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Gradient elution in normal-phase high-performance liquid chromatographic systems

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Abstract

Gradient elution is widely used for separation of complex samples in reversed-phase HPLC systems, but is less frequently applied in normal-phase HPLC, where it has a notoriously bad reputation for poor reproducibility and unpredictable retention. This behaviour is caused by preferential adsorption of polar solvents used in mixed mobile phases, which may cause significant deviations of the actual gradient profile from the pre-set program. Another important source of irreproducible retention behaviour is gradual deactivation of the adsorbent by adsorption of even traces of water during normal-phase gradient elution. To avoid this phenomenon, carefully dried solvents should be used. Finally, column temperature should be carefully controlled during normal-phase gradient elution if reproducible results are to be obtained. Working with dry solvents at a controlled constant temperature and using a sophisticated gradient-elution chromatograph, reproducibility of the retention data in normal-phase gradient elution better than 2% may be achieved even over several months of column use. The retention data in gradient elution can be calculated accurately if appropriate corrections are adopted for the gradient dwell volume and for the preferential adsorption of the polar solvents using experimental adsorption isotherms. The average error of prediction for the corrected calculated gradient retention data was lower than 2% for a silica gel column and lower than 3% for a bonded nitrile column, which may be suitable for the optimization of separation. Further, a simple approach is suggested for rapid estimation of changes in the retention induced by a change in the gradient profile in normal-phase HPLC. For such a rough estimation, it is not necessary to know the parameters of the dependence of the solute retention factors on the composition of the mobile phase.
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1. Introduction

Normal-phase adsorption chromatography (NPC) is the oldest liquid chromatographic mode, using either an inorganic adsorbent (silica or, less often, alumina) or a moderately polar bonded phase (cyanopropyl–$(CH_2)_3$–CN, diol–$(CH_2)_3$–O–CH_2–CHOH–CH_2–OH, or aminopropyl–$(CH_2)_3$–NH_2) chemically bonded on a silica gel support. As the retention on inorganic adsorbents originates in the interactions of the polar adsorption centres on the surface with polar functional groups of the analytes, this mode is also known as adsorption or liquid–solid chromatography. The mobile phase is a mixture of organic solvents of different polarities, such as *n*-hexane and 2-propanol, usually without added water. Even though since its introduction in late

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1960s reversed-phase (RPC) HPLC has become much more popular than NPC for separation of different classes of analytes, NPC is useful for separation of very hydrophilic compounds non-retained in RPC or of hydrophobic samples very strongly retained by RPC. NPC often has better selectivity for the separation of samples containing positional isomers or stereoisomers. Finally, if sample pre-treatment procedures involve the extraction into a non-polar solvent, direct injection on to a RPC column may cause problems, in contrast to NPC. Other advantages of NPC are: better stability of HPLC columns due to a lower pressure drop in non-aqueous mobile phases, larger possibilities of changing separation selectivity by selecting either the mobile phase or the column packing and better solubility or stability of some samples in non-aqueous solvents [1].

On the other hand, the control of retention in NPC by adjusting the composition of the mobile phase can be less reproducible and predictable than in RPC because of preferential adsorption (uptake) of polar organic solvents and of water by the column packing. These effects may become especially important during gradient elution where the composition of the mobile phase changes, unless the water contents in the mobile phase and temperature are carefully controlled. This is the main reason for a strong bias against the use of gradient elution in NPC by many practising chromatographers. However, gradient elution NPC can be appropriate for the separation of samples containing both isomers and compounds with different numbers of polar groups whose retention widely differs. It has been found especially useful for separation and characterisation of industrial polymer samples containing polar monomer units, where it usually provides better selectivity than gradient elution RPC [2,3].

If a column is not in equilibrium with a solvent mixture, preferential adsorption of the solvents with higher elution strengths occurs on the column and consequently the retention of analytes may significantly change with time, leading to irreproducible retention data. This phenomenon is known as ‘‘solvent demixing effect’’ and generally occurs in all HPLC modes. It was discussed by Quarry et al. [4] for RPC systems, but it is even more important in NPC because of greater retention of polar solvents on polar adsorbents in comparison to the retention of polar organic solvents on RPC columns in aqueous–organic mobile phases. The column uptake of polar solvents on silica gel and other strongly polar adsorbents in thin-layer and dry column chromatographic techniques [5,6] does not occur in isocratic NP HPLC if the column has been allowed to come to equilibrium with the mobile phase for a sufficiently long time. However, solvent demixing is still an important undesirable effect in contemporary gradient-elution NPC, as the adsorption of the polar solvent from the mobile phase on the polar adsorbent may cause deviations of the actual gradient profile from the pre-set program. To suppress this effect, gradients using a series of as many as 12 different solvents with gradually increasing polarities (so-called ‘‘incremental gradient elution’’) were suggested at the early stage of development of HPLC techniques [7–9]. This approach should allow a smooth increase in polarity during gradient elution and avoid excessive adsorption of solvents with only small differences in polarities, but is difficult to practice with modern commercial instrumentation for HPLC, which can form precise concentration gradients by mixing three to four solvents at maximum. With such gradients, preferential adsorption of polar solvents in gradient-elution NP HPLC is more significant if the gradient is started in a pure non-polar solvent than with gradients started at a non-zero concentration of the polar solvent [10].

Another practical complicating aspect of gradient elution in NP HPLC is the presence of traces of water in the solvents used as the components of the mobile phase. Because of the very high polarity of water, even if the concentration of water in the mobile phase is changed by only a few parts per million, the retention times of sample compounds in NPC may change by as much as an order of magnitude [11,12]. Hence, it is very important to control the water content in the chromatographic system. The water content in organic solvents used as the components of the gradient varies with respect to the purity, previous treatment and time of storage of the solvent, fluctuations of the ambient temperature and humidity of the air. To avoid undesirable changes in the contents of adsorbed water during gradient elution in NP HPLC, it was proposed to use all solvents in the mobile phase with the water concentration adjusted to equilibrium with the water content in the adsorbent (‘‘isohydric solvents’’) [13].

However, proper adjusting of the matching water concentrations in two or more different solvents is tedious and difficult to maintain over a longer time period. This also applies for partially (e.g. 50%) saturated solvents prepared intentionally by mixing dry and water-saturated solvents. Because of different saturation water concentrations in less and in more polar solvents, we obtain in such a case actually a ternary gradient of increasing concentration of the polar solvent and water, which may be difficult to reproduce. To obtain reproducible results, we found it more practical to use carefully dried solvents to mix during the gradient elution [10,14–16]. To summarize, variations in the retention data in gradient elution NPC and unacceptable changes in retention over time are due to various causes including mainly adsorption of water by the column, solvent demixing in the early stages of a gradient and variation in column temperature. These phenomena are investigated in present work with two objectives:

(1) To develop a simple procedure for rapid semi-quantitative estimation of the effects of changing gradient parameters on the retention data in gradient-elution NPC.

(2) To elaborate as accurate a prediction of retention in gradient-elution NPC as possible, taking into account various complicating phenomena, so that the calculation procedures could be suitable for predictive optimisation.

2. Theoretical

2.1. Description of the retention in normal-phase gradient elution

The effect of the composition of two-component (binary) mobile phases on the retention in normal-phase systems can be described using theoretical models of adsorption. The first model of retention in adsorption chromatography was developed by Snyder in the early 1960s [6,17,18]. The adsorption was understood as a competitive phenomenon between the molecules of the solute and of the solvent on the adsorbent surface. Later, corrections were introduced for preferential adsorption on localized adsorption centers [19,20]. Soczewiński [21,22] suggested a similar model of retention assuming adsorption in a mono-molecular 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 an identical equation describing the retention factor of an analyte, k, as a function of the concentration of the stronger (more polar) solvent, c, in binary mobile phases comprised of two solvents of different polarities [17,18,22,23]:

$$k = k_0 \cdot c^{-m} \tag{1}$$

where k_0 and m are experimental constants, k_0 being the retention factor in pure strong solvent.

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 [24,25]:

$$k = (a + b \cdot c)^{-m} \tag{2}$$

Here, 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 retention factor in pure non-polar solvent). If the retention in pure non-polar solvent is very high, the term a in Eq. (2) can be neglected and this equation becomes Eq. (1) [18].

A theoretical description of binary gradient elution in normal-phase systems was presented by Jandera and Churáček [25–27]. A linear gradient where the concentration of a polar solvent **B** in a less polar one, c, increases as the volume of eluate, V, increases from the initial concentration $c = A$ at the start to the final concentration $c = c_G$ in time t_G at a flow-rate F_m is described by Eq. (3):

$$c = A + \frac{c_G - A}{t_G \cdot F_m} \cdot V = A + B \cdot V \tag{3}$$

where 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 Eq. (1), the retention volume, V_R, of a sample compound in gradient-elution chromatography can be calculated as [26]:

$$V_R = \frac{1}{B}\left[(m+1)Bk_0V_0 + A^{(m+1)}\right]^{1/(m+1)} - \frac{A}{B} + V_0 \tag{4}$$

V_0 is the column hold-up volume, the parameter m in Eqs. (1) and (3) is the stoichiometric coefficient of the adsorption equilibrium between the analyte and the polar solvent **B** in a binary mobile phase, i.e. it

has the meaning of the number of molecules of solvent **B** necessary to displace one adsorbed molecule of the analyte. For many low-molecular mass compounds, one adsorbed molecule of analyte can be displaced by approximately one molecule of the solvent **B** and the value of m is very close to one, even though numerous exceptions from this rule have been observed. If the retention of a solute is adequately described by Eq. (1) and $m \approx 1$, the volume of the polar solvent **B** that should pass through the column to accomplish the elution of the analyte, $V_{solv} = k_0 \cdot V_0$, is constant and does not depend on the concentration of **B** in a binary mobile phase used for isocratic elution or on the gradient programme, as shown in Appendix A.

In this case, it can be derived from Eq. (4) that a change in the net retention volume caused by a change in the gradient programme can be very simply estimated as follows:

$$V_{solv} = (V'_{R1})^2 \cdot \frac{B_1}{2} + V'_{R1} \cdot A_1 = (V'_{R2})^2 \cdot \frac{B_2}{2} + V'_{R2} \cdot A_2 \quad (5)$$

This simplified equation enables rapid estimation of the change in retention volumes that can be expected when the steepness of the gradient is changed from B_1 to B_2 and (or) the initial concentration of the polar solvent **B** from A_1 to A_2, but Eq. (5) may not be valid for compounds whose parameter m of Eq. (1) differs significantly from 1. It should be noted that Eq. (5) cannot be used for reversed-phase gradient elution.

