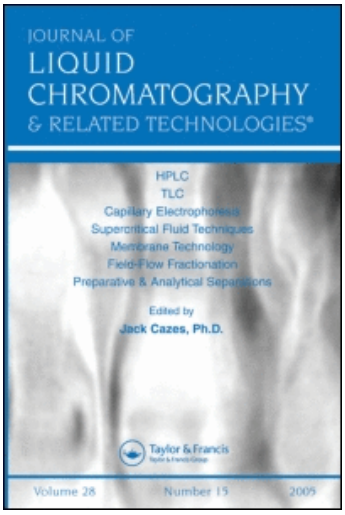

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COMPARISON OF REVERSED-PHASE AND NORMAL-PHASE COLUMN LIQUID CHROMATOGRAPHIC TECHNIQUES FOR THE SEPARATION OF LOW AND HIGH MOLECULAR WEIGHT COMPOUNDS

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ABSTRACT

Liquid chromatography of complex samples often requires gradient elution to separate components with great differences in retention properties. The theory of gradient elution has now been elaborated so that it allows predicting the retention and the resolution of sample compounds not only in reversed-phase, but also in various normal-phase (adsorption chromatography) systems, for almost any combination of gradient profile and relationship between the retention and mobile phase composition. Using this knowledge, parameters of binary or ternary linear and non-linear gradients can be adjusted by predictive calculations for the desired resolution and minimum separation time in various chromatographic systems.

In addition to accurate calculations of the gradient elution data, simple procedures can be employed for rapid estimation of

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the effects of the gradient program on the retention, both in reversed-phase and in normal-phase gradient chromatography. However, these procedures cannot be used for fine-tuning of separation, as they do not take into account possible effects of the gradient program on the elution order.

Non-ideal instrumental or phase system effects impair the reproducibility of the retention data and the accuracy of the retention prediction and separation optimization. The most important, are the migration of some compounds during the dwell period, before the start of the gradient and the preferential adsorption of strong solvents, or of the traces of water from the mobile phase on to the column during gradient run. These effects should be accounted for to increase the accuracy of the predicted gradient retention data and of the gradient optimization.

Both in reversed-phase and in normal-phase liquid chromatography, the strong solvent affects the retention of macromolecules much more strongly than the retention of small molecules. Consequently, less steep gradients, and much narrower gradient concentration ranges, are usually required for the separation of polymers and oligomers according to the molar mass distribution, than for the gradient-elution separation of small molecules.

INTRODUCTION

Many complex samples contain compounds that differ widely in retention, so that HPLC in isocratic elution mode, with a mobile phase of fixed composition, often does not yield successful separation of the individual solutes. To keep an acceptable time of analysis, the retention factors of the most strongly retained sample components, k, usually should be lower than 10. To obtain satisfactory separation of both weakly and strongly retained sample compounds, the operating conditions controlling the retention should be varied during the chromatographic run.[1–5] Gradient elution still remains the most widely used programming technique in liquid chromatography.[1] Gradually increasing elution strength of the mobile phase, i.e., increasing concentration(s) of one or more strong solvent(s) in a binary or in a more complex mobile phase, allows decreasing the retention factors, k, of small molecules by two to three orders of magnitude in a single gradient run, which results in shorter separation time, increased peak capacity, and more regular band spacing of compounds with larger differences in affinities to the stationary phase in comparison to isocratic techniques.

The profile of a continuous gradient is characterized by three adjustable parameters: (1) the gradient range, $\Delta\varphi$ (i.e., the difference between the initial, A, and the final, φ_G, concentrations of the strong solvent B, $\Delta\varphi = \varphi_G - A$); (2) the steepness of the gradient, (which is inversely proportional to the gradient time, t_G, and to the gradient volume, V_G), $B = \Delta\varphi/V_G$, $B' = \Delta\varphi/t_G$; and (3) the shape (curvature) of the gradient. All these parameters affect the elution time and the spacing of the peaks in the chromatogram and should be taken into account when developing gradient separations. Most frequently used are linear gradients controlled by Eq. (1)—linear gradient function describing the dependence of the volume fraction, φ, of the strong solvent B in a binary mobile phase on the time, t, elapsed from the start of the gradient, or on the volume of the eluate, V:[2,3]

$$\varphi = A + B't = A + \frac{\Delta\varphi}{t_G}t = A + \frac{B'}{F_m}V = A + BV = A + \frac{\Delta\varphi}{V_G}V \tag{1}$$

F_m is the flow rate of the mobile phase. The calculation of retention volumes in gradient elution is less straightforward than in isocratic chromatography, because it has to respect changing k in course of the elution. However, appropriate mathematical solutions are available for various gradient programs, and a variety of retention equations describing the dependencies of k on the mobile phase composition in various liquid chromatography modes can be found in the literature.[2,3,6–8]

Reversed-phase chromatography is by far the most widely used liquid chromatography mode, because it offers satisfactory separation of a great variety of samples, containing non-polar, polar, and even ionic compounds. Reversed-phase gradient elution is the technique of choice for separation of complex mixtures according to the different hydrophobicities of sample compounds.[9] Retention times in reversed-phase chromatography with binary mobile phases, are controlled by the volume fraction, φ, of the organic solvent in aqueous-organic mobile phases. The effect of φ on the retention factor, k, in a binary mobile phase can be often described by simple Eq. (2):[1–5,10–14]

$$\log k = \log k_0 - m\varphi = a - m\varphi \tag{2}$$

Here, k_0 is the retention factor of the sample solute extrapolated to pure water as the mobile phase.

The net retention volume V'_R in reversed-phase gradient-elution chromatography with linear gradients can be calculated from the Eq. (3), reported in various forms by several research groups:[2–4,6,11,12,15]

$$V_R = \frac{1}{mB}\log[2.31mBV_m 10^{(a-mA)} + 1] + V_m \tag{3}$$

Here, a and m are the parameters of the Eq. (2) and V_m is the hold-up volume of the column, i.e., the volume of the mobile phase in the column.

Chromatography on polar adsorbents suffers from a specific inconvenience—significant preferential adsorption of polar solvents; especially water, which may be connected with long equilibration times if the separation conditions are changed.[16] Hence, the control of retention in normal-phase chromatography by adjusting the mobile phase composition, can be less reproducible and less predictable than in reversed-phase chromatography. These effects may become especially important in gradient elution, where the composition of the mobile phase changes during the separation and are the reasons of a strong bias against using gradient elution in normal-phase chromatography. However, the column re-equilibration times after the end of the gradient can be short if dry mobile phases and non-localizing polar solvents B are used, such as dichloromethane, dioxane, or *tert*-butyl methyl ether.[8,17] In many normal-phase systems, a simple Eq. (4) can be used to describe the experimental dependencies of the retention factors, k, of sample compounds on the volume fraction, φ, of a polar solvent, B, in a binary mobile phase comprised of two organic solvents with different polarities:[10,18–24]

$$k = k_0 \varphi^{-m} \tag{4}$$

In normal-phase gradient-elution chromatography, the concentration of one or more polar solvents in a non-polar solvent increases. If the Eq. (4) applies in the chromatographic system used, the elution volume V_R of a sample solute in linear gradient-elution chromatography can be calculated from Eq. (5):[2,6]

$$V_R = \frac{1}{B}[(m+1)Bk_0V_m + A^{(m+1)}]^{1/(m+1)} - \frac{A}{B} + V_m \tag{5}$$

The Eq. (4) can be used only if the sample solute is very strongly retained in the pure less polar solvent, otherwise a three-parameter retention Eq. (6) should be used to describe the effect of the concentration of the polar solvent on retention:[2,20]

$$k = (a + b\varphi)^{-m} \tag{6}$$

In this case, the gradient retention volumes can be calculated from Eq. (7):[7,8]

$$V_R = \frac{1}{bB}\{(m+1)bBV_m + [a+Ab]^{(m+1)}\}^{1/(m+1)} - \frac{a+Ab}{bB} + V_m \tag{7}$$

a, b, k_0, and m in the Eqs. (4)–(7) depend on the nature of the solute and on the chromatographic system, but are independent of the concentration of the strong solvent B, φ, in the mobile phase.

