difficulties when attempting to separate 2-methoxyphenol from 4-fluorophenol in binary mobile phases composed of methanol and water and, on the other hand, the separation of 2-phenylphenol from 3-*tert.*-butylphenol is not possible in water–acetonitrile binary systems. This is illustrated by examples of separations using methanol–water (Fig. 2) and acetonitrile–water gradients (Fig. 3). The separation of a mixture of nine phenols shown in these examples is suitable for the application of gradient elution, because the retentions of the first and the last compound eluted differ approximately 12-fold.



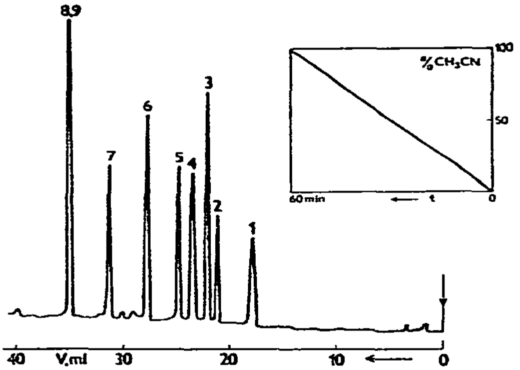


Fig. 2. Separation of a mixture of phenols using a linear binary concentration gradient of methanol in water (gradient I in Table II). Column as in Table I. Flow-rate, 1.0 ml/min. Detection: UV (254 nm) 1.0 a.u.f.s. Compounds: 1 = 4-cyanophenol; 2 = 2-methoxyphenol; 3 = 4-fluorophenol; 4 = 3-fluorophenol; 5 = *m*-cresol; 6 = 4-chlorophenol; 7 = 4-iodophenol; 8 = 2-phenylphenol; 9 = 3-*tert*.-butylphenol.

Fig. 3. Separation of a mixture of phenols using a linear binary concentration gradient of acetonitrile in water (gradient II in Table II). Conditions and compounds as in Fig. 1.

However, the application of a ternary gradient, where the concentrations of methanol and acetonitrile in the mobile phase were increased with time simultaneously in a constant ratio (gradient III in Table II), did not significantly improve the separation (Fig. 4). This would be expected, as we would expect a better resolution of 2-methoxyphenol from 4-fluorophenol in a mobile phase relatively rich in acetonitrile and that of 2-phenylphenol from 3-*tert*.-butylphenol in a mobile phase containing methanol in excess over acetonitrile. Consequently, the ternary gradient should be designed in such a way that the concentration of methanol in the mobile phase increases with time, while the concentration of acetonitrile decreases simultaneously.

It remains to find the optimal parameters of the gradient functions (A_x, B_x, A_y and B_y in eqns. 8 and 9) for each organic solvent. Here, calculations of retention volumes as shown above may be useful in saving time and solvents necessary for trial-and-error experiments, because V_R for critical pairs of compounds can be calculated

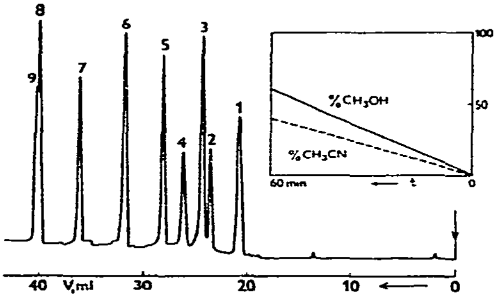


Fig. 4. Separation of a mixture of phenols using a linear ternary concentration gradient of methanol and acetonitrile in water (gradient III in Table II). Conditions and compounds as in Fig. 1.

TABLE III

DIFFERENCES IN RETENTION VOLUMES (ΔV_R) FOR THE TWO "CRITICAL" PAIRS OF COMPOUNDS 4-FLUOROPHENOL/2-METHOXYPHENOL (1) AND 3-*tert.*-BUTYLPHENOL/2-PHENYLPHENOL (2) UNDER DIFFERENT GRADIENT ELUTION CONDITIONS

Numbers of gradients and conditions as in Table II. ΔV_R values are taken from calculated V_R values in Table II.

Parameter	*Gradient*					
	I	*II*	*III*	*IV*	*V*	*VI*
ΔV_R (1) (ml)	0.0	2.3	0.6	0.3	0.8	0.6
ΔV_R (2) (ml)	1.0	0.5	0.7	1.1	1.3	1.2

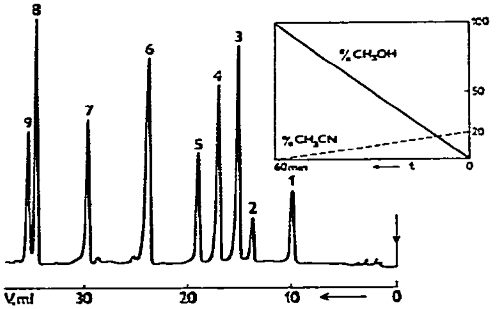
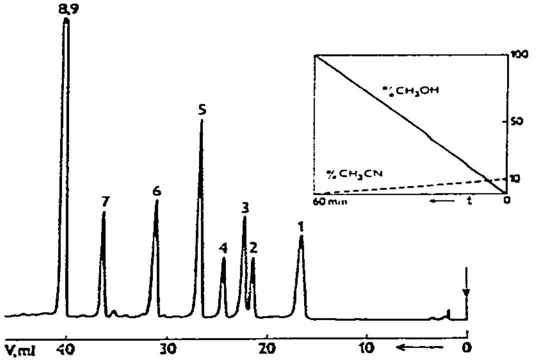


Fig. 5. Separation of a mixture of phenols using a linear ternary concentration gradient of methanol and acetonitrile in water (gradient V in Table II). Conditions and compounds as in Fig. 1. Optimized ternary gradient.

for different ternary gradients and from these results the optimal gradient can be chosen.

