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Prediction of retention in gradient-elution normal-phase high-performance liquid chromatography with binary solvent gradients[1]

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Abstract

Chromatographic behaviour of phenylurea herbicides on a silica gel column with binary gradient elution using mobile phases containing dry 2-propanol, *n*-heptane and dioxane was investigated. With dried solvents and the temperature controlled to ±0.1°C, differences between the original retention volumes and the data from repeated experiments measured on the same column after ten months of use were less than 0.2 ml for 85% of the values compared. Experimental adsorption isotherms of 2-propanol and of dioxane in *n*-heptane suggest that the concentration of the polar solvents in the stationary phase is practically independent of their concentration in mobile phases containing more than 1–3% (v,v) polar solvent. In gradient elution starting at this (or at a higher) concentration of the polar solvent, the effect of the preferential adsorption is small and the differences between the calculated gradient volumes and the experimental data were in most cases less than 0.25 ml, or 2%. Predictive calculations were based on two- and three-parameter equations describing the dependence of the sample capacity factors (isocratic) on the concentration of the polar solvent in the mobile phase, the latter yielding slightly better agreement with the experiment. Possible migration of sample compounds in the mobile phase of initial composition in the gradient dwell volume was accounted for.

Keywords: Gradient elution; Retention prediction; Mobile phase composition; Pesticides; Phenylurea pesticides

1. Introduction

Chromatography in reversed-phase systems is the most popular mode used in contemporary practice of liquid chromatography. This is justified by general applicability of reversed-phase chromatography for separation of different classes of compounds ranging from aromatic hydrocarbons and fatty acid esters to ionizable or ionic compounds such as carboxylic acids, nitrogen bases, amino acids, peptides, proteins and sulphonic acids. The main advantage is unrivalled "hydrophobic selectivity" of this method for compounds with even minor differences in the structure of the hydrophobic part of their molecules or for compounds with functional groups differing in polarities. On the other hand, columns packed with polar adsorbents usually show better separation selectivities than alkylsilica columns for various positional isomers of moderately polar compounds [1,2], or for some oligomers containing repeat polar groups [3]. Further, columns packed with unmodified adsorbents are not subject to "bleeding", i.e., gradual loss of the bonded stationary phase which usually

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occurs during the lifetime of the column, decreasing slowly the retention and impairing the separation.

Chromatography on polar adsorbents suffers from another inconvenience – preferential adsorption of more polar solvents may occur, especially of water, which is often connected with long equilibration times if separation conditions are changed. This phenomenon may be very important in gradient elution, where the concentration of a polar solvent in a non-polar one is increasing during the gradient run and preferential adsorption may lead to important deviations of the actual gradient profile from the pre-set mobile phase composition program. Reproducibility of gradient-elution retention data in normal-phase systems with mobile phases comprised of two organic solvents – a polar and a non-polar one – depends on a number of experimental factors that should be controlled. To obtain reproducible results it is necessary to keep a constant adsorbent activity [4]. It is very important to work at a constant temperature and water content in the mobile phase. To maintain a constant content of water in the mobile phase, "isohydric" organic solvents with equilibrium water concentrations were recommended [5]. In a closed "constant moisture system" with a certain volume of solvent containing the required concentration (a few ppm) of water the eluent from the column and detector is re-cycled through a large column packed with alumina adsorbent back to the solvent reservoir, so that constant adsorbent activity is guaranteed [6]. However, equilibrating such a system takes a long time and after a certain number of cycles the mobile phase becomes excessively contaminated with the sample solutes, so that it is necessary to exchange both the solvent and the adsorbent in the large column and re-equilibrate the system. This procedure is tedious and not practical in the gradient-elution mode where equilibrium water contents should be maintained in all the solvents used to form the gradient. Therefore we preferred to use dehydrated solvents kept dry over activated molecular sieves and filtered just before the use to improve the reproducibility and to work under controlled temperature conditions. In some separation problems using dry solvents could lead to excessive band broadening or even to unsymmetrical peak shapes. It is assumed that water fills up the micropores in the adsorbent responsible for this undesirable behaviour. Adsorbents with no or low contents of micropores should not be subject to this problem which can also be diminished by using strongly polar organic solvents such as alcohols as mobile phase components in gradient elution. Further, these solvents are likely to "buffer" possible effects of small residual trace concentrations of water present in the solvents even after careful drying.

More than twenty years ago, we started our systematic investigation of gradient elution in normal-phase systems with silica gel columns and binary gradients formed using solvents with large differences in polarities. However, the instrumentation available at that time did not allow very precise determination of the retention data and it was not possible to discriminate various sources of instrumental inaccuracies. The objective of the present work was to investigate possible origins of inaccuracies in prediction of retention in normal-phase gradient elution if dry solvents, controlled temperature and a modern instrument with high accuracy and reproducibility of solvent mixing and mobile phase delivery are used. For this purpose, the following points were investigated:

1. Reproducibility of the retention data in repeated gradient-elution experiments after long-term column use.
2. Adsorption isotherms characterizing the distribution of polar solvents between the mobile phase and the adsorbent in the column used and breakthrough curves of the solvents in blank gradients, which allow to estimate the effects of the preferential adsorption of the polar solvent on the precision of the results.
3. Two- and three-parameter equations describing the isocratic retention were compared as the basis for calculation of gradient elution data.
4. Different methods were studied to account for the isocratic step before the start of the gradient induced by the mobile phase of initial composition contained in the gradient dwell volume.
5. Finally, the calculated and the experimental retention data of the compounds studied were compared as the test of suitability of prediction methods.