To first approximation, the bandwidths in gradient elution are equal to the isocratic bandwidths in the mobile phase of the instantaneous composition at the column outlet at the elution time of the band maximum. Hence, the gradient bandwidths w_g can be predicted from Eq. (8) and the resolution R_S from Eq. (9),

introducing the value of the instantaneous retention factor k_e in the mobile phase with the concentration of the strong eluent (solvent B), φ_e, at the elution time of the band maximum. k_e can be calculated from the elution volume introducing the gradient function $\varphi_e = f(V_R)$ into the appropriate equation $k_e = f'(\varphi_e)$ describing the dependence of the retention on the concentration of B in the chromatographic system:[2–4,6,25]

$$w_g = \frac{4V_m(1 + k_e)}{\sqrt{N}} \tag{8}$$

and

$$R_s = \frac{V_{R(2)} - V_{R(1)}}{w_g} \tag{9}$$

N is the number of theoretical plates determined under *isocratic* conditions. It should be kept in mind that the equations commonly used to describe the band broadening in isocratic chromatography, are based on the assumption of a constant retention factor for each sample compound during the whole elution run and do not apply in gradient elution, where the retention factors defined as the sample mass ratio in the stationary and in the mobile phases, respectively, decrease gradually. Hence, neither retention factors nor the plate number can be evaluated directly from the gradient retention volumes and bandwidths using the approaches commonly used in isocratic chromatography.

EXPERIMENTAL

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) was used for all measurements. The gradient dwell volume of the instrument was 0.40 mL, or 0.50 mL when an in-line filter was used. Glass cartridge columns, 150 mm × 3.3 mm I.D., packed with silica gel Separon SGX, 7 μm, ($V_m = 0.90$ mL), Separon SGX Nitrile, 7 μm, ($V_m = 0.97$ mL), and Separon SGX C18, 7 μm, ($V_m = 0.87$ mL) were obtained from Tessek, Prague, Czech Republic. Lichrospher 60RP-select B, 5 μm, 125 mm × 4 mm I.D. ($V_m =$ 0.95 mL) and Purospher Star RP-18e, 3 μm, 30 mm × 4 mm I.D. ($V_m = 0.21$ mL) stainless steel cartridge columns and Chromolith Speed Rod RP 18e monolithic column, 50 × 4.6 mm I.D. ($V_m = 0.70$ mL), were obtained from Merck, Darmstadt, Germany, stainless steel column, 75 × 2.1 mm I.D. ($V_m = 0.26$ mL), packed with Poroshell 300SB-C18 superficially porous octadecyl silica material, 5 μm, from

Agilent Technologies, Palo Alto, CA, and Novapak Silica 60, 4 μm, stainless steel cartridge column, 150 × 3.9 mm I.D., were from Waters, Milford, MA. The flowrate of the mobile phases was kept at 1 mL/min and the temperature at 40°C in all experiments.

Mobile Phases and Samples

Propan-2-ol, *n*-heptane, and dioxane, all of HPLC grade (Baker, Deventer, The Netherlands), before being used in normal-phase chromatographic experiments, were dried and kept in tightly closed dark bottles over molecular sieve beads Dusimo 5Å (Lachema, Brno, Czech Republic), previously activated at 300°C (cca 30–40 g/L). Methanol, propan-2-ol, and acetonitrile (Lichrosolv grade, Merck, Darmstadt, Germany) were used as obtained in reversed-phase experiments. Water was double distilled in glass. All solvents were filtered using a Millipore 0.45 μm filter and degassed in an ultrasonic bath immediately before use. Mobile phases, continuously stripped by a stream of helium, were prepared by mixing, in appropriate volume ratios, directly into the HP 1090M instrument.

Phenylurea and triazine herbicides were obtained from Lachema, Brno, Czech Republic, polystyrenes from Waters, Milford, MA, oligoethyleneglycol nonylphenyl ethers from Servo, Delden, The Netherlands, and alkylbenzenes from Merck, Darmstadt, Germany. The sample solutes were dissolved in the mobile phase to provide adequate response of the UV detector (approximately 10–20 μg/mL). Five microliter sample volumes were injected in each experiment.

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 propan-2-ol or of dioxane in heptane, hexane, or in dichloromethane. The parameters of the retention Eqs. (2), (4), and (6) were determined from the isocratic retention factors, $k = (V_R/V_m - 1)$ using linear or non-linear regression, as described elsewhere.[7] 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, both in normal-phase and in reversed-phase gradient elution. The column hold-up volumes, V_m, were determined using a non-retained marker compound, uracil in reversed-phase systems and trichloroethylene in normal-phase systems.

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

RESULTS AND DISCUSSION

Transfer of Gradient Methods

For rational gradient method development, transfer, and optimization, it is very important to know how various adjustable gradient parameters affect the retention and resolution. The gradient variable parameters involve the gradient time t_G or the gradient volume V_G, the gradient range, i.e., the change in the concentration of the strong solvent B during the gradient time, $\Delta\varphi$ and the concentration of the strong solvent B at the start of the gradient, A. Equations (3), (5), and (7) show that the effects of the gradient time and gradient range on the retention are coupled via the gradient steepness parameter, B, defined by the Eq. (1). Hence, adjusting the gradient time (or the gradient volume) at a constant gradient range has the same effect on the retention as adjusting the gradient range, while keeping a constant gradient time, as long as all sample compounds elute within the gradient time. The lower limit of the gradient range, i.e., the concentration of the solvent, B, at the start of the gradient, A, has very important effect on the retention. The upper limit of the gradient range can be conveniently set in practice to the concentration at which the last sample component (or any important impurity possibly interfering in the next run) elutes from the column. Once the gradient range is set in this way, the gradient volume, V_G, determines the steepness of the gradient, B.

The gradient retention volumes decrease as either the gradient steepness, B, or the initial concentration of the solvent, B, A, increase [Eqs. (3), (5), and (7)]. At a constant gradient range, the gradient steepness, B, is inversely proportional to the gradient volume, V_G, i.e., both to the gradient time, t_G, and to the mobile phase flowrate, F_m [Eq. (1)]. This means that any change in the flowrate should be compensated by appropriate change in the gradient time; otherwise, the decrease (or the increase) in the gradient retention times is not directly proportional to the increase (or decrease) in the flowrate. The example in Fig. 1 shows that increasing F_m causes less than proportional decrease in the retention times, only when decreasing gradient steepness is compensated by appropriate decrease in the gradient time.