Eq. (1) does not describe accurately enough the effect of the mobile phase on the retention of some compounds and the three-parameter Eq. (2) is necessary for this purpose. In this case, a slightly more complex equation should be used to calculate the retention volumes in gradient elution [10,25]:

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

2.2. Effect of the dwell volume on retention

Eqs. (4) or (6) can be used if the volume between the gradient former and the column (i.e. the ‘‘gradient dwell volume’’) V_D is low enough so 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 often not the case and with some instruments the gradient dwell volume can be quite significant, even a few milliliters. At the start of the gradient, this volume in the instrument is filled with the mobile phase of the composition corresponding to the initial gradient conditions and consequently the ‘‘dwell volume’’ of the mobile phase should flow through the column before the starting gradient profile arrives at the top of the column. Hence, the expected gradient elution is delayed and some sample solutes, especially weakly retained ones, may migrate certain distance along the column during this unintended initial isocratic step, which contributes in this way to the elution volume. This behaviour can be described as two-step elution with the first, isocratic (dwell volume) step, followed by the second, gradient, step. The dwell volume may differ from one instrument to another and may cause difficulties if a gradient HPLC method is transferred from one instrument to another one. To avoid these problems and to make possible precise prediction of the gradient elution data by calculation, the gradient dwell volume should be accounted for in method development and appropriate correction should be adopted for the instrumental gradient delay in calculations [10,25–28], as described in Appendix B [10]. This correction results in slight modification of Eq. (4):

$$V'_R = V_D \frac{1}{1 + \frac{A^m}{k_0}} + \frac{1}{B}\left[(m+1)Bk_0\left(V_0 - \frac{V_D}{(1 + k_0 A^{-m})}\right) + A^{m+1}\right]^{1/(m+1)} - \frac{A}{B} \quad (7)$$

or of Eq. (6):

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

2.3. Effect of the adsorption of polar solvents on retention

As discussed in the Introduction, possible uptake of polar solvent(s) from mixed mobile phases on the column can significantly change the actual gradient and affect the separation in NP gradient-elution chromatography. The errors in calculated retention volumes caused by this effect are less important with gradients that start at a non-zero initial concentration, A, of the polar solvent **B** [10]. If for some reason a gradient should start at $A = 0$, an empirical correction approach was suggested consisting in adding the experimentally determined breakthrough volume of the strong solvent to the calculated V'_R [15]. However, this approach has several drawbacks: (1) it is justified only if sample compounds do not migrate significantly along the column prior to the breakthrough of the polar solvent **B**; (2) it necessitates experimental determination of the breakthrough volumes for each gradient program used, which is not very practical and accurate with solvents that do not absorb light in the UV region. Hence, in this work, another more general approach was investigated, based on the experimentally determined adsorption isotherm describing the distribution of the polar solvent between the binary mobile phase and the column used.

The distribution equilibrium of a binary solvent mixture can often be described by a simple Everett's equation [29], which is equivalent to the two-parameter Langmuir isotherm [30] if one solvent is strongly adsorbed, as is usual in adsorption NP chromatography:

$$q = \frac{q_s \cdot b_1 \cdot c}{(1 + b_1 \cdot c)} = \frac{a_1 \cdot c}{(1 + b_1 \cdot c)} \tag{9}$$

Here, q is the concentration of the sample compound in the stationary and c that in the mobile phases, a_1, b_1 are the coefficients of the isotherm and q_s is the column saturation capacity. In our earlier work, we found that the Langmuir model does not describe satisfactory the distribution of some binary solvent mixtures in normal-phase systems [31,32] and we introduced the following isotherm equation describing two-layer adsorption of the polar solvent B on a polar adsorbent:

$$q = \frac{q_{1s} \cdot b_1 \cdot c}{(1 + b_1 \cdot c)} \cdot (1 + b_2 \cdot c) = \frac{A_1 \cdot c}{1 + B_1 \cdot c} + A_2 \cdot c \tag{10}$$

where q_{1s} is the adsorbent saturation capacity for the adsorption in the first layer and b_1, b_2, A_1, A_2, B_1, are other isotherm parameters.

The retention volume in normal-phase gradient-elution chromatography can be corrected for the uptake of the polar solvent on the column by taking into account that the volume of the pure polar solvent **B** which is necessary to elute sample compounds, V_{solv}, should be increased to include the volume of **B** adsorbed on the column from the start of the gradient till the elution of the peak maximum, V_{ads}. V_{ads} can be calculated from the appropriate adsorption isotherm, as shown in Appendix C. Using this approach, Eq. (4) is modified as follows:

$$V'_R = \frac{1}{B}\left[(m+1)\cdot B \cdot k_0 \cdot V_0 + (A^2 + 2B \cdot V_{ads})^{(m+1)/2}\right]^{1/(m+1)} - \frac{A}{B} \tag{11}$$

or Eq. (6) to:

$$V'_R = \frac{1}{b \cdot B}\left\{(m+1)\cdot b \cdot B \cdot V_0 + \left[a + b \cdot (A^2 + 2B \cdot V_{ads})^{1/2}\right]^{(m+1)}\right\}^{1/(m+1)} - \frac{a + A \cdot b}{b \cdot B} \tag{12}$$

The adsorbed volume is introduced into Eqs. (11) or (12) from one of the Eqs. (C.6), (C.7), (C.10) or (C.11) in Appendix C, whichever is more appropriate with respect to the isotherm controlling the distribution of the polar solvent in the normal-phase chromatographic system used.

3. Experimental

3.1. Equipment

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 thermostated column compartment and a Series 7994A workstation (Hewlett-Packard, Palo

Alto, CA, USA) was used to acquire the elution data. The experimental gradient dwell volume was 0.505 ml. Glass cartridge columns, 150 mm×3.3 mm I.D., packed with silica gel Separon SGX, 7.5 μm (V_0 = 0.905 ml) and Separon SGX Nitrile, 7.5 μm (V_0 = 0.966 ml) were obtained from Tessek (Prague, Czech Republic). The flow-rate of the mobile phases was kept at 1 ml/min and the temperature at 40°C in all experiments.

3.2. Mobile phases and samples

2-Propanol, *n*-heptane and dioxane, all of HPLC grade, were purchased from Baker (Deventer, The Netherlands). The solvents were dried and kept in tightly closed dark bottles over Dusimo 5 Å molecular sieve beads (Lachema, Brno, Czech Republic), previously activated at 300°C (ca. 30–40 g/l), filtered using a Millipore 0.45-μm filter and degassed in an ultrasonic bath immediately before use. Mobile phases were prepared directly in the HP 1090M instrument from the components continuously stripped by a stream of helium.

Phenylurea herbicides sample compounds were obtained from Lachema (Brno, Czech Republic). Their structures are given in Table 1. The solutes were dissolved in the mobile phase to provide adequate response of the UV detector (approximately 10–20 μg/ml); 5-μl sample volumes were injected in each experiment.

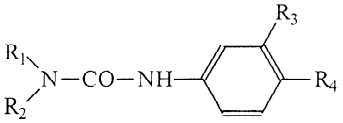
3.3. Methods

The columns were first equilibrated with approximately 20 column hold-up volumes of the mobile phase and then the retention volumes, V_R, of the sample compounds were measured under isocratic conditions in mobile phases with different concentrations of 2-propanol or of dioxane in heptane, hexane or in dichloromethane. The parameters of the retention Eqs. (1) and (2) were determined from the isocratic retention factors, $k = (V_R/V_0 - 1)$ using linear or non-linear regression, as described previously [33]. In gradient-elution experiments, a 5-min reversed gradient (to speed-up the column re-equilibration) and a 5-min isocratic equilibration time with the starting mobile phase were used after the end of each experiment to re-equilibrate the column.

Using this procedure, the reproducibility of the retention times among replicate runs was 1.5% or better. The column dead (hold-up) volume, V_0, was determined using trichloroethylene as a non-retained marker compound.

To acquire the data necessary for the determination of the equilibrium isotherms by frontal analy-

Table 1
Chemical structures of phenylurea herbicides and related compounds studied



Compound		R_1	R_2	R_3	R_4
PHU	Phenuron	$-CH_3$	$-CH_3$	–H	–H
DPU	Desphenuron	$-CH_3$	–H	–H	–H
NBU	Neburon	$-CH_3$	$-C_4H_9$	–Cl	–Cl
DCU	Deschlorometoxuron	$-CH_3$	$-CH_3$	–H	$-OCH_3$
IPU	Isoproturon	$-CH_3$	$-CH_3$	–H	$-CH(CH_3)_2$
CMU	bis-*N*,*N*′-(3-Chloro-4-methylphenyl)urea	3-Chloro-4-methylphenyl	–H	–Cl	$-CH_3$
FMU	Fluometuron	$-CH_3$	$-CH_3$	$-CF_3$	–H
CTU	Chlorotoluron	$-CH_3$	$-CH_3$	–Cl	$-CH_3$
DIU	Diuron	$-CH_3$	$-CH_3$	–Cl	–Cl
MOU	Monuron	$-CH_3$	$-CH_3$	–H	–Cl

sis method [32,34], the HP 1090M liquid chromatograph was used with the mobile phase placed in one reservoir flask and the sample solution in another one. In each experiment, the ratio of the flow-rates of the two solutions was adjusted from 0 to 100% in successive 10 or 5% steps. Time was allowed for the stabilisation of the detector signal after each concentration change. The flow-rate (1 ml/min) and the column temperature (40°C) were kept constant during all the experiments. The solute concentration in the stationary phase was determined from the appropriate integral mass balance equation [34] using (A) the experimental concentrations of the sample components at the plateaus of the frontal analysis curve and (B) the retention volumes corresponding to the inflection points on the breakthrough curve, corrected for the volume of the tubing between the mixing point of the liquids pumped in each channel and the column top (0.31 ml). All experiments were repeated at least twice.

All calculations were performed in the spreadsheet form using the Quattro Pro 5.0 table editor, except for modelling of breakthrough curves, which was performed by numerical simulations using a home-written program in Basic 4.