We calculated the retention volumes of the two pairs of phenols for three gradients differing in the initial concentration of acetonitrile. From the comparison of



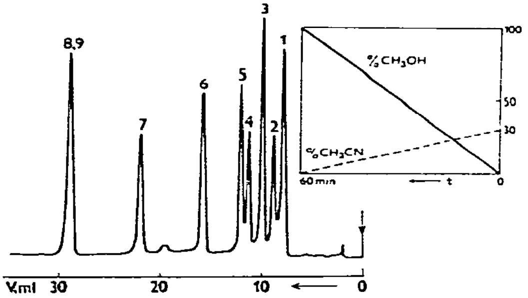


Fig. 6. Separation of a mixture of phenols using a linear ternary concentration gradient of methanol and acetonitrile in water (gradient IV in Table II). Conditions and compounds as in Fig. 1.

Fig. 7. Separation of a mixture of phenols using a linear ternary concentration gradient of methanol and acetonitrile in water (gradient VI in Table II). Conditions and compounds as in Fig. 1.

the retention volumes in Table II and their differences in Table III, it follows that the optimal separation can be expected in gradient V (maximal differences in V_R), where 20 % acetonitrile in water is chosen as solvent A and 100 % methanol as solvent B (t = 60 min). In practice, this gradient indeed yielded a better separation (Fig. 5) than the other ternary gradients tested (Figs. 6 and 7).

CONCLUSIONS

Retention volumes in reversed-phase chromatography using ternary mobile phases can be predicted by calculations with acceptable precision, either under isocratic conditions or in gradient elution chromatography with ternary gradients. An error in calculated retention volumes of *ca.* 3–5 % relative is to be expected, with some possible exceptions. Iteration methods are necessary for calculations.

Ternary gradients can be useful for the separation of certain complex mixtures, where the separation of all sample compounds is not possible in chromatography with binary solvent gradients. Sometimes, the selectivity and resolution are increased and the separation problem may be solved by using a ternary solvent gradient.

The prediction of retention volumes in ternary gradients by calculation can sometimes avoid the need for trial-and-error experiments and save time and money.

A wide variety of ternary gradients can easily be formed in the gradient elution equipment, making possible the mixing of only two liquids if solvent mixtures are used in one or each of the solvent reservoirs.

The retention behaviour in gradient elution chromatography using ternary gradients is more complex than when binary solvent gradients are used. The present theory is an approximate one, as it neglects possible mutual interactions between the two organic solvents used in the aqueous mobile phase and their influence upon retention. It was found that such effects, if they exist, can be neglected with the systems and solutes studied in this work, but it is possible that they may become more important in certain other systems. However, we hope that the present theory may provide a basis for a better understanding of retention behaviour in ternary mobile phases under isocratic and gradient conditions.

The present treatment can easily be extended to quaternary and even more complex solvent gradients, if necessary. It seems, however, that ternary solvent systems would be sufficient for almost all practical liquid chromatographic applications.

REFERENCES

1 S. R. Bakalyar, R. McIlwick and E. Roggendorf, *J. Chromatogr.*, 142 (1977) 353.
2 L. B. Sybrandt and E. F. Montoya, *Int. Lab.*, July/Aug. (1977) 51.
3 P. Jandera and J. Churáček, *J. Chromatogr.*, 91 (1974) 223.
4 P. Jandera, J. Churáček and L. Svoboda, *J. Chromatogr.*, 174 (1979) 35.
5 P. Jandera and J. Churáček, *J. Chromatogr.*, 192 (1980) 1.
6 P. Jandera and J. Churáček, *J. Chromatogr.*, 192 (1980) 19.
7 K. Karch, I. Sebestian, I. Halász and H. Engelhardt, *J. Chromatogr.*, 122 (1976) 171.
8 B. L. Karger, J. R. Gant, A. Hartkopf and P. H. Weiner, *J. Chromatogr.*, 128 (1976) 65.
9 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
10 R. A. Hartwick, C. M. Grill and P. R. Brown, *Anal. Chem.*, 51 (1979) 34.
11 L. R. Snyder, J. W. Dolan and J. R. Gant, *J. Chromatogr.*, 165 (1979) 3.
12 J. W. Dolan, J. R. Gant and L. R. Snyder, *J. Chromatogr.*, 165 (1979) 31.
13 P. J. Schoenmakers, H. A. H. Billiet, R. Tijssen and L. de Galan, *J. Chromatogr.*, 149 (1978) 519.
14. H. Colin, P. Jandera and G. Guiochon, in preparation.
15 P. Jandera, J. Churáček and L. Svoboda, *J. Chromatogr.*, 192 (1980) 37.
16 I. Halász and I. Sebestian, *Chromatographia*, 7 (1974) 371.