The gradient program can be easily modified to obtain the expected retention data when changing the operation conditions. In all equations for gradient-elution times or volumes [for example, Eqs. (3), (5), (7)], the product of the net elution volume and of the gradient steepness parameter, $V'_R B$, is constant

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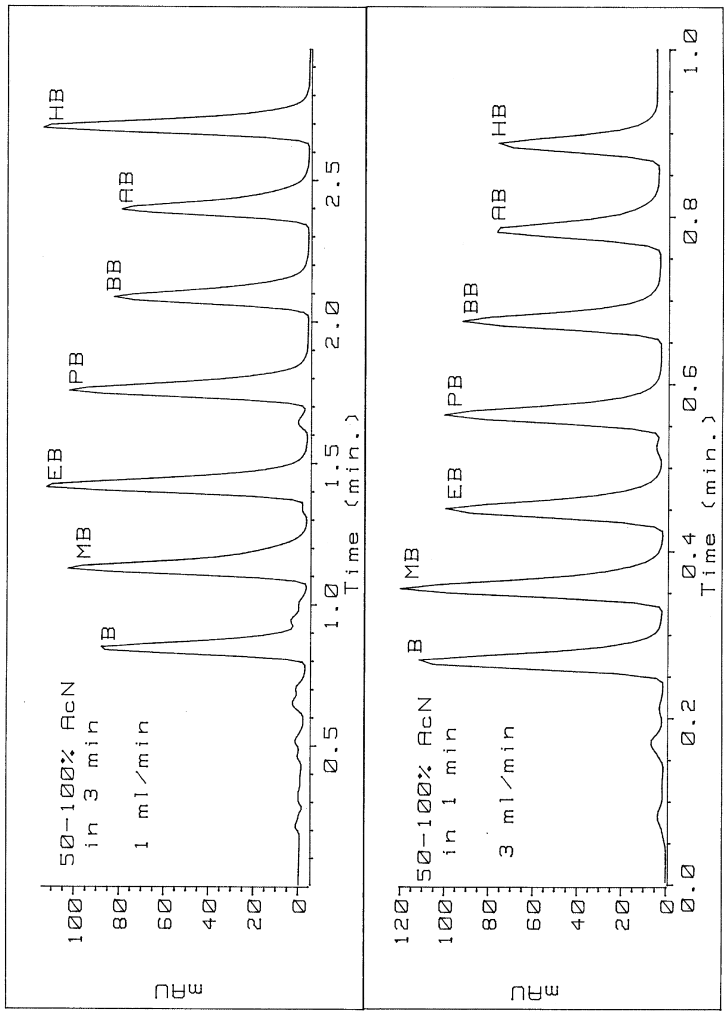

Figure 1. Flowrate effects on gradient-elution reversed-phase separation of alkylbenzenes. Purospher Star RP-18e, 3 μm, column (30 × 4 mm I.D.). Linear gradient 50–100% acetonitrile in 3 min at 1 mL/min and at 3 mL/min, in one minute, 40°C, detection UV, 254 nm. B—benzene, MB—toluene, EB—ethyl benzene, PB—propyl benzene, BB—butyl benzene, AB—amyl benzene, HB—hexyl benzene.

as long as the product $V'_m \cdot B$ does not change. This means that at a constant gradient range (i.e., with constant concentrations of the stronger eluent at the start, A, and at the end, φ_G, of the gradient) the ratio of the net retention volumes to the gradient volume does not change if the gradient volume, V_G, expressed in the number of the column hold-up volumes is constant. Hence, any change in the mobile phase flowrate, F_m, or in the column length, L, and diameter, d_c, at a constant gradient range should be compensated by appropriate change in the gradient time, t_G, to keep the original ratio V_m/V_G unchanged:[3,5]

$$\frac{V_m}{V_G} = \frac{V}{t_G F_m} = \frac{d_c{}^2 L}{t_G F_m} = \text{const} \tag{10}$$

This condition has several important practical consequences:

1. If the flowrate of the mobile phase increases from F_1 to F_2, the gradient time should be decreased by the factor F_2/F_1 to keep the steepness parameter B constant and to decrease the elution times in the proportion F_2/F_1.
2. If the column inner diameter increases or decreases from $d_{c,1}$ to $d_{c,2}$ by a factor $f = d_{c,2}/d_{c,1}$ (such as when transferring an analytical method to a preparative or to a microbore column), a constant product $V_m B$ should be maintained by adjusting the flowrate by the factor f^2. In such a case, the retention volumes change by the factor f^2, but the retention times and the operating pressure do not change.
3. If the separation efficiency is adjusted by increasing the length of the column from L_1 to L_2, the gradient time should be increased by the factor $f = L_2/L_1$ at a constant flowrate. Then the retention times and the retention volumes increase by the same factor f. The gradient time, t_G, should be decreased if a shorter column is used when rapid separation methods are developed. Of course, increasing or decreasing the column length affects not only the column plate number and the resolution, but also the operating pressure.

The effects of the gradient slope, $B = \Delta\varphi/(t_G F_m)$, and of the initial concentration of the strong solvent, A, on the retention in various gradient-elution liquid chromatography modes, can be determined exactly by calculation using e.g., Eqs. (3), (5), or (7), as appropriate. For this purpose, the constants a and m of the retention equation controlling the chromatographic system should be determined in at least two independent gradient or isocratic experiments.

However, it is possible to use a simple approach for rapid rough estimation of the change in retention volumes from $V'_{R,2}$ to $V'_{R,1}$ caused by increasing or decreasing the steepness of the gradient from B_1 to B_2 and (or) the initial concentration of the polar solvent B from A_1 to A_2, from a single gradient experiment.

In reversed-phase chromatography, $m \cong 3$ for many low-molecular mass sample solutes such as simple benzene derivatives. With this assumption, Eq. (3) can be modified to Eq. (11) enabling the estimation of the retention volume $V'_{R,2}$ at a new gradient program from the retention volume $V'_{R,1}$ measured with the original gradient:

$$V'_{R,2} \cong \frac{1}{3B_2} \log\left[\frac{B_2}{B_1} 10^{3(A_1-A_2)}(10^{3B_1 V'_{R,1}} - 1) + 1\right] \tag{11}$$

Figure 2 shows that using simplified Eq. (11) instead of the accurate Eq. (3) causes the prediction errors < 0.2 mL in the gradient retention volumes of three alkylbenzenes, with m ranging from 2.6 to 3.6. The errors increase for higher molecular weight compounds with greater parameters, m.

A similar approach can also be used in normal-phase chromatography, assuming the validity of Eq. (4) with the parameter $m \cong 1$ for the displacement model of adsorption, where one molecule of the solute is displaced from the adsorbent surface by one molecule of the strong (more polar) solvent, B.[9] A constant volume of the polar solvent, V_{solv}, is assumed to accomplish the elution of a sample compound using various gradient programs:

$$V_{\text{solv}} = \left(V'_{R,1}\right)^2 \frac{B_1}{2} + V'_{R,1}\, A_1 = \left(V'_{R,2}\right)^2 \frac{B_2}{2} + V'_{R,2}\, A_2 \tag{12}$$

For gradients starting at zero concentration of the polar solvent B, $A_1 = A_2 = 0$ and the Eq. (12) simplifies to:

$$V'_{R,2} = V'_{R,1}\sqrt{\frac{B_1}{B_2}} \tag{13}$$

In Table 1, experimental normal-phase gradient elution volumes of a few phenylurea herbicides are compared with the elution volumes calculated using Eq. (12) for gradients of propan-2-ol in hexane. The simple calculation yields more or less underestimated elution volumes for gradients starting at 0% polar solvent, and overestimated data for gradients starting at a non-zero concentration of propan-2-ol, 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 average error of prediction of the retention times reported in Table 1 is approximately 7% or less. The retention volumes calculated from the Eq. (12) may be subject to significant errors for compounds whose parameters, m, differ significantly from 1. Equation (12) cannot be used for reversed-phase gradient elution.