4. Results and discussion

4.1. Possibilities of simple rapid prediction of retention data in normal-phase gradient elution

As shown in the theoretical part, the volume of the pure polar solvent *B* necessary to elute a sample solute, V_{solv}, should be independent of the profile of a linear gradient if the retention is controlled by the adsorption equilibrium where one molecule of **B** replaces one molecule of the solute. If so, the changes in the retention volumes corresponding to a change in either the gradient steepness or the initial concentration of **B** can be directly predicted from a simple Eq. (5). To check the validity of this assumption, Eq. (5) was used to calculate the volumes V_{solv} of 2-propanol and dioxane from the experimental net retention volumes of phenylurea compounds in gradient elution with various gradient programs on a silica gel and on a bonded nitrile column with heptane, hexane and dichloromethane as weak solvents. The results are shown in Table 2.

The volumes V_{solv} are generally greater on a silica gel than on a bonded nitrile column, with dioxane than with 2-propanol as the polar solvent and with heptane or hexane than with dichloromethane as the non-polar solvent. This is in agreement with the differences in polarities of the column packing materials and of the mobile phase components — stronger adsorption is expected on more polar silica gel adsorbent and with less polar solvents used as the components of the mobile phase. V_{solv} are approximately independent of the initial concentration of the polar solvent B at the start of the gradient in all the systems studied except 2-propanol/heptane/silica gel. V_{solv} increase by 20–30% in the 2-propanol/heptane/silica gel system, by 30–50% in the dioxane/heptane/silica gel system and by 15–25% in the dioxane/hexane/bonded nitrile system when the gradient steepness decreases three times. On the other hand, V_{solv} are not significantly affected by the steepness of the gradient in the 2-propanol/dichloromethane/silica gel and in the 2-propanol/hexane/nitrile systems. The agreement between the values of V_{solv} found in the latter systems is surprisingly good, as the experimental values of the parameter *m* in Eq. (1) for the sample compounds studied vary from 0.6 to 2, and for many solutes Eq. (1) is less suitable than Eq. (2) to describe the dependence of the retention factors on the concentration of the polar solvent under isocratic conditions. The variation of V_{solv} with gradient steepness in other systems can be at least partly attributed to a significant effect of the preferential adsorption of the polar solvent, as discussed in detail in Section 4.2.

To further investigate possibilities of simple prediction of retention data in NP gradient-elution HPLC on silica gel and on bonded nitrile columns, the experimental elution volumes of several phenylurea herbicides in the most steep gradients (0–50% 2-propanol in 30 min or 0–100% dioxane in 30 min, respectively) were used to predict V_R for other gradients — less steep or starting at a non-zero concentration of the polar solvent **B** — by calculation using Eq. (5). In most cases, the simple calculation yields underestimated elution volumes for gradients starting at 0% polar solvent and over-estimated data for gradients starting at a non-zero

Table 2
Volume of the pure polar solvent, V_{solv} (ml), necessary to accomplish the elution of a sample compound in normal-phase HPLC

Silica gel column, gradients of 2-propanol (P) in n-heptane							
Solute	0–50% P	0–25% P	0–16.7% P	3–50% P	6–50% P	9–50% P	V_{solv}, A[a]
DPU	0.79	0.94	1.04	0.78	0.74	0.69	0.75±0.03
CMU	0.67	0.80	0.89	0.66	0.62	0.57	0.63±0.03
IPU	0.76	0.84	0.89	0.75	0.73	0.70	0.73±0.03
DCU	1.50	1.75	1.90	1.51	1.48	1.43	0.48±0.03
Silica gel column, gradients of dioxane (D) in n-heptane							
Solute	0–100% D	0–50% D	0–33.3% D	3–100% D	6–100% D	9–100% D	V_{solv}, A[a]
DPU	2.97	3.90	4.52	3.00	3.00	2.98	2.99±0.02
CMU	2.49	3.31	3.90	2.51	2.50	2.47	2.49±0.01
IPU	1.47	1.84	2.08	1.47	1.46	1.42	1.46±0.02
DIU	1.58	1.98	2.25	1.58	1.57	1.53	1.56±0.02
Silica gel column, gradients of 2-propanol (P) in dichloromethane							
Solute	1–50% P	1–25% P	1–16.7% P				V_{solv}, A
PHU	0.082	0.087	0.091				0.09±0.004
MOU	0.077	0.080	0.081				0.08±0.002
CMU	0.18	0.21	0.22				0.20±0.02
DPU	0.23	0.26	0.29				0.26±0.02
Bonded nitrile column, gradients of 2-propanol in n-hexane							
Solute	0–50% P	0–25% P	0–16.7% P	3–50% P	6–50% P	9–50% P	V_{solv}, A
FMU	0.33	0.35	0.35	0.33	0.32	0.30	0.33±0.02
CTU	0.39	0.41	0.42	0.39	0.38	0.36	0.39±0.02
PHU	0.52	0.53	0.53	0.53	0.52	0.50	0.52±0.01
CMU	0.13	0.14	0.14	0.13	0.13	0.12	0.13±0.007
Bonded nitrile column, gradients of dioxane in n-hexane							
Solute	0–100% D	0–50% D	0–33.3% D	3–100% D	6–100% D	9–100% D	V_{solv}, A
NBU	0.50	0.56	0.59	0.50	0.49	0.47	0.52±0.04
FMU	0.86	0.99	1.06	0.86	0.85	0.82	0.91±0.08
CTU	1.11	1.29	1.39	1.12	1.10	1.08	1.18±0.12
CMU	0.98	1.13	1.21	0.98	0.97	0.94	1.04±0.09

Gradient time = 30 min, 1 ml/min, 40°C. V_{solv} calculated from Eq. (A4). Solutes as in Table 1. V_{solv}, A, average value±SD.
[a] Except gradients ending at less than 50% P or 100% D.

concentration of 2-propanol or dioxane (Table 3), probably due to the preferential adsorption of polar solvents during gradient elution and to other effects that are not accounted for in the calculation.

The main advantage of using simple Eq. (5) is that it does not necessitate the determination of the parameters of the retention equations (of the dependencies of k on c) and can be used for rapid prediction of retention in gradient-elution NPC just from the retention data measured experimentally in another gradient-elution run. The average error of prediction of the retention times reported in Table 3 is approximately 7%, which is acceptable for rapid rough estimate of the effect of changing gradient profile on the retention. However, for full method optimisation, more rigorous calculation approaches are required as discussed in Sections 4.2 and 4.3.

4.2. Preferential adsorption and breakthrough of polar solvents in gradient-elution NP HPLC

In our earlier study, we have found that the isotherm describing the distribution of binary solvent mixtures between liquid phase and polar adsorbents

Table 3
Retention volumes of phenylureas in normal-phase HPLC

Solute		Silica gel column, gradients of 2-propanol (P) in heptane					
		0–50% P	0–25% P	0–16.7% P	3–50% P	6–50% P	9–50% P
IPU	V_R(E)	11.18	16.01	19.50	9.47	8.00	6.60
	V_R(C)	–	15.23	18.34	9.75	8.67	7.46
DPU	V_R(E)	11.31	16.68	21.66	9.53	8.01	6.68
	V_R(C)	–	15.41	18.56	9.88	8.64	7.59
CMU	V_R(E)	10.55	15.55	16.69	8.82	7.25	5.89
	V_R(C)	–	14.34	17.24	9.11	7.89	6.88
DCU	V_R(E)	14.86	22.02	27.78	13.27	11.90	10.71
	V_R(C)	–	20.43	27.71	13.50	12.23	11.07
Avg. error (%)			−6.9	−5.9	+2.9	+5.9	+11.7

Solute		Silica gel column, gradients of dioxane (D) in heptane					
		0–100% D	0–50% D	0–33.3% D	3–100% D	6–100% D	9–100% D
IPU	V_R(E)	10.93	16.41	20.82	10.07	9.26	8.43
	V_R(C)	–	14.85	17.86	10.19	9.50	8.85
DIU	V_R(E)	11.14	17.04	21.45	10.36	9.47	8.66
	V_R(C)	–	15.15	18.22	10.40	9.71	9.06
CMU	V_R(E)	13.57	21.12	27.50	12.87	11.96	11.19
	V_R(C)	–	18.58	22.43	12.86	12.18	11.53
DPU	V_R(E)	14.70	22.90	30.04	13.83	13.09	12.32
	V_R(C)	–	20.18	24.39	14.00	13.33	12.68
Avg. error (%)			−11.4	−16.6	+0.7	+2.2	+3.9

Solute		Silica gel column, gradients of 2-propanol (P) in dichloromethane		
		1–50% P	1–25% P	1–16.7% P
CMU	V_R(E)	5.03	6.53	7.62
	V_R(C)	–	6.47	7.34
DPU	V_R(E)	5.39	7.12	8.30
	V_R(C)	–	6.88	7.96
Avg. error (%)			−3.0	−3.9

Solute		Bonded nitrile column, gradients of 2-propanol (P) in hexane					
		0–50% P	0–25% P	0–16.7% P	3–50% P	6–50% P	9–50% P
CMU	V_R(E)	6.34	8.26	9.84	4.29	3.03	2.34
	V_R(C)	–	8.39	9.95	4.93	3.98	3.37
FMU	V_R(E)	8.55	11.49	13.74	6.67	5.21	4.08
	V_R(C)	–	11.51	13.78	7.10	5.96	5.10
CTU	V_R(E)	9.10	12.13	14.70	7.21	5.58	4.56
	V_R(C)	–	12.29	14.73	7.65	6.48	5.57
PHU	V_R(E)	10.16	14.02	16.71	8.46	7.04	5.71
	V_R(C)	–	13.79	16.57	8.72	7.50	6.52
Avg. error (%)			+1.4	+0.6	+7.6	+17.0	+26.3

Solute		Bonded nitrile column, gradients of dioxane (D) in hexane					
		0–100% D	0–50% D	0–33.3% D	3–100% D	6–100% D	9–100% D
NBU	V_R(E)	8.01	11.28	12.98	6.93	6.00	5.09
	V_R(C)	–	10.72	12.80	7.25	6.57	5.97
FMU	V_R(E)	9.56	13.42	16.26	8.52	7.66	6.77
	V_R(C)	–	12.91	15.49	8.81	8.12	7.49
CTU	V_R(E)	10.25	14.98	18.26	9.53	8.64	7.78
	V_R(C)	–	13.89	16.68	9.52	8.81	8.17
CMU	V_R(E)	10.07	15.57	17.50	9.06	8.20	7.34
	V_R(C)	–	13.63	16.37	9.32	8.63	7.99
Avg. error (%)			−6.9	−5.3	+2.8	+5.1	+10.3