2. Theoretical

The retention in normal-phase systems as a function of the composition of two-component (binary) mobile phases can be described using theoretical models of adsorption. The first model of retention in adsorption chromatography was developed by Snyder in the early 1960s [4,7,8]. Flat adsorption in a monomolecular layer on a homogeneous adsorption surface was assumed. The adsorption was understood as a competition phenomenon between the molecules of the solute and of the solvent on the adsorbent surface. The interactions in the mobile phase were assumed less significant and neglected. Later, corrections were introduced for preferential adsorption on localized adsorption centers [9,10]. Soczewinski [11,12] suggested a model of retention assuming adsorption in a monomolecular layer on a heterogeneous surface of adsorbent and cancellation of the solute–solvent interactions in the mobile and in the stationary phases. With some simplification, both models lead to identical equation describing the retention (capacity factor, k') as a function of the concentration of the stronger (more polar) solvent, φ, in binary mobile phases comprised of two solvents of different polarities [7,12,13]:

$$k' = k'_0 \cdot \varphi^{-m} \tag{1}$$

where k'_0 and m are experimental constants, k'_0 being the capacity factor in pure strong solvent. This equation has become known as the Snyder–Soczewinski model equation [8].

Scott and Kucera [14–17] in their model of adsorption defined the distribution coefficient of a solute between the stationary and the mobile phases as the ratio of the forces acting on the solute in the two phases, set to the product of the probability of interactions and the interacting forces in each phase. In mobile phase, both polar and dispersive forces were considered, but dispersive forces on the adsorbent surface were neglected. The number of the adsorption sites was considered independent of the composition of the mobile phase when the concentration of the polar solvent in the mobile phase was above 3% and decreasing retention with increasing concentration of the more polar solvent in these mobile phases was attributed mainly to increasing interactions in the mobile phase. With these assumptions, they derived retention equation different from Eq. (1):

$$\frac{1}{k'} = a' + b' \cdot \varphi \tag{2}$$

where a' and b' are experimental constants.

It is interesting to note that both Eqs. (1,2) can be derived on the basis of molecular statistical–mechanical theory of adsorption chromatography [18].

Based on the original Snyder concept of adsorption as a competitive phenomenon but with less simplification than in derivation of Eq. (1), another retention equation was derived [19,20]:

$$k' = (a + b \cdot \varphi)^{-m} \tag{3}$$

Here again, a, b and m are experimental constants depending on the solute and on the chromatographic system [$a = 1/(k'_a)^m$, where k'_a is the capacity factor in pure non-polar solvent]. If the retention in pure non-polar solvent is very high, the term a in Eq. (3) can be neglected and this equation becomes Eq. (1) [13]. On the other hand, if the molecule of polar solvent occupies approximately equal area on the adsorbent surface as the molecule of solute, $m = 1$, Eq. (3) is then identical to Eq. (2) [19,20].

Theoretical description of linear binary gradient elution in normal-phase systems was presented by Jandera and Churáček [20–22]. In these gradients the concentration of a polar solvent, φ, increases as the volume of eluate, V, increases:

$$\varphi = A + B \cdot V \tag{4}$$

Here, A is the initial concentration of the strongly polar organic solvent in the mobile phase and B is the slope (steepness) of the gradient in concentration units per ml of the eluate. If the retention in a normal-phase system can be described by the two-parameter retention Eq. (1), the retention volume, V_R, of a sample compound in gradient-elution chromatography can be calculated as [21]:

$$V_R = \frac{1}{B}[(m+1)Bk'_0V_0 + A^{(m+1)}]^{\frac{1}{m+1}} - \frac{A}{B} + V_0 \tag{5}$$

On the other hand, if the three-parameter Eq. (3) is necessary to describe adequately the retention in a given normal-phase system, a slightly more complex equation should be used to calculate the retention volumes in gradient elution [20,21]:

$$V_R = \frac{1}{b \cdot B}[b \cdot B(m+1)V_0 + (a + A \cdot b)^{(m+1)}]^{\frac{1}{m+1}} - \frac{a + A \cdot b}{b \cdot B} + V_0 \quad (6)$$

These single-step gradient equations can be used if the gradient dwell volume V_D is so low that it can be neglected or if the injection is delayed with respect to the start of the gradient to compensate for the dwell volume. Unfortunately, this is not the case in most practical separation problems. For reversed-phase systems, Snyder suggested to add the gradient dwell volume to the retention volume calculated from the single-step gradient equation. This approach is in principle applicable also for calculation of the retention data in normal-phase gradient elution, if the gradient elution is started at a low concentration of the polar organic solvent in the mobile phase. However, if the gradient dwell volume is large and (or) the gradient is started at a high concentration of the polar solvent, sample solutes are eluted for some time under isocratic conditions before the gradient is started and can move a significant distance along the column, which may cause considerable errors in gradient elution data predicted from the single-step gradient equations, even if the dwell volume is eventually added to the predicted V_R. To calculate the retention data in this case, both the gradient and the preceding isocratic elution steps should be taken into account. For this purpose, gradient dwell volume was added to the retention volume calculated from the equation considering a proportional part of the column migrated in the gradient elution step. This approach was first described by Jandera and Churáček [20], [22] and later by Quarry et al. [23]. In the present work we used slightly different approach, which considers the two-step elution as the elution on two columns, the first in the isocratic and the second in the gradient mode. The modification of the Eqs. (5,6) in this case is described in Appendix A.

3. Experimental

An HP 1090M liquid chromatograph equipped with a UV diode- array detector, operated at 230 nm, an automatic sample injector, a 3DR solvent delivery system, a thermostatted column compartment and a Series 7994A workstation (Hewlett-Packard, Palo Alto, CA, USA) was used to acquire the elution data. A glass cartridge column, 150 mm×3.3 mm I.D., packed with silica gel Separon SGX, 7.5 μm, was obtained from Tessek (Prague, Czech Republic). The flow-rate of the mobile phases was kept at 1 ml/min and temperature at 40°C in all experiments.