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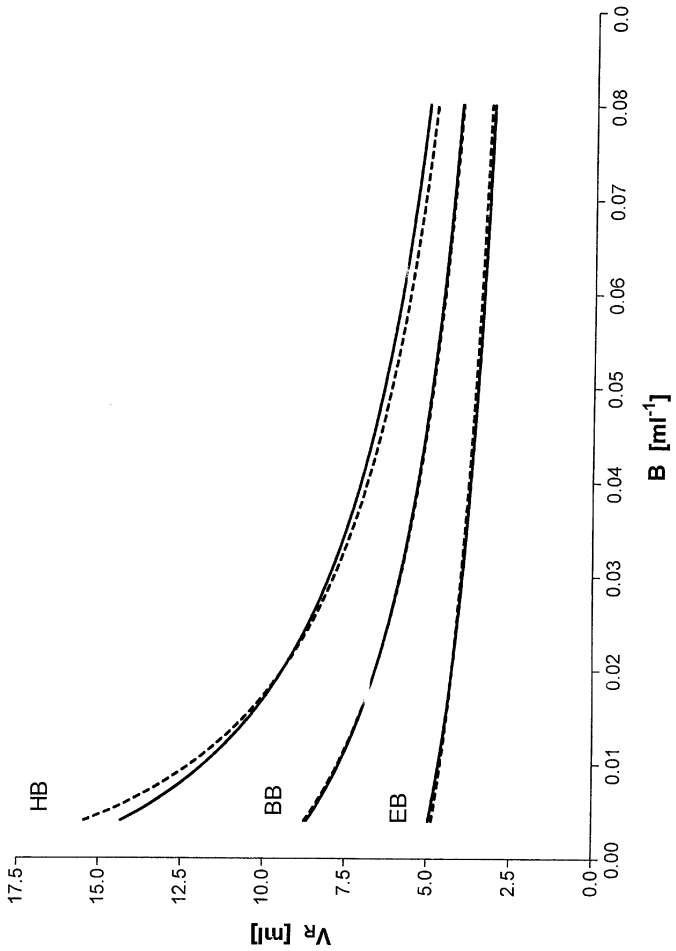

Figure 2. The effect of changing gradient steepness parameter, $B = \Delta\varphi/V_G$, on the net retention volumes, V'_R, in reversed-phase chromatography. Gradients from 50 to 90% acetonitrile in water. Dashed lines—calculated from Eq. (3), full lines—calculated from the retention volumes in the 20 min gradient (1 mL/min) using Eq. (11). Sample compounds: ethylbenzene (EB, $m = 2.60$), butylbenzene (BB, $m = 3.09$) and hexylbenzene (HB, $m = 3.59$). Column: Lichrospher 60RP-select B, 5 µm (125 × 4 mm I.D., $V_m = 0.90$ mL).

Table 1. Retention Volumes of Herbicides in Normal-Phase Gradient Elution HPLC

Solute		0–50% P	0–25% P	0–16.7% P	3–50% P	6–50% P	$V_{solv,\ average}$
DPU	V_{solv}	0.79	0.94	1.04	0.78	0.74	0.75 ± 0.03
	V_R (E)	11.31	16.68	21.66	9.53	8.01	
	V_R (C)	—	15.41	18.56	9.88	8.64	
CMU	V_{solv}	0.67	0.80	0.89	0.66	0.62	0.63 ± 0.03
	V_R (E)	10.55	15.55	16.69	8.82	7.25	
	V_R (C)	—	14.34	17.24	9.11	7.89	
IPU	V_{solv}	0.76	0.84	0.89	0.75	0.73	0.73 ± 0.03
	V_R (E)	11.18	16.01	19.50	9.47	8.00	
	V_R (C)	—	15.23	18.34	9.75	8.67	
DCU	V_{solv}	1.50	1.75	1.90	1.51	1.48	1.48 ± 0.03
	V_R (E)	14.86	22.02	27.78	13.27	11.90	
	V_R (C)	—	20.43	27.71	13.50	12.23	
Avg. error, %		—	−6.9	−5.9	+2.9	+5.9	

Silica gel Separon SGX column (150×3.3 mm I.D.), gradients of propan-2-ol in heptane. Phenylurea herbicides and related compounds: DPU—desphenuron, CMU—*bis*-*N*, *N*′-(3-chloro-4-methylphenyl) urea; IPU—isoproturon; DCU—deschlorometoxuron.

Gradient time 30 min, 1 mL/min, 40°C. $V_{solv} = V_m k_0$ (mL)—volume of pure propanol necessary for the elution of a solute (average value ± SD, except gradients ending at <50% P); V_R (E), mL, experimental retention volumes; V_R (C), mL, retention volumes calculated from Eq. (12) using V_R (E) values measured in the gradients from 0 to 50% P.

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Optimization of Gradient Elution

Commercially available optimization software (such as "DryLab G" developed by Snyder et al.)[1,26] usually are focused on the calculation of the gradient time providing adequate resolution for all important sample compounds. The gradient range is optimized in the second step, which is often limited to finding a suitable final gradient concentration, φ_G, whereas the initial concentration of the solvent B, A, is set to 0.[1,26,27] However, this approach neglects the fact that the effect of the initial concentration, A, on the retention and on the resolution is equally important as the effect of the gradient steepness, B, and the two parameters of the gradient profile show synergistic effects on separation. Not only the retention volumes decrease, but also the separation selectivity and even the elution order of some sample components may change when A increases (see examples in Fig. 3). Setting an appropriate non-zero initial concentration, A, can not only significantly shorten the analysis time, but usually also facilitates the column equilibration in between the gradient runs.