V_R(E), ml, experimental values, V_R(C), ml, values calculated from Eq. (5) using V_R(E) values in the steepest gradient (to 50% 2-propanol or to 100% dioxane). Solutes as in Table 1, gradient time 30 min, temperature 40°C, 1 ml/min.

depends on the type of the adsorbent and of the solvents [32]. The distribution of 2-propanol between heptane or hexane and both a silica gel column and a bonded nitrile column is adequately described by strongly curved Langmuir isotherms (Eq. (9)) with rather steep initial slopes and plateaus corresponding to the column saturation occurring at low concentrations of 2-propanol in the mobile phase. On the other hand, the isotherm of dioxane in hexane on a bonded nitrile column is almost linear and the saturation of the adsorbent capacity does not take place in binary solvent mixtures containing up to 50% dioxane. This is also the case with the distribution of 2-propanol between dichloromethane and a silica gel column, which is not adequately described by the Langmuir model. The experimental distribution can probably be explained by multi-layer adsorption on the surface of the adsorbent. An associative isotherm model (Eq. (10)) derived assuming two-layer adsorption on the surface of the adsorbent describes very well the adsorption of 2-propanol from dichloromethane on a silica gel column [32]. Here, the isotherm has a slightly sigmoidal (S-shape) profile and the saturation of the adsorbent with 2-propanol does not occur in the binary mobile phases containing up to 12% 2-propanol in dichloromethane. The experimental observation suggests that multi-layer adsorption behaviour is more likely in the systems with lower polarity differences between the adsorbent and (or) the two components of a binary solvent mixture, which could explain, e.g. the differences in the uptake of 2-propanol on a silica gel column from heptane and from dichloromethane illustrated by curves 1 and 3 in Fig. 1.

Hence, the type of the adsorption isotherm generally has a strong impact on the uptake of the polar solvent **B** on the column during gradient elution. This is illustrated by the data in Table 4 and by several examples in Fig. 1, showing the volume of pure polar solvents, V_{ads}, adsorbed on a silica gel and on a bonded nitrile chromatographic columns in equilibrium with binary mobile phases of varying composition under isocratic conditions: The silica gel column is almost completely saturated with the polar solvent in mobile phases containing more than approximately 1% 2-propanol or 2% dioxane in heptane ($V_{ads} = 0.09$ ml, i.e. 10% of V_0 for 2-propanol and 0.08 ml, i.e. 9% of V_0 for dioxane). Lower breakthrough volumes of dioxane with respect to 2-propanol are obviously caused by stronger adsorption of the latter, more polar, solvent. The bonded nitrile column is almost saturated by adsorption of 0.06 ml of 2-propanol (6% of V_0) from 2-propanol–hexane mobile phases.

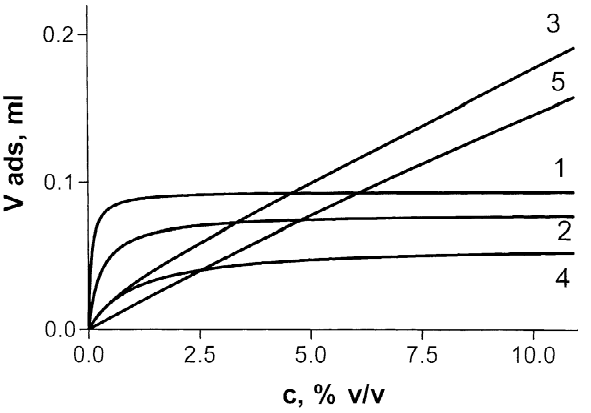


Fig. 1. Volume of polar solvent adsorbed on a chromatographic column, V_{ads}, in equilibrium with the concentration c of the polar solvent in the mobile phase. Plots (1–3) — Column: Silica gel, Separon SGX, 7.5 μm, 150×3.3 mm I.D. ($V_0 = 0.905$ ml, phase ratio $\Phi = 0.418$). Plots (4, 5) — Bonded nitrile, Separon SGX Nitrile, 7.5 μm, 150×3.3 mm I.D. ($V_0 = 0.966$ ml, phase ratio $\Phi = 0.328$). Binary mobile phases: 2-propanol–heptane (1), dioxane–heptane (2), 2-propanol–dichloromethane (3), 2-propanol–hexane (4), dioxane–hexane (5).

The volume of the pure polar solvent adsorbed in the course of gradient elution starting at 100% less polar solvent ($A = 0$) is equal to the saturation volume of the column, V_{sat} and does not depend on the steepness of the gradient. However, the polar solvent uptake steeply decreases if the gradient is started at $A > 0$ and it drops to 1–7 μl (less than 1% of V_0) for gradients starting at 3–9% 2-propanol or of dioxane on the silica gel column and to 15, 9 and 6 μl, i.e. to 26, 15 and 10% of the full saturation volume for the gradients starting at 3, 6 and 9%, respectively, 2-propanol in hexane (Table 4). With gradients of 2-propanol in dichloromethane on the silica gel column and of dioxane in hexane on the bonded nitrile column, V_{sat} could not be determined as the column does not get fully saturated during the gradient elution (curves 3 and 5 in Fig. 1).

The breakthrough curves under isocratic condi-

Table 4
Volumes of polar solvents, V_{sat} (ml), necessary to saturate the column calculated from Eq. (C.7) assuming validity of the Langmuir isotherm (Eq. (9), parameters a_1, b_1 [l/l]), breakthrough volumes, V_B (ml), and breakthrough concentrations, c_B (v/v), of the polar solvents (I): determined from the breakthrough curves calculated by numerical simulations (Figs. 2–4) and (II); calculated using Eqs. (C.2) and (C.3) on a silica gel (S) and on a bonded nitrile (N) columns

Column Silica	Gradient (30 min) Propanol/heptane (%)	V_{sat} (ml)	V_B (ml) I	V_B (ml) II	c_B (%v/v) I	c_B (%v/v) II
Langm.	0–50	0.094	4.90	4.76	5.0	5.6
$a_1 = 354$,	0–25	0.094	6.30	6.15	3.8	3.9
$b_1 = 1424$	0–16.7	0.094	7.32	7.22	3.0	3.2
	3–50	0.002	1.45	1.47	3.03	3.11
	6–50	0.001	1.40	1.42	6.03	6.03
	9–50	<0.001	1.40	1.41	9.03	9.01
Silica	Dioxane/heptane (%)					
Langm.	0–100	0.079	3.58	3.58	6.7	7.2
$a_1 = 69.4$,	0–50	0.079	4.44	4.48	4.7	5.1
$b_1 = 332$	0–33.3	0.079	5.09	5.18	3.9	4.2
	3–100	0.007	1.56	1.62	3.05	3.70
	6–100	0.004	1.45	1.47	6.07	6.19
	9–100	0.003	1.45	1.43	9.10	9.09
Silica[a]	Propanol/CH_2Cl_2 (%)					
Langm.	1–50		5.26	4.75[a]	1.05	4.13[a]
$a_1 = 4.69$,	1–25		5.56	5.84[a]	1.01	4.49[a]
$b_1 = 12.6$	1–16.7		5.60	6.40[a]	1.02	4.96[a]
Nitrile	Propanol/hexane (%)					
Langm.	0–50	0.058	3.78	4.09	3.0	4.3
$a_1 = 17.0$,	0–25	0.058	4.55	5.17	2.0	3.1
$b_1 = 93.0$	0–16.7	0.058	5.06	6.00	1.4	2.5
	3–50	0.015	1.79	1.92	3.01	3.7
	6–50	0.009	1.56	1.61	6.01	6.2
	9–50	0.006	1.49	1.53	9.01	9.1
[b]	Dioxane/hexane (%)	[b]		[b]		[b]
Nitrile	0–100		3.13		0.1	
	0–50		3.17		0.1	
	0–33.3		3.20		0.1	
	3–100		3.00		3.01	
	6–100		2.87		6.02	
	9–100		2.78		9.02	

Conditions as in Table 2.

[a] Langmuir isotherm does not fit well the data, associative isotherm (Eq. (10)) applies, $q_{1s} = 0.078$, $b_1 = 19.67$, $b_2 = 147.0$ — the values calculated using the Langmuir isotherm are underestimated.

[b] The isotherm is almost linear, saturation capacity is not achieved up to 50% dioxane in the mobile phase, calculation approach II cannot be applied.

tions can be easily calculated from the retention factor of the polar solvent, the column hold-up volume and efficiency. This is not the case in gradient elution, but if the constants of the adsorption isotherm are known, we can still calculate the profile of the breakthrough curve, the breakthrough volume, V_B, and the breakthrough concentration, c_B, of the polar solvent during gradient

elution. For example, if the Langmuir model applies and the column becomes fully saturated with the polar solvent at an early stage of gradient elution, V_B and c_B can be calculated directly using Eqs. (C.2), (C.3) and (C.6) in Appendix C. Otherwise, numerical solution of the basic differential mass balance equation of the polar solvent on the column can be employed, as described elsewhere for the band profiles in overloaded gradient elution [35]. In the present work, rapid equilibrium with fast mass transfer kinetics was assumed, to allow the equilibrium-dispersive model of chromatography to be employed for numerical solution using a modified Rouchon finite difference algorithm, however with modified boundary conditions taking into account that the feed (mobile phase) with continuously increasing concentration of the polar solvent is being introduced on to the column for the whole time of elution. This approach allows us to simulate breakthrough curves for any gradient program, as has been verified by comparison of several experimental and simulated breakthrough curves (not shown). Hence, tedious and often inaccurate experimental monitoring of rather volatile solvents that do not absorb in the UV region is not necessary. The calculated breakthrough curves in Figs. 2–4 represent the gradient profiles at the column outlet accounting for the column uptake of the polar solvent. The breakthrough curves of 2-propanol and of dioxane on a silica gel column for gradients starting at 100% heptane steeply increase to the breakthrough concentration, then their profiles are almost linear with

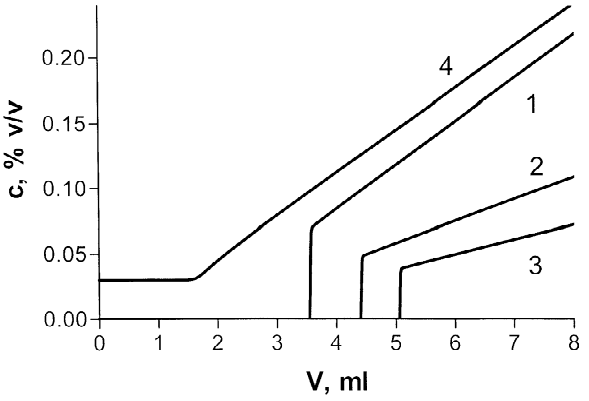


Fig. 3. Breakthrough curves of dioxane in heptane on a Separon SGX silica gel column in normal-phase gradient-elution HPLC, simulated by numerical calculations using the experimental isotherm data and assuming $N=5000$. Gradient dwell volume = 0.50 ml. Gradients: 0–100% dioxane in 30 min (1), 0–50% dioxane in 30 min (2), 0–33.3% dioxane in 30 min (3), 3–100% dioxane in 30 min (4). *c*, concentration of dioxane in the eluate; *V*, volume of the eluate from the start of the gradient.