2-Propanol, *n*-heptane and dioxane, all of HPLC grade, were purchased from Baker, (Deventer, Netherlands). The solvents were dried and kept in tightly closed dark bottles over molecular sieve beads Dusimo 5 Å (Lachema, Brno, Czech Republic), previously activated at 300°C (ca. 30–40 g/l), filtered using a Millipore 0.45-μm filter and degassed by ultrasonication immediately before the use. Mobile phases were prepared directly in the HP 1090M instrument from the components continuously stripped by a stream of helium according to a gradient pre-set program. Phenylurea herbicides sample compounds were obtained from Lachema and are listed in Table 1. The solutes were dissolved in the mobile phase to provide adequate response of the UV detector. Sample volumes of 5 μl were injected in each experiment.

Table 1
Compounds studied: phenylurea herbicides

Compound No.	Name
1	Phenuron
2	Diuron
3	Chlorotoluron
4	Fluometuron
5	Desphenuron
6	Metoxuron
7	Linuron
8	Deschlorometoxuron
9	Isoproturon
10	Metobromuron
11	N'-(4-Methyl-3-chlorophenyl)-N-methylurea
14	Monuron
15	Chlorobromuron
16	Neburon

The columns were first equilibrated with the mobile phase and then the retention volumes, V_R, of the sample compounds were measured at different gradient profiles. A 5-min reversed gradient and a 5-min equilibration time were used after the end of each experiment to re-equilibrate the column. The mean values of V_R from three repeated experiments are compared with the data calculated using Eqs. (5–8) and the parameters of the retention Eqs. (1,3) determined from the isocratic capacity factors, $k' = (V_R/V_0 - 1)$ as described previously [24]. Column dead (hold-up) volume, V_0, was determined using trichloroethylene as the marker ($V_0 = 0.905$ ml).

The isotherms of 2-propanol and of dioxane in *n*-heptane were determined using the frontal-analysis method as described previously [25], except for using a Waters–Millipore R 401 refractometric detector instead of the UV-absorbance one.

4. Results and discussion

To test the reproducibility of the retention data in normal-phase chromatography with binary solvent gradients prepared from dehydrated solvents kept dry over molecular sieves before the use, we compared the retention volumes of several phenylurea herbicides on a fresh silica gel column with the data from the experiments repeated on the same column after ten months of use. The results with linear gradients of 2-propanol in *n*-heptane and of dioxane in *n*-heptane are shown in Table 2. The differences between the retention volumes measured in the experiments repeated in this way are lower than 0.2 ml or 2% of V_R, except for five values of 120 tested. This reproducibility of retention is at least comparable with that usually achieved with most bonded phases.

Fig. 1 shows the adsorption isotherms of 2-propanol and of dioxane in *n*-heptane. The isotherms are very steep and the silica gel column becomes approximately 87% saturated with 2-propanol and 67% saturated with dioxane in mobile phase with 0.5% polar solvent in *n*-heptane. An increase in the concentration of the polar solvents in the mobile phase from 3% to a higher value does not result in a significant increase of its concentration in the stationary phase. Consequently, little preferential adsorption occurs after a gradient with initial concentration of 3% or more of 2-propanol or dioxane in *n*-heptane has been started. This has been confirmed by the breakthrough volumes, V_B, determined from the detector response recorded at a low vawelength (210 nm) in blank gradients (Table 3). The net breakthrough volumes corrected for the column hold-up volume and for the gradient dwell volume of the instrument are 0.2 ml or less in gradients starting at 3% or more 2-propanol and 0.26 ml or less in gradients starting at 6% or more dioxane. 2-Propanol has approximately twice the elution strength of dioxane, which explains a less steep isotherm and somewhat higher breakthrough volumes. Consequently, the starting concentration of 6% dioxane has approximately the same effect on the retention in gradient elution as the starting concentration of 3% 2-propanol.

The results of various methods of predictive calculations of retention volumes of phenylurea herbicides in normal-phase chromatography with binary gradients of 2-propanol in *n*-heptane and of dioxane in *n*-heptane are compared with the experimental data in Table 4 for gradients with different slopes, B, and starting concentrations, A, of the polar solvents. To account for the gradient dwell volume and for possible effects of preferential adsorption of the polar solvent, three methods of calculation were compared:

1. Two-step calculations using Eq. (8) based on three parameter retention Eq. (3) – $V_R(C_1)$ – or Eq. (7) derived using the two-parameter retention Eq. (1) – $V_R(C_2)$, both considering possible migration of sample compounds along the column in the isocratic step before the start of the gradient induced by the gradient dwell volume.
2. Single-step gradient volume calculations using Eq. (6) with gradient dwell volume added to the result – $V_R(C_3)$.
3. Single-step gradient volume calculations using Eq. (6) with gradient dwell volume and the breakthrough volume V_B added to the result to account for possible effects of preferential adsorption. The retention volumes calculated in this

Table 2
Reproducibility of gradient-elution retention volumes of phenylurea herbicides, V_R (ml)