Therefore, we developed a simple approach for the optimization of the gradient volume and gradient range by adjusting, simultaneously, both the initial concentration and the gradient steepness. The procedure is essentially based on the "window diagram" strategy, takes into account the separation of all important individual sample components, and can be performed on a PC using a simple spreadsheet editor, such as Quattro Pro, Excel, or another similar readily available software. First, we set a desired time for gradient elution, i.e., the gradient volume, V_G, in which a pre-set final concentration of the polar solvent, φ_G, should be achieved. With a pre-set V_G, the slope, B, of the gradient and the initial concentration of solvent, B, A, are linked by the Eq. (14):[28–30]

$$B = \frac{(\varphi_G - A)}{V_G} \tag{14}$$

With this assumption, the elution volumes of sample components, V_R, depend on a single parameter, A, and can be calculated after introducing the Eq. (14) into the Eqs. (3), (5), or (7), as appropriate. In the calculation, V_G is first selected for an acceptable analysis time, e.g., 20 mL at 2 mL/min for a gradient finishing at 100% strong solvent B, $\varphi_G = 1$. The setting of V_G usually does not significantly affect the results. The differences between the V_R of compounds with adjacent peaks or the resolution R_s of the adjacent bands are plotted vs. A in the form of a "window diagram" from which the optimum initial concentration A can be selected, which provides the resolution desired in minimum time. Using the optimized A, gradient steepness B is calculated using the Eq. (14). Then, the final gradient concentration, φ_G, is adjusted at a constant gradient steepness B, for the end of the gradient immediately after the elution of the last sample band, which can be easily determined from the corresponding elution volume calculated using

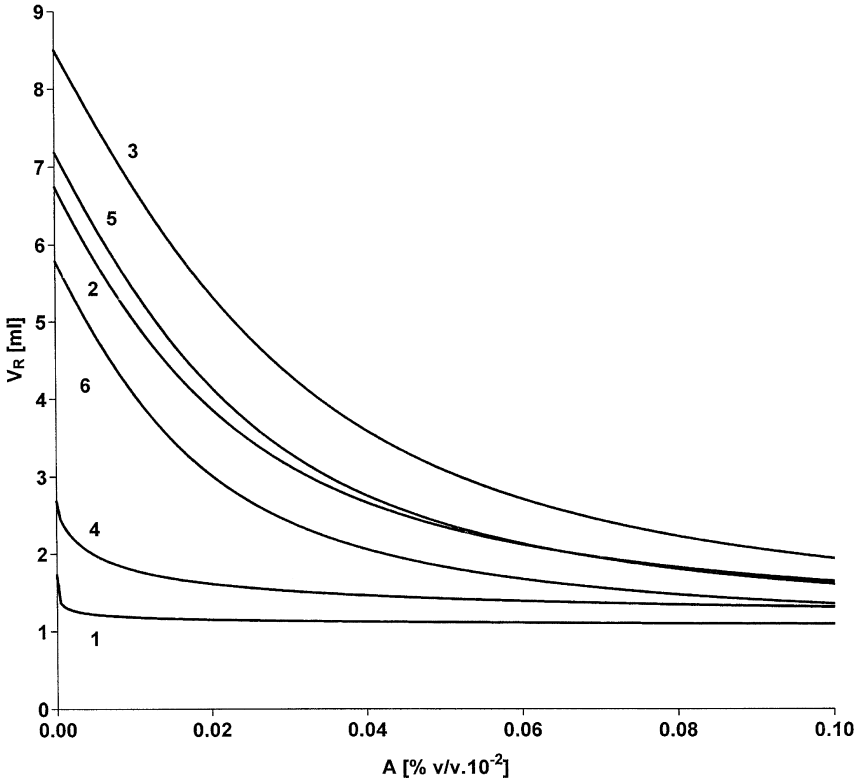

Figure 3. The dependencies of the retention volumes of nitrophenols, V_R, on the initial concentration of methanol, A, in normal-phase gradient elution chromatography. Column: Slica gel, Separon SGX , 150 mm × 3.3 mm I.D.; gradients of propan-2-ol in heptane, from $A \cdot 10^2$ to 15% in 30 min at 1 mL/min. $V_D = 0.4$ mL. Sample compounds: 2-nitrophenol (1), 3-nitrophenol (2), 4-nitrophenol (3), 3-methyl-2-nitrophenol (4), 4-methyl-3-nitrophenol (5) and 2-methyl-5-nitrophenol (6).

one of the Eqs. (3), (5), or (7). The selection of the highest value of A at which the desired resolution (e.g., $R_{s,g} = 1.5$) is achieved for all compounds in the sample mixture, in most cases automatically minimizes the time of the analysis, as the elution volumes decrease with increasing A (Fig. 3). The optimization can be repeated for a higher pre-set V_G, or increased column efficiency (a longer column or finer packing particles) if the window diagram shows that the desired resolution cannot be achieved. The optimization approach can be used either in reversed-phase or in normal-phase gradient elution operation, and is illustrated by an example in Fig. 4 for reversed-phase separation of 12 phenylurea herbicides.

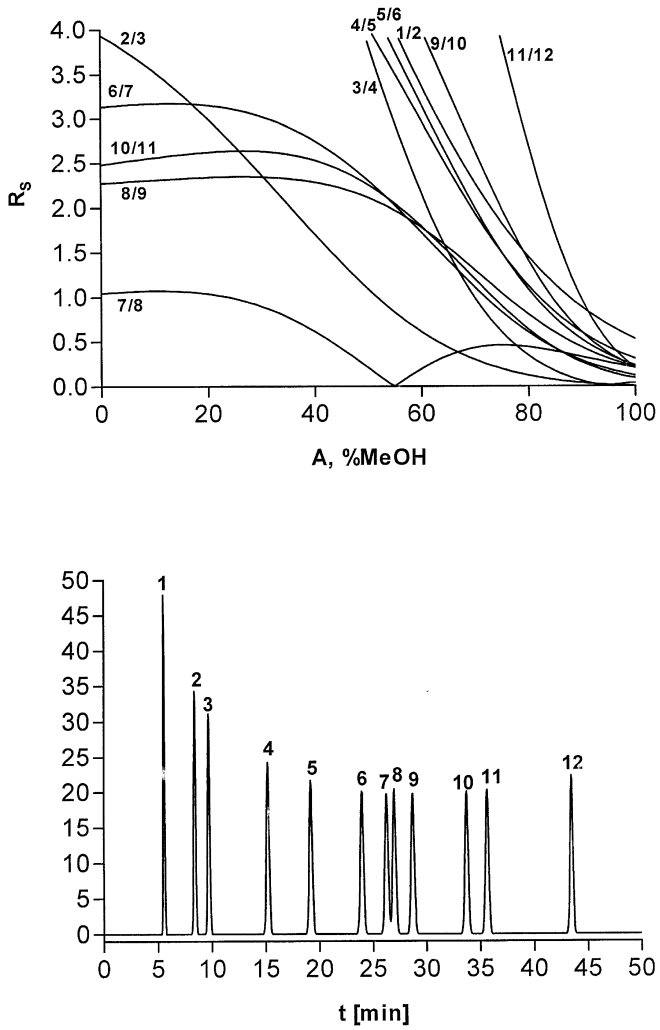

Figure 4. Top: The resolution window diagram for the gradient-elution separation of a mixture of 12 phenylurea herbicides on a Separon SGX C18, 7.5 μm, column (150 × 3.3 mm I.D.) in dependence on the initial concentration of methanol in water at the start of the gradient, A, with optimum gradient volume $V_G = 73$ mL. Column plate number $N = 5000$; sample compounds: hydroxymetoxuron (1), desphenuron (2), phenuron (3), metoxuron (4), monuron (5), monolinuron (6), chlorotoluron (7), metobromuron (8), diuron (9), linuron (10), chlorobromuron (11), neburon (12). Bottom: The separation of the 12 phenylurea herbicides with optimised binary gradient from 24 to 100% methanol in water in 73 min. Flowrate 1 mL/min.