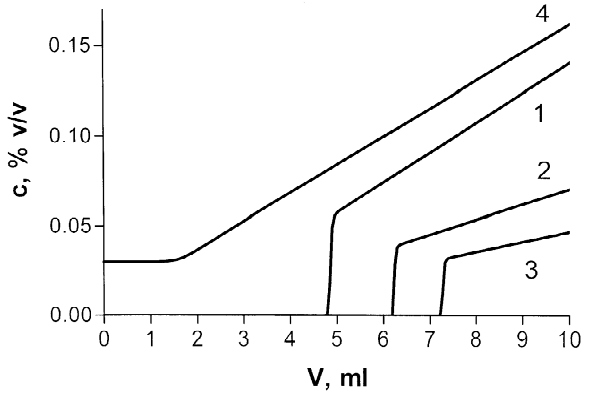


Fig. 2. Breakthrough curves of 2-propanol in heptane on a Separon SGX silica gel column in normal-phase gradient-elution HPLC, simulated by numerical calculations using the experimental isotherm data and assuming $N=5000$. Gradient dwell volume = 0.50 ml. Gradients: 0–50% 2-propanol in 30 min (1), 0–25% 2-propanol in 30 min (2), 0–16.7% 2-propanol in 30 min (3), 3–50% 2-propanol in 30 min (4). *c*, concentration of 2-propanol in the eluate; *V*, volume of the eluate from the start of the gradient.

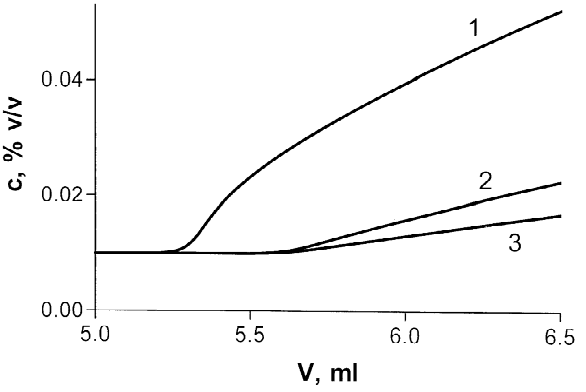


Fig. 4. Breakthrough curves of 2-propanol in dichloromethane on a Separon SGX silica gel column in normal-phase gradient-elution HPLC, simulated by numerical calculations using the experimental isotherm data and assuming $N=5000$. Gradient dwell volume = 0.50 ml. Gradients: 1–50% 2-propanol in 30 min (1), 1–25% 2-propanol in 30 min (2), 1–16.7% 2-propanol in 30 min (3). *c*, concentration of 2-propanol in the eluate; *V*, volume of the eluate from the start of the gradient.

the slopes equal to the programmed gradient steepness. If gradient elution is started at a non-zero initial concentration of polar solvent, the adsorbed volume of the pure solvent is very low, the breakthrough curve is linear and corresponds to a gradient delayed by the breakthrough time given by the sum of the column hold-up volume, V_0, and the gradient dwell volume, V_D (1.40 ml for the silica gel column and 1.46 ml for the bonded nitrile column, plots 4 in Figs. 2 and 3). For such gradients, it is not necessary to use any corrections for the preferential uptake of the polar solvent **B** on the column.

The breakthrough volumes, V_B, and the corresponding breakthrough concentrations, c_B, evaluated from the breakthrough curves of the polar solvents are in good agreement with the values calculated from Eqs. (C.2), (C.3) and (C.6) assuming the validity of the Langmuir isotherm and accomplishing full column saturation with the polar solvent **B** during gradient elution — Table 4. This suggests that in gradients starting at 100% non-polar solvent, the silica gel column becomes fully saturated at the early stage of gradient elution and the effect of the preferential adsorption on the retention can be corrected by adding the net breakthrough volume to V_R calculated using Eq. (11) or (12). For this purpose, V_{ads}, is calculated from Eq. (C.6).

The profiles of the breakthrough curves of 2-propanol from hexane on a Separon SGX Nitrile column (not shown) are very similar as on the silica gel column, hence the same conclusions concerning the correction for the uptake of 2-propanol can be adopted. Because of a weaker polarity of the bonded nitrile column, the breakthrough volumes are lower than on the silica gel column. For gradients starting at 9, 6 and 3% of 2-propanol, the differences between the breakthrough volumes and the sum of V_0 and V_D are 0.03, 0.10 and 0.33 ml, respectively (Table 4).

The profiles of the breakthrough curves of 2-propanol from dichloromethane on a silica gel column (Fig. 4) differ significantly from the profiles obtained with heptane as the weak solvent and are significantly curved (convex) even though the gradients are started at 1% of 2-propanol.

The reason is that the Langmuir isotherm does not describe well the experimental distribution, for which the two-layer associative isotherm (Eq. (10)) provides a better description. Hence, the correction of the retention for the uptake of 2-propanol on the column requires using Eqs. (11) or (12) with V_{ads} calculated from Eq. (C.11) (see Appendix C).

The breakthrough curves of dioxane from heptane on a bonded nitrile column (not shown) are almost linear. The breakthrough volumes slightly decrease as the gradient steepness increases or as the initial concentration of dioxane at the start of the gradient decreases and the breakthrough volumes are significantly greater than the sum of V_0 and V_D (Table 4), due to the almost linear shape of the adsorption isotherm, which prevents the saturation of the column adsorption capacity during any gradient of dioxane on the bonded nitrile column. Consequently, V_{ads} calculated from Eq. (C.10) should be introduced into Eqs. (11) or (12) to correct the calculated elution volumes for the uptake of dioxane on the column.

4.3. Exact calculation of corrected elution volumes in NP gradient-elution HPLC

Earlier, we found that using controlled column temperature and dehydrated organic solvents kept dry over molecular sieves before the use, reproducible retention data could be obtained in normal-phase gradient-elution HPLC on a silica gel column used for over 10 months, with the differences between the elution volumes measured in the repeated experiments lower than 0.2 ml or 2% of V_R, as it is documented by experimental data in Ref. [10]. Further, the elution volumes could be accurately predicted by calculation from the retention factors determined under isocratic conditions [10]. The accuracy of prediction was better for the data calculated from Eq. (6) based on the three-parameter retention Eq. (2) than for the data calculated from Eq. (4) relying on the two-parameter retention Eq. (1). The agreement between the experimental data and the retention volumes calculated in this way was better than 0.25 ml. Hence we used Eqs. (6) and (2) for all predictive calculations in this study.

In previous work, corrections were considered for the migration of sample compounds before the start of the real gradient due to the gradient dwell volume (see Appendix B). The effect of the preferential

uptake of the polar solvent on the prediction of retention was accounted for by adding the experimentally determined breakthrough volumes to the calculated elution volumes. However, the experimental breakthrough volume of a non-UV absorbing polar solvent may be subject to errors because of the difficulties connected with the detection, as discussed in Section 4.2. The errors in predicted retention data were greater in gradients starting at 0% than in gradients starting at 3% or more of the polar solvent.

To eliminate these errors, the adsorption isotherms of the polar solvents were determined experimentally and used in the calculation of corrected elution volumes, as explained in Section 2.3. The elution volumes in gradient elution starting at zero concentrations of 2-propanol in heptane on the silica gel column (Table 5), of 2-propanol in hexane on the bonded nitrile column (Table 6) and of dioxane in heptane on the silica gel column (Table 7) corrected for the uptake of polar solvent **B**, $V_R(C)$, were calculated from Eq. (12) with the saturation volume of **B**, V_{sat}, introduced from Eq. (C.6) assuming full saturation of the column at the early stage of gradient elution. The corrected calculations resulted in improved average prediction error from 1.6 to 0.8% for gradients of propanol in heptane on the silica gel column. For the gradients of dioxane in heptane, the improvement of the corrected calculated data was only marginal, as — because of a lower uptake of dioxane — the accuracy of the uncorrected data (average error 0.7%) was satisfactory enough. This approach also did not bring any improvement for the gradients starting at 3–9% 2-propanol, where the

Table 5
Experimental elution volumes, $V_R(E)$, and calculated values: (a) uncorrected, from Eq. (6), $V_R(U)$, (b) corrected for the adsorption of polar solvent, from Eq. (12), $V_R(C)$, (c) corrected for the solute migration corresponding to the gradient dwell volume for gradients starting at $A>0$, from Eq. (8), $V_R(D)$, all in ml

Compound		Gradient					
		0–50% 30 min	0–50% 60 min	0–50% 90 min	3–50% 30 min	6–50% 30 min	9–50% 30 min
IPU	$V_R(E)$	11.18	16.01	19.50	9.47	8.00	6.60
$a = 0.0148$	$V_R(U)$	10.95	15.62	19.32	9.49	8.10	6.87
$b = 2.173$	$V_R(C)$	11.31	16.13	19.65	9.50	8.10	6.87
$m = 1.466$	$V_R(D)$				9.24	7.76	6.45
DPU	$V_R(E)$	11.31	16.68	21.66	9.53	9.01	6.68
$a = 0.0152$	$V_R(U)$	11.15	16.44	20.76	9.67	8.19	6.83
$b = 2.726$	$V_R(C)$	11.37	16.73	21.10	9.67	8.19	6.83
$m = 1.777$	$V_R(D)$				9.64	8.08	6.62
CMU	$V_R(E)$	10.55	15.55	19.69	8.82	7.25	5.89
$a = 0.011$	$V_R(U)$	10.40	15.29	19.29	8.89	7.40	6.09
$b = 3.089$	$V_R(C)$	10.64	15.60	19.65	8.90	7.40	6.09
$m = 1.749$	$V_R(D)$				8.85	7.28	5.86
DCU	$V_R(E)$	14.86	22.02	27.78	13.27	11.90	10.71
$a = 0.033$	$V_R(U)$	14.83	21.90	27.56	13.50	12.10	10.71
$b = 1.583$	$V_R(C)$	15.02	22.18	27.94	13.50	12.10	10.71
$m = 1.838$	$V_R(D)$				13.47	12.03	10.58
Average error of $V_R(U)$ (%)		1.3	1.5	2.0	1.0	1.8	2.4
Average error of $V_R(C)$ (%)		0.9	0.5	1.0	1.1	1.8	2.4
Average error of $V_R(D)$ (%)		–	–	–	1.4	1.2	1.2

Column: Separon SGX (silica gel), gradient 2-propanol in heptane, 1 ml/min, 40°C. Compounds as in Table 1. *a*, *b* and *m* are constants of Eq. (2).