Gradient	V_R (ml of solute No.)								
		1	2	3	4	6	7	9	11	15
0–50% P	(1)	11.94	11.73	11.08	11.20	14.36	6.50	10.98	10.43	6.38
in 30 min	(2)	12.02	11.88	11.13	11.32	14.44	6.50	11.18	10.55	6.43
0–50% P	(1)	17.18	17.16	15.89	16.19	21.33	8.20	15.63	15.44	8.12
in 60 min	(2)	17.28	17.23	16.02	16.37	21.44	8.40	16.01	15.55	8.24
0–50% P	(1)	21.41	21.33	19.85	20.26	26.93	9.55	19.46	19.35	9.42
in 90 min	(2)	21.08	21.15	19.68	20.43	26.62	9.75	19.50	19.69	9.44
3–50% P	(1)	10.34	10.22	9.46	9.61	12.89	4.86	9.40	8.82	4.81
in 30 min	(2)	10.23	10.17	9.36	9.59	12.87	4.85	9.47	8.82	4.74
6–50% P	(1)	8.85	8.70	7.92	8.11	11.37	11.69	7.90	7.19	3.83
in 30 min	(2)	9.01	8.72	8.02	8.12	11.50	11.90	8.00	7.25	3.95
9–50% P	(1)	7.59	7.30	6.64	6.73	9.93	10.26	6.58	5.76	3.20
in 30 min	(2)	7.60	7.24	6.66	6.82	9.87	10.51	6.60	5.89	3.26
0–100% D	(1)	11.47	11.08	10.86	10.18	13.62	6.63	10.85	13.74	6.86
in 30 min	(2)	11.61	11.14	10.62	10.17	13.50	6.65	10.93	13.57	6.78
0–100% D	(1)	17.31	16.93	15.84	15.33	20.78	9.22	16.49	21.18	9.33
in 60 min	(2)	17.34	17.04	15.86	15.41	20.77	9.24	16.41	21.12	9.30
0–100% D	(1)	21.86	21.81	20.11	19.80	26.78	11.16	20.86	27.26	11.61
in 90 min	(2)	22.05	21.45	20.22	19.36	27.07	11.02	20.82	27.50	11.18
3–100% D	(1)	10.69	10.45	9.72	9.44	12.52	5.81	10.08	12.91	5.93
in 30 min	(2)	10.77	10.36	9.81	9.37	12.70	5.78	10.07	12.87	5.92
6–100% D	(1)	9.89	9.67	8.90	8.67	11.72	5.04	9.26	12.04	5.10
in 30 min	(2)	9.91	9.47	8.84	8.49	11.80	4.96	9.26	11.96	5.03
9–100% D	(1)	9.06	8.79	8.07	7.76	10.92	4.31	8.43	11.29	4.38
in 30 min	(2)	9.16	8.66	8.07	7.67	11.07	4.26	8.43	11.19	4.36

(1) Original data, (2) data after 10 months. Column: silica gel Separon SGX, 7μm, 150×3.3 mm I.D.; 40°C; dried solvents: linear gradients of 2-propanol (P) or dioxane (D) in *n*-heptane. Numbers of compounds as in Table 1.

way were significantly higher than the experimental values and are not given in Table 4.

Table 4 shows the experimental and calculated retention volumes in all gradients tested for one compound taken as an example. In Table 5, mean values of the differences between the experimental and calculated retention volumes are calculated separately for gradients starting at zero (three gradients) and non-zero (3, 6 and 9%, nine gradients) concentrations of the polar solvent. Arithmetic means for the compounds tested are also given in this table.

The results of the fitting of two- and three-parameter retention Eqs. (1–3) are reported elsewhere [24]. The fit of the three-parameter retention Eq. (3) to the isocratic retention data was comparable or slightly better than the fit of the two-parameter Eq. (1), but Eq. (2) completely failed to describe the retention behaviour. The gradient retention volumes calculated on the basis of different retention equations (Eqs. (1,3)) – $V_R(C_1)$ and $V_R(C_2)$ are close to each other, but the values of $V_R(C_1)$ calculated using Eq. (8) based on the three-parameter k' versus φ dependence (Eq. (3)) show slightly better agreement with most experimental data (Table 5).

With gradients starting at 0% organic solvent, the retention volumes calculated from the two-step equation (Eq. (7)), $V_R(C_2)$, are the same as those calculated from the single-step equation (Eq. (4)) with

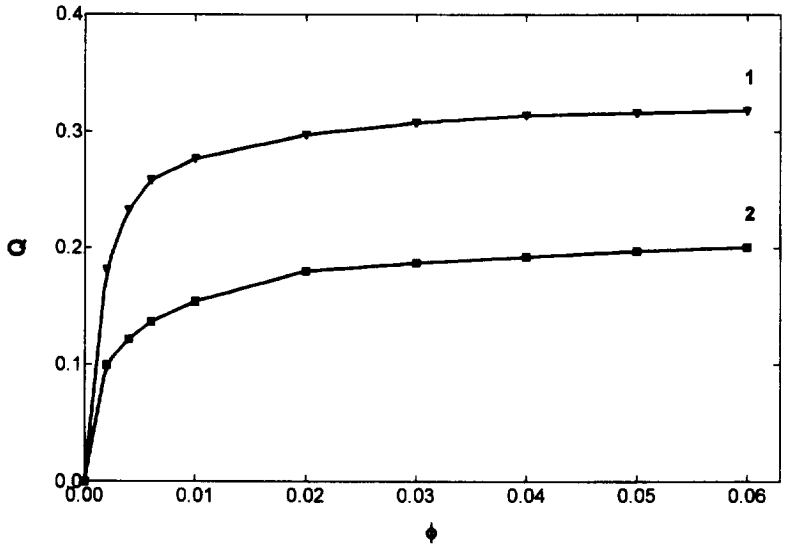

Fig. 1. Sorption isotherms of 2-propanol (1) and of dioxane (2) in *n*-heptane on a silica gel Separon SGX, 7.5 μm, column (150 mm×3.3 mm I.D.) at 40°C. φ: concentration of the polar solvent in the mobile phase in % (v/v)·10^{-2}; Q: concentration of the polar solvent in the stationary phase in % (v/v)·10^{-2}.

gradient dwell volume added, $V_R(C_3)$, as the two-parameter equation (Eq. (1)) assumes infinite retention and no mobility of sample compounds in the first isocratic gradient dwell volume step. The retention volumes $V_R(C_3)$ calculated for a single gradient step with the dwell volume added considering the three-parameter retention equation are slightly higher than the calculated two-step values, $V_R(C_1)$, because slight mobility of sample compounds in pure *n*-hexane is taken into account by the parameter a in Eq. (3).