From the window diagram (top), optimum separation is predicted for the gradient from 24 to 100% methanol in water in 73 min at 1 mL/min (bottom).[29]

Effects of the Instrumentation and of the Non-Ideal Retention Behavior on Gradient Elution

The irreproducibility of the retention data, or experimental deviations from predicted gradient retention data, can be caused by pump malfunction, imperfect matching of actual pump flow rates in high-pressure gradient systems, or by imprecise mixing of the pre-set volume ratios of the mobile phase components at high or low mixing proportions (with some instruments, irreproducible results can be avoided by starting gradients at more than 5% B and terminating at less than 95% B). Further, using gradient mixers to improve the mixing of mobile phase components and to suppress baseline fluctuations often impairs gradient rounding in the initial and final parts of the gradient, and always increases the gradient dwell volume.[1,31]

The gradient dwell volume, V_D, is the volume between the column and the place where the individual liquid streams of the gradient components merge together, and is responsible for a more or less significant delay between the arrival of the front of the gradient to the column top and the sample injection. The dwell volumes usually vary in between 0.1 and 5 mL in various commercial types of instruments and these differences can cause unexpected changes in the separation when a gradient method is transferred from one chromatograph to another. At the time of sample injection, the gradient dwell volume is filled with the initial mobile phase containing the strong solvent, B, in concentration A. The dwell volume of this mobile phase should first pass through the column before the front of the gradient arrives to the top of the column. Therefore, the sample compounds are subject to an unintentional dwell-volume isocratic pre-gradient step.[1–4,31]

Strongly retained compounds with the retention factor in the initial mobile phase $k_A > 20$ do not move along the column during the pre-gradient dwell volume step, which fully contributes to their elution volumes:[3,4]

$$V_R = V'_R + V_m + V_D \tag{15}$$

However, less strongly retained compounds can migrate a significant distance along the column before their zone is taken over by the front of the gradient.[2,3,7,8] This means that only a part of the hold-up volume, V_{mg}, remains available for the migration during the actual gradient elution:

$$V_{mg} = V_m - \frac{V_D}{k_A} \tag{16}$$

Hence, V_{mg} should be used instead of V_m in calculations of the gradient elution volumes using Eqs. (3), (5), or (7).[2,3,8] Some very weakly retained compounds can migrate along the whole column length and elute in the dwell volume step. The probability of such gradient pre-elution increases as the ratio of the dwell volume to the column hold-up volume, V_D/V_m, increases, as shown in Fig. 5. This behavior has a practical impact—standard gradient equipment with relatively large gradient dwell volumes usually is not suitable for operation with short and narrow diameter (especially capillary) HPLC columns. In some instruments, it is possible to delay the sample injection with respect to start of the gradient by the time V_D/F_m at the mobile phase flowrate F_m, but this can excessively increase the total time of analysis, so that fast gradients on the columns with small hold-up volumes require special instruments. The effects of the dwell volume on gradient elution are compared in Fig. 6 for the fast separation of a mixture of triazine herbicides on a conventional column (A) and on short monolithic (B) and superficially porous (C) columns in an instrument with a relatively low dwell volume ($V_D = 0.5$ mL). Whereas, all sample compounds elute during the gradient from the conventional Lichrospher column with $V_m = 0.95$ mL, simazine pre-elutes in the initial mobile phase during the isocratic dwell volume step from the monolithic Chromolith column ($V_m = 0.7$ mL) under the same gradient conditions, and four of seven triazine sample compounds pre-elute in the dwell volume step from the superficially porous Poroshell column with $V_m = 0.26$ mL, a hold-up volume smaller than V_D. To avoid the sample pre-elution and to decrease the dwell volume effects with instruments that do not allow delayed gradient operation, pre-column mobile phase flow splitting can be used. The contribution of the dwell volume to the retention can be corrected in calculations of the retention volumes by combining Eqs. (15) and (16) with Eqs. (3), (5), or (7).

It is well known that the components of the mobile phase with higher affinities to the stationary phase are adsorbed on the column more strongly than the components with lower elution strengths.[7,8,32,34] This preferential adsorption usually is not important in isocratic HPLC, where the column is in equilibrium with the mobile phase, but the uptake of strong solvent(s) during the gradient run can significantly change the properties of the stationary phase and, consequently, the elution volumes and the resolution. The adsorbed solvent is not effective as the eluent and the experimental elution volumes are higher than predicted by simple calculation using Eqs. (3), (5), or (7) if the solvent adsorption is important in the chromatographic system. The solvent uptake is usually more significant in normal phase chromatography than in reversed-phase systems, as polar solvents are much more strongly adsorbed on silica gel and other polar adsorbents from a non-polar solvent than methanol or acetonitrile are from water on bonded alkylsilica gel columns.[7,8,35]

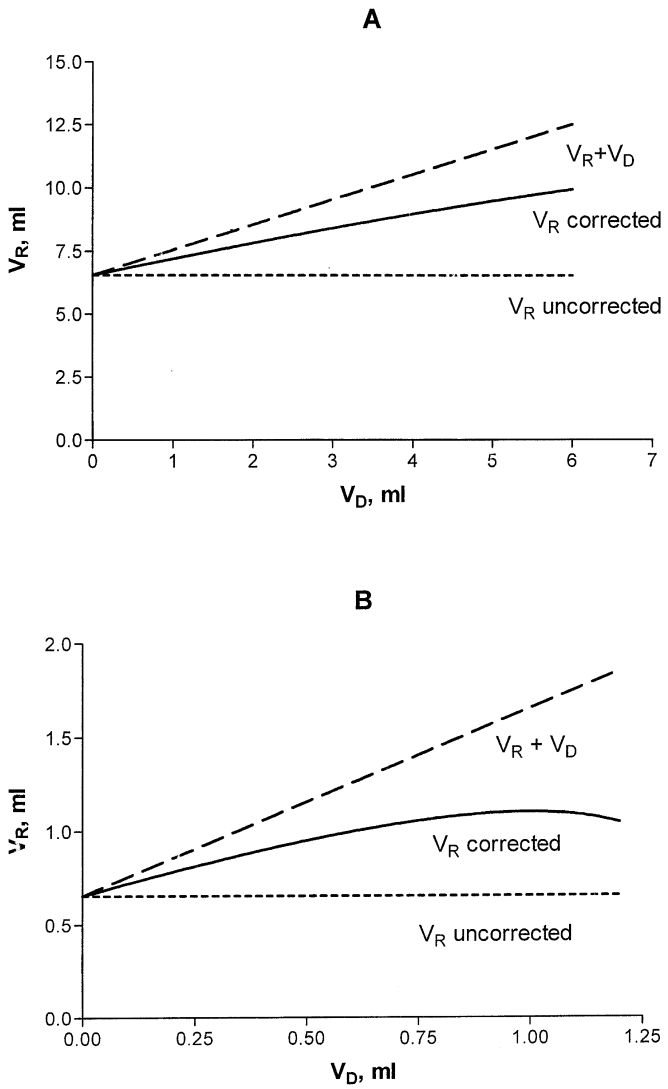

Figure 5. The effect of the gradient dwell volume, V_D, on the elution volume, V_R, in reversed-phase chromatography on a conventional analytical C18 column with the hold-up volume $V_m = 1$ mL (A) and on a microbore analytical C18 column with the hold-up volume $V_m = 0.1$ mL (B). Solute: neburon, retention Eq. (2) with parameters $a = 4$, $m = 4$. Linear gradients, 2.125% methanol/min at 1 mL/min from 75% to 100% methanol in water in 11.7 min (A) and at 0.1 mL/min (B), V_R *uncorrected*—calculated from Eq. (3), $V_R + V_D$—V_D added to V_R *uncorrected*, V_R *corrected*—calculated from Eq. (20) with $K_D = 0$.