Table 6
Experimental elution volumes, $V_R(E)$, and calculated values: (a) uncorrected, from Eq. (6), $V_R(U)$, (b) corrected for the adsorption of polar solvent, from Eq. (12), $V_R(C)$, (c) corrected for the solute migration corresponding to the gradient dwell volume for gradients starting at $A > 0$, from Eq. (8), $V_R(D)$, all in ml

Compound		Gradient					
		0–50% 30 min	0–50% 60 min	0–50% 90 min	3–50% 30 min	6–50% 30 min	9–50% 30 min
CMU	$V_R(E)$	6.34	8.26	9.84	4.29	3.03	2.34
$a = 0.015$	$V_R(U)$	5.48	7.24	8.59	4.08	3.20	2.70
$b = 8.086$	$V_R(C)$	6.19	8.25	9.84	4.27	3.30	2.75
$m = 1.162$	$V_R(D)$				4.08	2.91	2.29
FMU	$V_R(E)$	8.55	11.49	13.74	6.67	5.21	4.08
$a = 0.035$	$V_R(U)$	7.77	10.59	12.74	6.31	5.12	4.24
$b = 3.884$	$V_R(C)$	8.22	11.27	13.63	6.43	5.19	4.28
$m = 1.352$	$V_R(D)$				6.52	5.07	3.99
CTU	$V_R(E)$	9.10	12.13	14.70	7.21	5.58	4.56
$a = 0.060$	$V_R(U)$	8.33	11.39	13.69	6.90	5.66	4.69
$b = 3.445$	$V_R(C)$	8.76	12.08	14.62	7.00	5.72	4.73
$m = 1.497$	$V_R(D)$				7.13	5.66	4.50
PHU	$V_R(E)$	10.16	14.02	16.71	8.46	7.04	5.71
$a = 0.069$	$V_R(U)$	9.38	12.77	15.26	7.99	6.74	5.70
$b = 2.577$	$V_R(C)$	9.81	13.50	16.28	8.09	6.80	5.74
$m = 1.514$	$V_R(D)$				8.23	6.79	5.58
Average error of $V_R(U)$ (%)		9.7	8.8	8.9	5.0	3.3	5.6
Average error of $V_R(C)$ (%)		3.3	1.5	1.0	2.8	3.8	6.6
Average error of $V_R(D)$ (%)		–	–	–	2.7	2.5	2.0

Column: Separon SGX Nitrile, gradient dioxane in heptane, 1 ml/min, 40°C. Compounds as in Table 1. *a*, *b* and *m* are constants of Eq. (2).

columns are already almost completely saturated with the polar solvent. Here, the average error of prediction was 1.6% for the silica gel and 3.8% for the bonded nitrile column. The correction of the elution volumes $V_R(D)$ for possible band migration corresponding to the gradient dwell volume calculated from Eq. (8) slightly improved the average error of predicted retention volumes in gradient elution starting at a non-zero concentration of propanol to 1.3% for the silica gel column and to 2.4% for the bonded nitrile column (Tables 5 and 6).

The correction for the preferential polar solvent uptake significantly improved the predicted elution volumes of phenylurea herbicides in gradients starting at 0% dioxane in hexane on the bonded nitrile column, where the average error of prediction decreased from 8.6 to 2.5% (Table 8). Here, the values of V_{ads} calculated from Eq. (C.10) taking into account the validity of the Langmuir isotherm, but unsaturated column adsorption capacity, were introduced into Eq. (11) to correct the calculated elution volumes for the uptake of dioxane by the column. The calculated elution volumes were slightly overestimated for the gradients starting at 3–9% dioxane (average error of prediction 4.2%), which indicates that the band migration corresponding to the gradient dwell volume cannot be neglected for these gradients because of rather low retention of the sample solutes in the starting mobile phase. Indeed, the correction for possible band migration corresponding to the gradient dwell volume calculated from Eq. (8) resulted in significant improvement of the accuracy

Table 7
Experimental elution volumes, $V_R(E)$, and calculated values: (a) uncorrected, from Eq. (6), $V_R(U)$, (b) corrected for the adsorption of polar solvent, from Eq. (12), $V_R(C)$, (c) corrected for the solute migration corresponding to the gradient dwell volume for gradients starting at $A > 0$, from Eq. (8), $V_R(D)$, all in ml

Compound		Gradient					
		0–100% 30 min	0–100% 60 min	0–100% 90 min	3–100% 30 min	6–100% 30 min	9–100% 30 min
IPU	$V_R(E)$	10.93	16.41	20.82	10.07	9.26	8.43
$a = 0.045$	$V_R(U)$	10.80	16.25	20.76	10.07	9.32	8.56
$b = 1.678$	$V_R(C)$	10.86	16.34	20.88	10.08	9.32	8.56
$m = 2.228$	$V_R(D)$				10.07	9.29	8.50
FMU	$V_R(E)$	11.14	17.04	21.45	10.36	9.47	8.66
$a = 0.061$	$V_R(U)$	11.14	16.83	21.54	10.42	9.68	8.91
$b = 1.615$	$V_R(C)$	11.20	16.92	21.65	10.42	9.68	8.92
$m = 2.367$	$V_R(D)$				10.41	9.65	8.92
CMU	$V_R(E)$	13.57	21.12	27.50	12.87	11.96	11.19
$a = 0.032$	$V_R(U)$	13.64	21.33	27.89	12.98	12.28	11.55
$b = 1.364$	$V_R(C)$	13.65	21.35	27.91	12.98	12.28	11.55
$m = 2.596$	$V_R(D)$				12.98	12.27	11.53
DPU	$V_R(E)$	14.70	22.90	30.04	13.83	13.09	12.32
$a = 0.121$	$V_R(U)$	14.76	23.03	29.93	14.13	13.47	12.77
$b = 1.130$	$V_R(C)$	14.80	23.09	30.02	14.14	13.47	12.77
$m = 3.144$	$V_R(D)$				14.13	13.46	12.75
Average error of $V_R(U)$ (%)		0.5	1.0	0.6	0.9	2.1	2.8
Average error of $V_R(C)$ (%)		0.6	0.8	0.7	0.9	2.1	2.8
Average error of $V_R(D)$ (%)		–	–	–	0.9	1.9	2.5

Column: Separon SGX Nitrile, gradient 2-propanol in heptane, 1 ml/min, 40°C. Compounds as in Table 1. *a*, *b* and *m* are constants of Eq. (2).

of predicted elution volumes, $V_R(D)$, to the average error of 2.9%.

The correction for the adsorbed polar solvent using Eq. (12) also significantly improved the predicted elution volumes with gradients starting at 1% 2-propanol in dichloromethane on the silica gel column. The differences between the calculated corrected elution volumes and the experimental values were lower than 0.2 ml, with average error of prediction 1.7% in contrast to 12% error of prediction for uncorrected data (Table 9). This system is controlled by the two-layer associative isotherm and column saturation with 2-propanol is not accomplished during gradient elution, so that the values of V_{ads} vary with the elution volumes of sample compounds and had to be calculated from Eq. (C.11).

5. Conclusions

Good reproducibility of the retention data in normal-phase gradient-elution HPLC can be achieved at controlled temperature if carefully dried solvents are used to suppress the deactivation of polar adsorbent with trace water concentrations.

Simple calculations can be used for approximate predictions of the changes in retention caused by a change in the time (steepness) of a binary gradient and (or) in the initial composition of the mobile phase at the start of gradient elution, assuming that an approximately constant volume of pure polar solvent is necessary to accomplish the elution of an individual solute, independent of the gradient program. These calculations do not require the values of

Table 8
Experimental elution volumes, $V_R(E)$, and calculated values: (a) uncorrected, from Eq. (6), $V_R(U)$, (b) corrected for the adsorption of polar solvent, from Eq. (12), $V_R(C)$, (c) corrected for the solute migration corresponding to the gradient dwell volume for gradients starting at $A > 0$, from Eq. (8), $V_R(D)$, all in ml

Compound		Gradient					
		0–100% 30 min	0–100% 60 min	0–100% 90 min	3–100% 30 min	6–100% 30 min	9–100% 30 min
NBU	$V_R(E)$	8.01	11.28	12.98	6.93	6.00	5.09
$a = 0.081$	$V_R(U)$	6.97	9.69	11.80	6.20	5.46	4.80
$b = 2.925$	$V_R(C)$	8.18	10.99	13.15	7.19	6.25	5.40
$m = 1.776$	$V_R(D)$				6.56	5.69	4.87
FMU	$V_R(E)$	9.56	13.42	16.26	8.52	7.66	6.77
$a = 0.113$	$V_R(U)$	8.66	12.38	15.30	7.91	7.16	6.44
$b = 2.072$	$V_R(C)$	9.83	13.65	16.61	8.91	8.00	7.12
$m = 2.146$	$V_R(D)$				8.31	7.49	6.66
CTU	$V_R(E)$	10.25	14.98	18.26	9.53	8.64	7.78
$a = 0.088$	$V_R(U)$	9.64	13.90	17.27	8.90	8.16	7.42
$b = 1.748$	$V_R(C)$	10.78	15.15	18.57	9.89	9.00	8.14
$m = 2.064$	$V_R(D)$				9.33	8.52	7.71
CMU	$V_R(E)$	10.07	15.57	17.50	9.06	8.20	7.34
$a = 0.124$	$V_R(U)$	9.12	13.11	16.23	7.56	7.63	6.90
$b = 1.907$	$V_R(C)$	10.28	14.36	17.53	9.37	8.48	7.60
$m = 2.251$	$V_R(D)$				8.80	7.98	7.16
Average error of $V_R(U)$ (%)		9.4	11.2	6.9	10.2	7.0	5.3
Average error of $V_R(C)$ (%)		3.0	3.3	1.3	3.9	4.0	4.8
Average error of $V_R(D)$ (%)		–	–	–	3.0	2.8	2.7

Column: Separon SGX Nitrile, gradient dioxane in heptane, 1 ml/min, 40°C. Compounds as in Table 1. *a*, *b* and *m* are constants of Eq. (2).

the parameters of the equations describing the dependence of the retention factors on the composition of the mobile phase.