The differences between the experimental and the calculated retention volumes were in most cases higher with gradients starting at zero concentration of 2-propanol than with gradients starting at a higher concentration. This effect was not observed with dioxane–*n*-heptane gradients (Table 5), probably because of stronger polarity, higher elution strength and consequently stronger preferential adsorption of 2-propanol (Fig. 1).

If the gradient elution starts at a higher concentration of the organic solvent, the two-step calculation approach generally yields results in better agreement with the experimental retention volumes

Table 3
Breakthrough volumes V_B, of dry polar solvents in gradient elution on a silica gel column

	Gradient	V_B (ml)		Gradient	V_B (ml)		Gradient	V_B (ml)
1	0–50% P in 30 min	0.80	9	6–50% P in 90 min	0.16	17	3–100% D in 60 min	0.43
2	0–50% P in 60 min	0.90	10	9–50% P in 30 min	0.00	18	3–100% D in 90 min	0.48
3	0–50% P in 90 min	0.96	11	9–50% P in 60 min	0.00	19	6–100% D in 30 min	0.24
4	3–50% P in 30 min	0.06	12	9–50% P in 90 min	0.00	20	6–100% D in 60 min	0.26
5	3–50% P in 60 min	0.17	13	0–100% D in 30 min	1.02	21	6–100% D in 90 min	0.26
6	3–50% P in 90 min	0.21	14	0–100% D in 60 min	1.14	22	9–100% D in 30 min	0.13
7	6–50% P in 30 min	0.00	15	0–100% D in 90 min	1.29	23	9–100% D in 60 min	0.14
8	6–50% P in 60 min	0.09	16	3–100% D in 30 min	0.43	24	9–100% D in 90 min	0.14

Linear binary gradients of 2-propanol (P) or dioxane (D) in *n*-heptane. Column as in Table 2. V_0 = 0.905 ml; gradient dwell volume of the instrument, V_D = 0.50 ml. Temperature, 40°C; flow-rate, 1 ml/min. Note: the breakthrough volumes are corrected for the column dead volume and for the gradient dwell volume.

Table 4
Example of experimental retention volumes, $V_R(E)$, and the values calculated: (a) using two-step isocratic-gradient approach with three-parameter Eq. (8), $V_R(C_1)$; (b) using the same approach with two-parameter Eq. (7), $V_R(C_2)$; (c) using single-step gradient elution calculation with three-parameter retention Eq. (6) and gradient dwell volume ($V_D = 0.5$ ml) added, $V_R(C_3)$

Gradient	$V_R(E)$	$V_R(C_1)$	$V_R(C_2)$	$V_R(C_3)$
0–50% P in 30 min	11.13	10.92	10.77	10.77
0–50% P in 60 min	16.02	15.64	15.45	15.45
0–50% P in 90 min	19.68	19.35	19.09	19.09
3–50% P in 30 min	9.36	9.41	9.17	9.23
3–50% P in 60 min	12.54	12.59	12.23	12.31
3–50% P in 90 min	14.61	14.82	14.41	14.51
6–50% P in 30 min	8.02	7.92	7.71	7.86
6–50% P in 60 min	10.01	9.84	9.49	9.70
6–50% P in 90 min	10.89	10.98	10.56	10.81
9–50% P in 30 min	6.66	6.58	6.37	6.62
9–50% P in 60 min	7.72	7.62	7.32	7.64
9–50% P in 90 min	8.13	8.15	7.80	8.15
0–100% D in 30 min	10.62	10.48	10.39	10.39
0–100% D in 60 min	15.86	15.68	15.60	15.60
0–100% D in 90 min	20.22	19.94	19.95	19.95
3–100% D in 30 min	9.81	9.74	9.64	9.65
3–100% D in 60 min	14.05	14.13	14.03	14.04
3–100% D in 90 min	17.63	17.59	17.55	17.56
6–100% D in 30 min	8.84	8.96	8.85	8.88
6–100% D in 60 min	12.59	12.56	12.41	12.45
6–100% D in 90 min	15.23	15.23	15.11	15.16
9–100% D in 30 min	8.07	8.17	8.04	8.10
9–100% D in 60 min	11.06	11.00	10.80	10.90
9–100% D in 90 min	13.05	12.95	12.75	12.87

Compound No. 3, conditions and column as in Table 2. All retention volumes are in ml.
2-Propanol (P) in *n*-heptane; Eq. (1): $k'_0 = 0.31098$, $m = 1.412$; Eq. (3): $a = 0.03524$, $b = 2.3640$, $m = 1.6474$.
Dioxane (D) in *n*-heptane; Eq. (1): $k'_0 = 0.3251$, $m = 1.940$; Eq. (3): $a = 0.070396$, $b = 1.7139$, $m = 2.324$

than the single-step calculation with the dwell volume added and this approach is recommended for better precision of prediction. The differences between the values of $V_R(C_1)$ and $V_R(C_3)$ increase with increasing starting concentration of 2-propanol in *n*-heptane, but are almost independent of the starting

Table 5
Mean absolute values of differences between the experimental and calculated retention volumes $\Delta V_1 = V_R(E) - V_R(C_1)$; $\Delta V_2 = V_R(E) - V_R(C_2)$; $\Delta V_3 = V_R(E) - V_R(C_3)$; in ml, for phenylurea herbicides (numbers as in Table 1)