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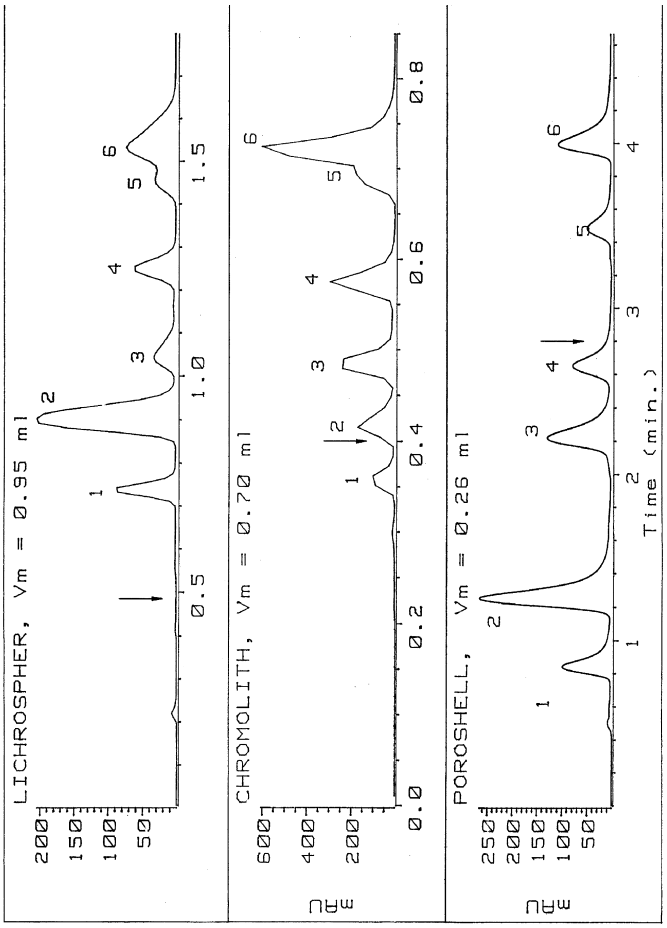

Figure 6. The effect of the column hold-up volume on the pre-elution in the dwell-volume step (0.5 mL). Columns: A—LICHROSPHER 60RP-select B, 5 µm, 125 mm × 4 mm I.D., $V_m = 0.95$ mL, gradient 50–70% acetonitrile in 2.6 min, 3 mL/min, no pre-elution; B—CHROMOLITH, 50 mm × 4.6 mm I.D., $V_m = 0.70$ mL, gradient 50–70% acetonitrile in 5 min, 3 mL/min, compound 1 pre-eluted in the dwell volume; C—POROSHELL, 5 µm, 2.1 × 75 mm I.D., $V_m = 0.26$ mL, gradient 20–40% acetonitrile in 7.1 min, 0.3 mL/min, compounds 1–4 pre-eluted in the dwell-volume; Sample compounds: triazine herbicides, simazine (1), atrazine (2), methoprotryne (3), terbutylazine (4), promethryne (5) and terbutryne (6), 40°C. Arrows indicate the start of the elution in the gradient period.

Another complication may occur when the column capacity for the strong solvent, B, gets saturated at a certain time during gradient elution. Then, the breakthrough of the solvent, B, is accompanied with a sudden increase of its concentration in the mobile phase, which can displace and sweep out from the column weakly retained sample compounds and (or) the impurities adsorbed previously from the mobile phase. Hence, a "ghost" peak may appear at the solvent, B, breakthrough time,[35] such as in Fig. 7(B), showing a record of a blank gradient of propan-2-ol in hexane on a silica gel column. The breakthrough curves in gradient elution HPLC can be predicted by numerical calculation if the adsorption isotherm of the strong solvent, B, between the column and the binary mobile phase is known, as shown in Fig. 7(A).

The experimentally determined constants of the adsorption isotherm equation can be used to correct the gradient elution volumes for the preferential uptake of solvent B. The distribution of polar organic solvents in reversed-phase systems, but also of some moderately polar solvents in normal-phase systems, can often be approximated by the linear isotherm:[36]

$$q = K_D\varphi \tag{17}$$

where K_D is the distribution constant of the solvent B. On the other hand, the distribution of polar solvents between a binary organic mobile phase and a polar column in normal-phase systems is usually reasonably well described by the Langmuir isotherm:[36]

$$q = \frac{q_s b\varphi}{1 + b\varphi} \tag{18}$$

where q and φ are the concentrations of the strong solvent, B, in the stationary and in the mobile phases, respectively, q_s is the column saturation capacity and b is the isotherm coefficient.

With linear isotherms, the strong solvent uptake decreases the actual gradient steepness, B, to a certain extent, which can be taken into account by introducing a corrected gradient steepness parameter, B_{corr}:

$$B_{\text{corr}} = B(1 - K_D\Phi) \tag{19}$$

where $\Phi = V_s/V_m$ is the phase ratio, i.e., the volume of the stationary and of the mobile phases in the column. Introducing the correction for the gradient dwell volume, V_D, and for the organic solvent uptake (if necessary) into the Eq. (3), the corrected reversed-phase gradient elution volumes can be calculated from Eq. (20):[8]

$$V_R = \frac{1}{mB(1 - K_D\Phi)}\log[2.31mB(1 - K_D\Phi)[V_m 10^{(a-mA)} - V_D] + 1] + V_D + V_m \tag{20}$$