For precise calculations of the elution volumes in normal-phase gradient elution from the isocratic data, an equation based on the three-parameter dependence of the retention factor on the concentration of the polar solvent (Eq. (2)) should be preferred to the two-parameter dependence (Eq. (1)).

The accuracy of both approximate and precise calculations of the retention data is significantly increased if possible effects of the uptake of the polar solvent on the column occurring during gradient elution and contribution of the gradient dwell volume to the retention are taken into account. To correct for the effect of the dwell volume, its value can be added to the calculated elution volumes only if the sample compounds are strongly retained in the initial mobile phase at the start of the gradient. Otherwise, the solutes may migrate a significant distance along the column before they are reached by the front of the gradient and the elution volumes have to be calculated as in the two-step gradient elution with an initial hold-up period corresponding to the dwell volume of the instrument.

Experimentally measured adsorption isotherms of polar solvents allow numerical calculation of the breakthrough curves during the elution with various gradient programs and determination of the breakthrough volumes and the breakthrough concentrations. The effect of the column uptake of the polar solvent during gradient elution on the elution data is controlled by the type of its adsorption isotherm, which depends on the nature of both the weak and

Table 9
Experimental elution volumes, $V_R(E)$, and calculated values: (a) uncorrected, from Eq. (6), $V_R(U)$, (b) corrected for the adsorption of polar solvent, from Eq. (12), $V_R(C)$, all in ml

Compound		Gradient		
		1–50% 15 min	1–50% 30 min	1–50% 45 min
MOU				
$a = 0.001$	$V_R(E)$		4.67	5.19
$b = 12.366$	$V_R(U)$		3.97	4.45
$m = 1.099$	$V_R(C)$		4.54	5.01
PHU				
$a = 0$	$V_R(E)$		4.79	5.40
$b = 12.463$	$V_R(U)$		4.12	4.67
$m = 1.228$	$V_R(C)$		4.69	5.24
CMU				
$a = 0.059$	$V_R(E)$	5.03	6.53	7.62
$b = 6.357$	$V_R(U)$	4.47	5.84	6.86
$m = 1.589$	$V_R(C)$	5.04	6.42	7.45
DPU				
$a = 0.058$	$V_R(E)$	5.39	7.12	8.30
$b = 5.820$	$V_R(U)$	4.85	6.52	7.81
$m = 1.794$	$V_R(C)$	5.41	7.10	8.40
Average error of $V_R(U)$ (%)		12.0	12.0	10.9
Average error of $V_R(U)$ (%)		0.3	1.7	2.5

Column: Separon SGX (silica gel), gradient 2-propanol in dichloromethane, 1 ml/min, 40°C. Compounds as in Table 1. *a*, *b* and *m* are constants of Eq. (2).

the strong solvents in the mobile phase and of the column packing material.

If the initial slope of the (Langmuir) isotherm is steep and the column is saturated at low concentrations of the polar solvent in the mobile phase, the effect of the column uptake usually can be neglected if the gradient is started at a non-zero concentration of the polar solvent. However, the solute migration in the initial isocratic dwell-volume period is more probable if the concentration of the polar solvent at the start of the gradient increases. For gradients starting at zero concentration of the polar solvent, the accuracy of predictive calculations of the elution data is significantly improved if the volume of the polar solvent taken up on the column is added to the volume of the solvent necessary to accomplish the elution of sample compounds.

If the isotherm (linear or multi-layer associative) does not allow rapid column saturation at an early stage of gradient elution, the volume of the polar solvent actually adsorbed on the column at the time of the elution of sample compounds should be used instead of the volume of the solvent necessary for the full saturation of the column in exact calculations of the retention data. The adsorbed volume can be determined from the isotherm parameters.

The correction of the predicted retention data for the uptake of polar solvents during gradient elution decreased the average error of prediction to 1% for gradients starting at 0% propanol or dioxane with a silica gel column and to 2–2.5% with a bonded nitrile column. When using gradients starting at a non-zero concentration of the polar solvent, it is more important to take into account a correction for band migration during the initial isocratic step induced by the gradient dwell volume. With this correction, the average error of predicted retention data was less than 2% for a silica gel column and 2.4–2.9% for a bonded nitrile column. With the silica gel column, these errors are probably low enough to allow using the corrected calculation approaches for optimisation of normal-phase gradient elution.

6. Nomenclature

A	concentration of the polar solvent in the mobile phase at the start of the gradient
A_1	constant of the two-layer associative isotherm (Eq. (10))
A_2	constant of the two-layer associative isotherm (Eq. (10))
B	gradient steepness (Eq. (3))
B_1	constant of the two-layer associative isotherm (Eq. (10))
F_m	flow-rate of the mobile phase
N	number of theoretical plates of the column
V	volume of eluate from the start of elution
V_B	breakthrough volume of the polar solvent in the mobile phase
V'_B	net breakthrough volume of the polar solvent in the mobile phase, $V'_B = V_B - V_0$

V_D gradient dwell volume of the instrument
V_M volume of the mobile phase in the column
V_R elution volume of a sample compound
V'_R net elution volume of a sample compound, $V'_R = V_R - V_0$
$V'_R(C)$ net elution volume corrected for the polar solvent uptake on the column
$V'_R(D)$ net elution volume corrected for the migration during the isocratic step corresponding to the gradient dwell volume
$V'_R(U)$ uncorrected net elution volume
V'_{R1} part of the net elution volume of a sample compound contributed by the first, isocratic step in two-step elution
V'_{R2} part of the net elution volume of a sample compound contributed by the second, gradient step in two-step elution
V_S volume of the stationary phase in the column
V_{ads} volume of the pure polar solvent adsorbed on the column at the time of elution of a sample compound
V_{sat} volume of the pure polar solvent necessary for full saturation of the column
V_{solv} volume of the pure polar solvent necessary to accomplish the elution of a sample compound
V_0 column hold-up volume
V_{01} part of the column hold-up volume migrated by a sample compound in the first, isocratic step of the two-step elution
V_{02} part of the column hold-up volume migrated by a sample compound in the second, gradient step of the two-step elution
a constant in Eq. (2)
a_1 constant of the Langmuir (Eq. (9)) and two-layer associative (Eq. (10)) isotherms
b constant in Eq. (2)
b_1 constant of the Langmuir (Eq. (9)) and two-layer associative (Eq. (10)) isotherms
b_2 constant of the two-layer associative isotherm (Eq. (10))
c concentration of the polar solvent in the mobile phase (or in the eluate)
c_B breakthrough concentration of the polar solvent in the eluate
c_f concentration of the polar solvent in the eluate at the time of elution of a sample compound
c_G concentration of the polar solvent in the eluate at the end of the gradient (in time t_G)
k retention factor of a sample solute, $k = (V_R - V_0)/V_0$
k_a k in pure non-polar solvent
k_f instantaneous k at the time of elution of a sample compound
k_0 k in pure polar solvent, constant in Eq. (1)
k_1 k in the isocratic step prior to the gradient step in two-step elution
m constant in Eqs. (1) and (2)
q concentration of the adsorbed polar solvent in the stationary phase
q_s saturation capacity concentration of the adsorbed polar solvent in the stationary phase
t_G time of the gradient
w_g bandwidth of a solute in gradient-elution HPLC (Eq. (D.1))
Φ phase ratio in the column, $\Phi = V_S/V_M$

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Appendix A. Justification of the assumption of a constant volume of polar solvent necessary to accomplish the elution of a compound at 1:1 adsorption equilibrium stoichiometry

In NP chromatography, a molecule of the analyte adsorbed on a polar adsorbent can often be displaced by a single molecule of a polar solvent in a mixed mobile phase. In this case, $m = 1$ and Eq. (1) for the retention factor of the analyte is simplified to:

$$k = \frac{k_0}{c} \quad (A.1)$$

In isocratic elution chromatography, the volume of the polar solvent **B**, V_{solv}, necessary for the elution of the analyte with reduced retention volume V'_R is equal to:

$$V_{solv} = c \cdot V'_R = \frac{c \cdot V_0.k_0}{c} = V_0 \cdot k_0 \quad (A.2)$$

Hence, the value of V_{solv} is independent of the concentration of the polar solvent in the mobile phase, c.

In gradient-elution chromatography with a linear gradient controlled by Eq. (3), V_{solv} can be determined by integration:

$$V_{solv} = \int_0^{V'_R} c \cdot dV = A \cdot V'_R + \frac{B}{2} \cdot V'^2_R \quad (A.3)$$

For a compound with $m = 1$, Eq. (A.3) for the net elution volume in gradient-elution NP HPLC, V'_R, can be written as:

$$V'_R = \frac{1}{B}\left[2B \cdot V_0 \cdot k_0 + A^2\right]^{1/2} - \frac{A}{B} \quad (A.4)$$

Introducing Eq. (A.4) for V'_R into Eq. (A.3) we obtain:

$$V_{solv} = V_0 \cdot k_0 \quad (A.5)$$

which means that V_{solv} in this case does not depend on the gradient and is the same as in isocratic elution — Eq. (A.2).