Gradient	*P, a*			*P, b*		*D, a*			*D, b*	
Solute	ΔV_1	ΔV_2	ΔV_3	ΔV_1	$\Delta V_2 = \Delta V_3$	ΔV_1	ΔV_2	ΔV_3	ΔV_1	$\Delta V_2 = \Delta V_3$
3	0.096	0.320	0.128	0.307	0.530	0.067	0.130	0.096	0.200	0.253
4	0.060	0.152	0.060	0.343	0.353	0.152	0.093	0.190	0.073	0.127
5	0.104	0.070	0.150	0.433	0.496	0.350	0.423	0.436	0.103	0.153
7	0.200	0.097	0.304	0.526	0.463	0.131	0.184	0.063	0.127	0.143
11	0.040	0.048	0.147	0.270	0.313	0.318	0.354	0.370	0.227	0.273
15	0.119	0.157	0.242	0.390	0.393	0.083	0.231	0.059	0.107	0.143
Mean	0.103	0.141	0.172	0.378	0.425	0.183	0.236	0.202	0.140	0.182

Gradients of 2-propanol in *n*-heptane (P) and of dioxane in *n*-heptane (D) starting at 3–9% of the polar solvent (*a*, nine gradients) and at 0% of the polar solvent (*b*, three gradients). Details of the gradient elution conditions are given in Table 4.
M = mean values for all the solutes.

Table 6
Comparison of experimental retention volumes, $V_R(E)$, with the values calculated using two-step isocratic-gradient approach with three-parameter equation (Eq. (8)), $V_R(1)$, and single gradient step equation (Eq. (6)) + dwell volume ($V_D = 1.28$ ml), $V_R(2)$ for the instrument with a gradient mixer

Solute	Gradient =	0–50% in 30 min	0–50% in 60 min	0–50% in 90 min	3–50% in 30 min	6–50% in 30 min	9–50% in 30 min
	A =	0	0	0	0.03	0.06	0.09
	B =	0.016667	0.0083333	0.0055556	0.0156667	0.0146667	0.0136667
3	$V_R(1)$	11.67	16.39	20.10	10.08	8.47	6.98
	$V_R(2)$	11.70	16.43	20.15	10.25	8.85	7.59
	$V_R(E)$	11.54	16.32	20.02	10.01	9.15	6.87
4	$V_R(1)$	11.91	16.79	20.64	10.33	8.72	7.21
	$V_R(2)$	11.93	16.83	20.69	10.49	9.08	7.80
	$V_R(E)$	11.69	16.31	20.46	10.20	8.52	7.04
5	$V_R(1)$	11.92	17.21	21.53	10.35	8.67	7.06
	$V_R(2)$	11.93	17.22	21.54	10.45	8.97	7.61
	$V_R(E)$	11.67	16.86	20.99	10.07	8.41	6.85
7	$V_R(1)$	6.93	8.59	9.81	4.99	3.91	3.26
	$V_R(2)$	6.96	8.62	9.85	5.69	4.89	4.38
	$V_R(E)$	6.90	8.69	9.95	5.16	4.07	3.33
8	$V_R(1)$	15.60	22.66	28.31	14.19	12.68	11.13
	$V_R(2)$	15.61	22.68	28.34	14.28	12.88	11.49
	$V_R(E)$	14.95	22.00	27.57	13.71	12.12	10.57
9	$V_R(1)$	11.72	16.38	20.07	10.10	8.48	7.01
	$V_R(2)$	11.73	16.40	20.10	10.27	8.88	7.65
	$V_R(E)$	11.55	16.33	19.88	9.90	8.27	6.82
11	$V_R(1)$	11.17	16.06	20.06	9.56	7.85	6.26
	$V_R(2)$	11.18	16.07	20.07	9.67	8.19	6.87
	$V_R(E)$	11.08	15.96	19.98	9.51	7.77	6.24

Gradients of 2-propanol in *n*-heptane: concentrations of 2-propanol in % (v/v)·10^{-2}. Other conditions as in Table 4.
A, B: initial concentration and slope of the gradient (Eq. (4)).

concentration in the gradients of dioxane in *n*-heptane. (Another method of calculation of V_R using simple addition of the breakthrough volume to the single-step gradient retention volumes yields unrealistically high values of retention volumes and fails to account for possible preferential solvent adsorption.) Of the calculation methods tested, the Eq. (8) using three-parameter dependence of k' on the concentration of the polar solvent in the mobile phase, φ, and two-step calculations yields calculated retention volumes, $V_R(C_1)$, in the best agreement with the experimental values, better than 0.25 ml or than 3% of the retention volume. The mean difference for various compounds with gradients starting at 3% or more of the polar solvent was 0.1 ml and 0.18 ml for gradients of 2-propanol and of dioxane, respectively. It should be noted that this difference is higher for gradients starting at zero concentration of 2-propanol, but does not increase with gradients starting at zero concentration of dioxane, which could probably apply also for other polar solvents with the elution strength comparable to or lower than that of dioxane. More experimental work will be necessary to verify this assumption. These results indicate that the preferential adsorption and the consequent solvent-demixing effect are sufficiently suppressed in gradients starting with at least 3% 2-propanol and that these effects are not very significant with gradients starting at even lower concentrations of dioxane (and possibly of other weaker solvents).

The results summarized in Tables 4 and 5 were measured using the instrument with a low gradient dwell volume (0.5 ml). Most gradient instruments have higher dwell volumes. To investigate the behaviour in such systems, we inserted the gradient mixer (i.e., a short column packed with small particle stainless-steel beads) between the gradient pump and the injector, which increased the gradient dwell volume to 1.28 ml. Insertion of the gradient mixer is recommended if high sensitivity of detection is required as it allows to suppress small concentration

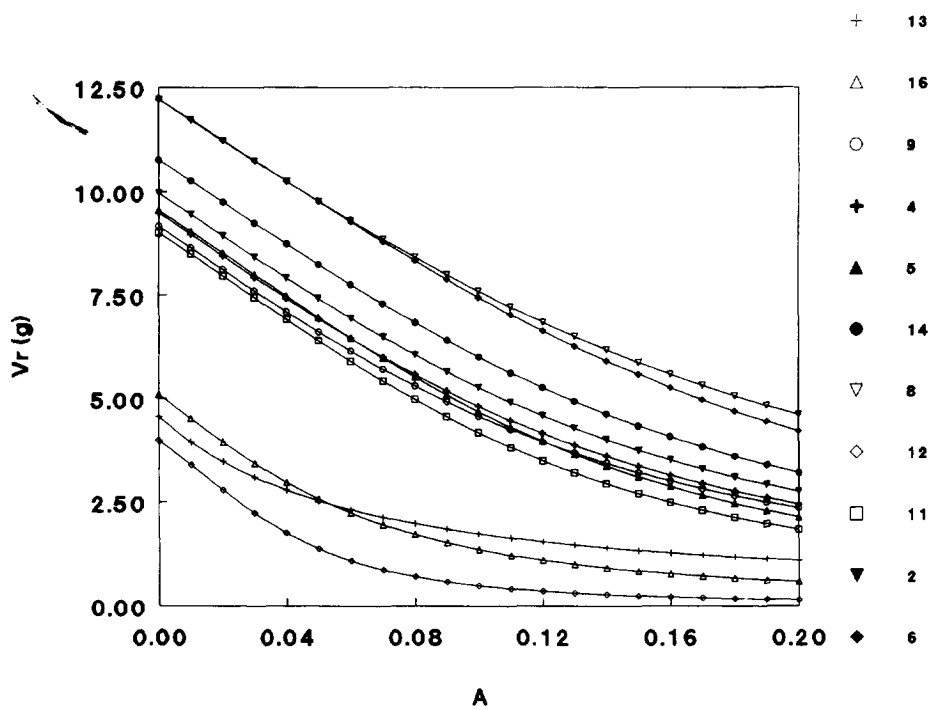

Fig. 2. Dependence of the retention volumes, $V_r(g)$ in gradient elution chromatography on the initial concentration of 2-propanol in *n*-heptane, A (in % (v/v)·10^{-2}) in gradients with gradient time of 30 min. Column as in Fig. 1; flow-rate, 1 ml/min; numbers of compounds as in Table 1.

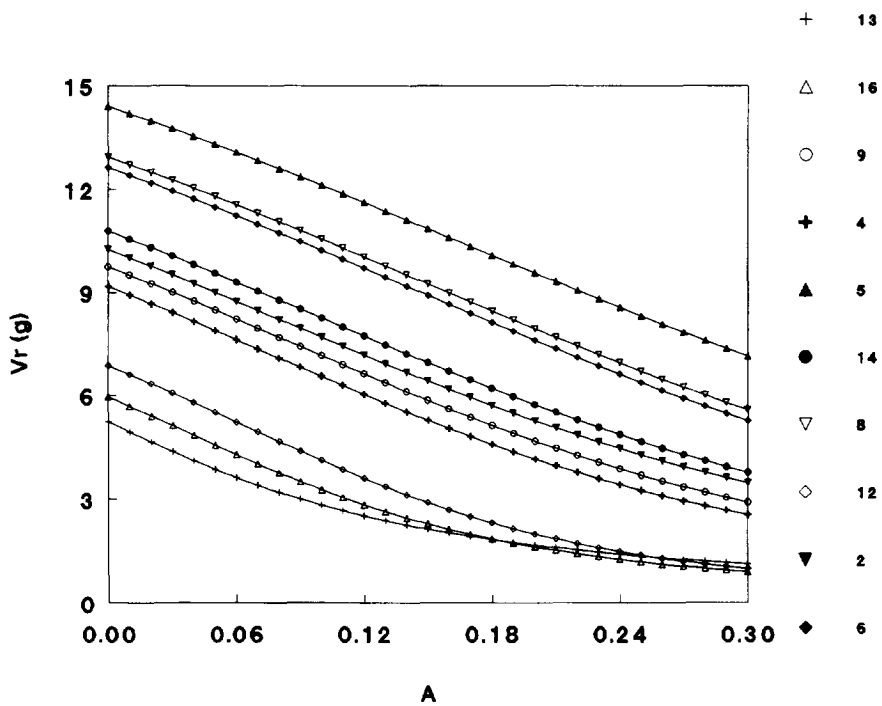

Fig. 3. Dependence of the retention volumes, V_r(g) in gradient elution chromatography on the initial concentration of dioxane in *n*-heptane, A (in % (v/v)·10^{-2}) in gradients with gradient time of 30 min. Column as in Fig. 1; flow-rate, 1 ml/min; numbers of compounds as in Table 1.

fluctuations in the mobile phase, which may increase the detector noise. The results obtained with this system are presented in Table 6. With higher gradient dwell volume system, the differences between the calculated gradient elution volumes using the two-step and the single-step plus the gradient dwell volume approaches are higher than in the device with low dwell volume and two-step calculation improves the prediction more significantly.

Figs. 2 and 3 illustrate the effect of the initial concentrations of 2-propanol and of dioxane, *A*, on retention volumes in gradient elution. The retention volumes decrease with increasing *A* like with increasing concentration of the organic solvent in isocratic elution chromatography and the elution order can be changed in gradient elution starting at different concentrations of the polar solvent. This effect was more apparent with 2-propanol–*n*-heptane than with dioxane–*n*-heptane gradients. Similar behaviour can be observed also as a function of the gradient slope. Such behaviour is typical for gradient elution not only in normal-phase but also in reversed-phase and ion-exchange systems, as demonstrated by earlier published separations (see, e.g., [22]). This means that gradient method development should take into account simultaneous effects of changing gradient slope and initial concentration. Possibilities of this simultaneous adjustment of gradient elution parameters are being investigated and the results will be published later.