Appendix B. Correction of the elution volume in gradient-elution NP HPLC for band migration along the column during the isocratic dwell-volume step

The ''gradient dwell volume'', V_D, can sometimes significantly contribute to the total retention volume of the solute, especially when V_D is large and the gradient is started at a non-zero concentration of the polar solvent **B**. The reason is that the dwell-volume part of the instrument is filled with the mobile phase of the composition used at the start of the gradient elution and the mobile phase volume equal to V_D has to pass through the column before the actual gradient programme can start. At the time when a sample compound is taken by the front of the gradient, it has already migrated a part of the column hold-up volume, V_{01}, at the initial isocratic conditions, so that the part of the column hold-up volume, V_{02}, available for its migration during the actual gradient elution is lower than the actual column hold-up volume, V_0: $V_{02} = V_0 - V_{01}$. V_{01} is related to V_0 in the same proportion as the gradient dwell volume V_D to the (hypothetical) elution volume of the solute under initial isocratic conditions where the retention factor of the solute is k_1. Hence, the gradient part of the hold-up volume available for each sample compound, V_{02}, is:

$$\frac{V_{01}}{V_0} = \frac{V_D}{V_0(1+k_1)}; V_{02} = V_0 - \frac{V_D}{(1+k_1)} \quad (B.1)$$

The gradient volume can be calculated as in two-step gradient elution with an initial hold-up period, i.e. the final gradient elution volume is comprised of: (1) the contribution of the gradient step to the net retention volume, V'_{R2}, which can be calculated from Eqs. (4) or (6) using V_{02} instead of V_0, and (2) the isocratic contribution of the gradient dwell volume, $V'_{R1} = V_D - V_{01}$:

$$V_R = V'_{R1} + V'_{R2} + V_0 = V_D - V_{01} + V'_{R2} + V_0 = \frac{V_D}{1 + \frac{1}{k_1}} + V'_{R2} + V_0 \quad (B.2)$$

By this approach, Eq. (4) is slightly modified to Eq. (10) and Eq. (6) to Eq. (11).

Appendix C. Correction of the retention volume in NP HPLC for the column uptake of polar solvents during gradient elution (solvent-demixing effect)

A polar solvent **B** is adsorbed from a mixed organic mobile phase by a polar adsorbent during gradient elution, so that its concentration in the mobile phase is lower than expected for the programmed gradient profile and the column effluent contains only the pure less polar solvent until the eventual breakthrough of the polar solvent into the

mobile phase occurs. The net breakthrough volume, V'_B, can be calculated as the volume of the mobile phase necessary to bring the column into equilibrium with the mobile phase by adsorption of the volume V_{ads} of pure solvent **B**. A linear gradient running from the initial concentration of **B**, $c = A$, to the final concentration of **B**, $c = c_G$, in the time t_G at a flow-rate F_m is described by Eq. (3) and V'_B can be determined by integration of a simple equation:

$$V_{ads} = \int_0^{V'_B} c \cdot dV = A \cdot V'_B + \frac{B}{2} \cdot V'^2_B \quad (C.1)$$

from which we obtain Eq. (C.2) for the total breakthrough volume, V_B:

$$V_B = V'_B + V_0 + V_D = \frac{\sqrt{A^2 + 2B \cdot V_{ads}}}{B} + V_0 + V_D \quad (C.2)$$

and Eq. (C.3) for the corresponding breakthrough concentration of the solvent *B*, c_B:

$$c_B = A + B \cdot V'_B = A + \sqrt{A^2 + 2B \cdot V_{ads}} \quad (C.3)$$

(V_0 is the column hold-up volume and V_D is the gradient dwell volume.)

For a gradient starting from zero concentration of the polar solvent **B**, $A = 0$:

$$V_B = \sqrt{\frac{2V_{ads}}{B}} + V_0 + V_D \quad (C.4)$$

$$c_B = \sqrt{2B \cdot V_{ads}} \quad (C.5)$$

V_{ads} can be determined from the experimental adsorption isotherm of the polar solvent **B** between the column packing material and a two-component mobile phase. If the distribution of the polar solvent **B** between the stationary and the mobile phase is controlled by a Langmuir isotherm with a steep initial slope and column saturation (plateau concentration of **B** in the stationary phase) is achieved at a low concentration of **B** in the mobile phase (a high value of the isotherm parameter b_1) and the gradient is started at $A = 0$, V_{ads} can be calculated as the volume of **B**, V_{sat}, necessary to reach the column saturation capacity concentration, q_s:

$$V_{ads} = V_{sat} = \frac{\Phi \cdot V_0 \cdot a_1}{b_1} = \Phi \cdot V_0 \cdot q_s \quad (C.6)$$

where Φ is the phase ratio, i.e. the ratio of the volumes of the stationary, V_S, and of the mobile, $V_M = V_0$, phases in the column.

In some cases, the distribution isotherm does not allow accomplishing the full saturation of the column with the polar solvent **B** at the time of elution of sample compounds during the gradient run. If so, the volume of the polar solvent adsorbed on the column, V_{ads}, is controlled by the actual elution volume, V'_R, which depends on the individual solute and gradient program.

Then, V_{ads} can be determined by integrating the product of the volume of the stationary phase in the column and of a differential increase in the adsorbed concentration of **B**, q, from the initial equilibrium value at the start of the gradient, q_0, to the adsorbed concentration at the solute elution time, q_f:

$$V_{ads} = \int_{q_0}^{q_f} V_S \cdot dq = \Phi \cdot V_0 \int_A^{c_f} \left(\frac{dq}{dc}\right) \cdot dc \quad (C.7)$$

Here, q is expressed as the concentration of **B** in the whole volume of the stationary phase in the column, V_S, which is — for simplicity sake — set equal to the part of the volume of the column that is not occupied by the mobile phase. The volume of the mobile phase in the column, V_M, is equal to the column hold-up volume, V_0, $\Phi = V_S/V_M$, is the column phase ratio and (dq/dc) is the first derivation of the adsorption isotherm for the solvent **B** on the column packing. The first derivation of the Langmuir isotherm, Eq. (9), is described by the following equation:

$$\left(\frac{dq}{dc}\right) = \frac{a_1}{(1 + b_1 \cdot c)^2} \quad (C.8)$$

and the first derivation of the associative bi-layer isotherm, Eq. (10), by:

$$\left(\frac{dq}{dc}\right) = \frac{a_1 \cdot (b_1 - a_2)}{b_1 \cdot (1 + b_1 \cdot c)^2} + \frac{a_1 \cdot a_2}{b_1} \quad (C.9)$$

After introducing the appropriate equation for (dq/dc) we can solve Eq. (C.7) for V_{ads}:

$$V_{ads} = \frac{\Phi \cdot V_0 \cdot a_1}{b_1} \left[\frac{1}{1 + b_1 \cdot A} - \frac{1}{1 + b_1 \cdot (A + B \cdot V'_R)} \right] \quad (C.10)$$

for NP systems where the Langmuir isotherm, Eq. (9), applies, or:

$$V_{ads} = \frac{\Phi \cdot V_0 \cdot a_1 \cdot (b_1 - a_2)}{b_1^2} \cdot \left[\frac{1}{1 + b_1 \cdot A} - \frac{1}{1 + b_1 \cdot (A + B \cdot V'_R)} \right] + \frac{\Phi \cdot V_0 \cdot a_1 \cdot a_2 \cdot B \cdot V'_R}{b_1} \quad (C.11)$$

for the systems controlled by the two-layer associative isotherm (Eq. (10)).

The uptake of the polar solvent **B** on the column occurring during gradient elution can be accounted for by assuming a constant volume of the polar solvent **B** necessary to accomplish the elution, V_{solv}, as shown in Appendix A. Hence, the volume of **B** adsorbed on the column, V_{ads}, should be added to V_{solv} to correct the elution volume, $V'_R(U)$, calculated from Eqs. (4) or (6) for the adsorption effect. In this way, we obtain the following equation for the corrected elution volume, $V'_R(C)$:

$$V_{solv} = [V'_R(U)]^2 \cdot \frac{B}{2} + V'_R(U) \cdot A + V_{ads} = [V'_R(C)]^2 \cdot \frac{B}{2} + V'_R(C) \cdot A \quad (C.12)$$

in Eqs. (4) or (6), which are thus slightly modified to Eqs. (11) and (12). The retention volumes calculated from Eqs. (7) and (8) can be corrected in a similar way if necessary, however, the uptake of the polar solvent on the column is less important with gradients starting at a non-zero initial concentration of **B** than with gradients using $A = 0$.

Appendix D. Calculation of bandwidths in gradient-elution HPLC

To first approximation, the band broadening in gradient-elution chromatography can be set equal to the band broadening in isocratic elution with a mobile phase of the composition corresponding to the instantaneous composition of the mobile phase at the elution time of the band maximum [26]. Once the retention volume in gradient elution is known, the appropriate instantaneous retention factor k_f at the elution of the peak maximum can be calculated from Eq. (3) and from the appropriate equation describing the isocratic retention behaviour (Eq. (1) or Eq. (2)). The approximate value of w_g can then be calculated from Eq. (D.1) [27]:

$$w_g = \frac{4V_0(1 + k_f)}{\sqrt{N}} \quad (D.1)$$

using c_f as the instantaneous concentration of the polar solvent at the outlet of the column at the time the band maximum elutes from the column and N as the number of theoretical plates of the column measured for the same compound under isocratic conditions. Bandwidths w_g in gradient elution are generally narrower than under isocratic conditions because of band-compression by increasing concentration of the solvent **B** during a gradient run. If the retention volume in gradient elution can be calculated using Eq. (4), we obtain the following equation for the solute bandwidth:

$$w_g = \frac{4V_0}{\sqrt{N}} \cdot [1 + k_0[(m + 1)Bk_0V_0 + A^{(m+1)}]^{-m/(m+1)}] \quad (D.2)$$

If Eq. (6) should be used to calculate V_R, the solution yields Eq. (D.3):

$$w_g = \frac{4V_0}{\sqrt{N}} \cdot [1 + [b \cdot B(m + 1) \cdot V_0 + (a + A \cdot b)^{(m+1)}]^{-m/(m+1)}] \quad (D.3)$$

Eqs. (4) and (8) or Eqs. (6) and (9) can be used for the calculation of resolution and for optimisation of normal-phase gradient elution, as shown elsewhere [16].

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