5. Conclusions

Using dry organic solvents and controlled temperature in normal-phase chromatography, reproducible gradient-elution data may be obtained over long time of the column use. The retention in binary mobile phases comprised of solvents with different polarities can be accurately described by a three-parameter retention equation. In some instances, two-parameter retention equation based on the Snyder–Soczewinski model of retention can be used for characterization of retention without significant loss

of accuracy, but generally calculations based on three-parameter (Eq. (3)) instead on two-parameter (Eq. (1)) retention equation usually improves the agreement of the calculated gradient retention volumes with the experimental values.

The description of retention in isocratic solvent systems can be used for predicting retention in gradient elution with binary gradients of a strongly polar solvent in a non-polar one, such as of 2-propanol or dioxane in *n*-heptane. The accuracy of prediction can be affected by the preferential sorption of the more polar solvent on the column packed with a polar adsorbent, but the errors caused by this effect are small if the gradient is started at 3% or more of the organic solvent. With less strong polar solvents, the effects of preferential adsorption and of solvent-demixing could be even lower, but the safe minimum starting concentration should be verified in each particular system.

Best precision of predicted gradient elution volumes can be achieved if three-parameter retention equation is used and if the isocratic step connected with the gradient dwell volume before the start of gradient elution is respected in the calculation. The results of this calculation approach agree better with the experiment than simple addition of the gradient dwell volume to the retention volume calculated from a single-step gradient equation, especially if the gradient is started at a higher concentration of the polar solvent and (or) if the gradient dwell volume is large.

6. List of the symbols used

A concentration of the strong (polar) solvent at the start of the gradient [% (v/v)·10^{-2}]

B slope (steepness) of the gradient [% (v/v)·10^{-2}/ml] of the eluate

V volume of the eluate from the sample injection (ml)

V_B breakthrough volume of the polar solvent in gradient elution (ml)

V_D gradient dwell volume of the instrument (ml)

V_R retention volume (ml)

V'_{R1} contribution of the isocratic (dwell volume) step to the net retention volume

V'_{R2} contribution of the gradient step to the net retention volume

V_0 column dead (hold-up) volume

V_{01} contribution of the isocratic (dwell volume) step to V_0

V_{02} contribution of the gradient step to V_0

a parameter of the retention Eq. (3)

a' parameter of the retention Eq. (2)

b parameter of the retention Eq. (3)

b' parameter of the retention Eq. (2)

k' capacity factor of a sample solute

k'_a capacity factor of a sample solute in pure non-polar solvent

k'_0 capacity factor of a sample solute in pure polar solvent – parameter of the retention Eq. (1)

k'_1 capacity factor of a sample solute in the mobile phase in the dwell volume step, i.e., at the start of the gradient

m parameter of the retention Eq. (1)

φ concentration of the strong (polar) solvent in the mobile phase (isocratic) or instantaneous concentration of this solvent at the top of the column corresponding to the volume V of the eluate

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Appendix A

If the gradient dwell volume V_D cannot be neglected and a sample solute moves some distance along the column before the front of the gradient reaches the top of the column, the elution occurs in two steps: first, isocratic and the second, gradient. This situation is equivalent to the elution on two columns in series, where the first is eluted in the isocratic mode in the mobile phase containing the strong solvent in concentration A (starting concentration in gradient elution) and the second in the gradient mode. The contribution of the first part (column) to the total retention volume of the solute $V_1 = V_D$. The part of the column through which the solute has migrated at the end of the first step, i.e., at the time when it is taken by the front of the gradient,

has the dead (hold-up) volume V_{01} corresponding to the proportional part of the total column dead volume, V_0: $V_{01}/V_0 = V_D/[V_0(1+k'_1)]$, where k'_1 is the capacity factor in the mobile phase of initial composition. Then $V_{01} = V_D/(1+k'_1)$ and the second part (column) which remains responsible for the gradient elution step has the dead volume $V_{02} = V_0 - V_D/(1+k'_1)$. The total retention volume is $V_R = V'_{R1} + V'_{R2} + V_0$, where V'_{R2} is the contribution of the gradient step to the net retention volume, which can be calculated from Eq. (5) or Eq. (6) after subtracting V_0 and using V_{02} instead of V_0. V'_{R1} is related to the gradient dwell volume: $V'_{R1} = V_D - V_{01} = V_D/[1+(k'_1)^{-1}]$.

Applying this approach to the systems where the retention is controlled by the two-parameter equation (Eq. (1)), the equation (Eq. (5)) for the retention volume in gradient elution with solute migrating along the column in the gradient dwell volume is modified to Eq. (7):

$$V_R = \frac{1}{B}[(m+1)Bk'_0\left(V_0 - \frac{V_D}{1+k'_0A^{-m}}\right) + A^{(m+1)}]^{\frac{1}{m+1}} - \frac{A}{B} + \frac{V_D}{1+(k'_0)^{-1}A^m} + V_0 \quad (7)$$

In the same way, Eq. (6) is changed to Eq. (8):

$$V_R = \frac{1}{b\cdot B}\left\{b\cdot B(m+1)\left[V_0 - \frac{V_D}{1+(a+b\cdot A)^{-m}}\right] + (a+b\cdot A)^{(m+1)}\right\}^{\frac{1}{m+1}} - \frac{a+A\cdot b}{b\cdot B} + \frac{V_D}{1+(a+b\cdot A)^m} + V_0 \quad (8)$$

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