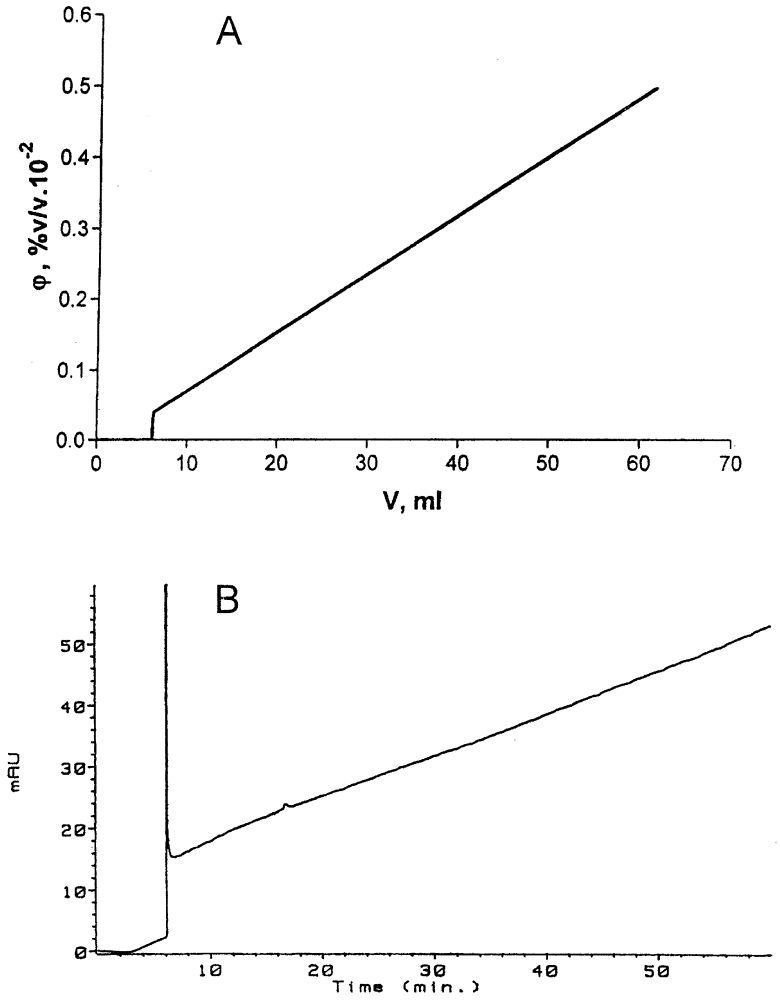

Figure 7. (A) Calculated breakthrough curves in normal-phase gradient-elution HPLC. Simulated calculation using the experimental isotherm data and assuming $N = 5000$. Gradient dwell volume = 0.50 mL. (B) The record of the blank gradient showing the breakthrough of propan-2-ol at 6 min and a "ghost peak" of impurities displaced at the breakthrough volume. Column: silica gel Separon SGX (7.5 μm), 150 × 3.3 mm I.D., 1 mL/min, 40°C. Gradient: 0–50% 2-propanol in 30 min (φ—concentration of propan-2-ol in the eluate, V—volume of the eluate from the start of the gradient).

In normal-phase chromatography, the distribution of the polar solvent, B, between the polar column and the binary organic mobile phase is often controlled by the Langmuir isotherm equation (18), from which the adsorbed volume of the polar solvent, V_{ads}, can be calculated at any time in the course of the gradient.[8] Modifications of the Eq. (5) to respect the sample migration during the gradient dwell period, and possible preferential solvent uptake, yield Eq. (21):

$$V_R = \frac{1}{B}[(m+1)B(k_0 V_m - V_D A^m) + (A^2 + 2BV_{ads})^{(m+1)/2}]^{1/(m+1)} - \frac{A}{B} + V_D + V_m \quad (21)$$

A similar approach can be adopted to modify the Eq. (7). Table 2 illustrates the errors of prediction for normal-phase gradient elution volumes in uncorrected and corrected calculation approaches. The results show that the average error 6.7% in uncorrected retention volumes of chlorotoluron calculated using Eq. (5), is reduced to 2.2–2.6% with correction for the gradient dwell volume, and to 1.6% with correction for both the dwell volume and for the preferential adsorption of propan-2-ol during the gradient elution. The preferential adsorption affects, significantly, only the elution volumes with gradients starting in pure heptane, where its neglection leads to significant underestimation of the calculated elution volumes. When the gradients start with 3%–9% propan-2-ol, the effect of the preferential adsorption on the retention volumes is negligible, and simple addition of V_D to the retention volumes calculated using Eq. (5) yields the results comparable with accurate calculations using Eq. (21). This can be probably explained by a relatively strong retention in the dwell volume mobile phase, which is connected with only little migration along the column during the dwell volume step in the gradients employed (to 1% of the column volume in 3% propan-2-ol with $k = 44$; to 3% of the column volume in 6% propan-2-ol with $k = 17$ and to 5% of the column volume in 9% propan-2-ol with $k = 9.3$). However, the errors caused by simple addition of V_D to the elution volumes calculated using Eq. (5) (or other appropriate equations) are much more significant for sample compounds less strongly retained at the start of the gradient.

Gradient Elution Chromatography of Polymers and Oligomers

Large molecules can be partially or completely excluded from the pores of the packing material by size exclusion. Therefore, wide-pore column packing materials are preferred for separation of biopolymers and synthetic polymers. Size-exclusion may limit the pore accessibility for large molecules. In calculations of the gradient-elution retention volumes, the size-exclusion effect can be

Table 2. Experimental (E) and Predicted Retention Volumes (A–D) in Normal-Phase Gradient Elution Chromatography

	Grandient Range	A $\%v \times 10^{-2}$	B $\%v\,mL^{-1} \times 10^{-2}$	E	1	2	3	4
V_R (mL)	0–50%	0	0.01667	11.13	10.26	10.76	10.76	11.08
Δ (mL)					−0.87	−0.37	−0.37	−0.05
V_R (mL)	0–25%	0	0.00833	16.01	14.95	15.45	15.45	15.86
Δ (mL)					−1.06	−0.56	−0.56	−0.14
V_R (mL)	0–16.7%	0	0.00556	19.68	18.71	19.21	19.21	19.69
Δ (mL)					−0.97	−0.47	−0.47	+0.01
V_R (mL)	3–50%	0.03	0.01567	9.36	8.77	9.27	9.22	9.23
Δ (mL)					−0.59	−0.09	−0.14	−0.13
V_R (mL)	3–26.5%	0.03	0.00783	12.54	11.56	12.06	12.30	12.31
Δ (mL)					−0.98	−0.48	−0.24	−0.23
V_R (mL)	3–18.7%	0.03	0.00523	14.61	14.07	14.57	14.48	14.49
Δ (mL)					−0.54	−0.04	−0.13	−0.12
V_R (mL)	6–50%	0.06	0.01467	8.02	7.36	7.86	7.73	7.73
Δ (mL)					−0.66	−0.26	−0.29	−0.29
V_R (mL)	9–50%	0.09	0.01367	6.66	6.13	6.63	6.40	6.41
Δ (mL)					−0.53	−0.03	−0.26	−0.25
Δ average, %					6.7	2.2	2.6	1.6

The column and the operation conditions are as in Table 1. Sample solute: chlorotoluron, $k_0 = 0.311$, $m = 1.412$ [Eq. (4)], 30 min linear gradients of propan-2-ol in hexane, 1 mL/min, 40°C. Calculated retention volumes: A—from Eq. (5), no correction; B—as in A, $V_D = 0.5$ added; C—from Eq. (21) assuming no propanol adsorption; D—from Eq. (21) with correction for V_D and adsorption of propan-2-ol controlled by Langmuir isotherm (18), $b = 1424\%v^{-1} \times 10^2$, $q_s = 0.2486\%v \times 10^{-2}$. Δ = deviation from the experimental V_R.

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corrected for by using the size-exclusion volume, V_{SEC}, determined in a strong mobile phase where the adsorption is completely suppressed, instead of the column hold-up volume, V_m, in Eqs. (3), (5), or (7),[37] assuming that the size exclusion does not affect the phase ratio in the column.

However, the most important difference between the retention behavior of small and large molecules is caused by the effect of the size of the molecules on their distribution between the stationary and the mobile phases. Generally, various structural elements in the molecule contribute additively to the free energy of adsorption and, hence, to the logarithm of the retention factors (Martin rule),[38] so that the retention of polymers and oligomers increases with increasing number of repeat monomer units, n, according to a second order polynomial equation (22):[39]

$$\log k = \log \beta + n \log \alpha + n^2 \log \gamma \tag{22}$$

The term, α, characterizes the repeat unit chromatographic selectivity, the term, β, the contribution of the end groups to the retention, and the quadratic term, γ, is a measure of occasionally observed deviations caused by conformation and other effects. The constant γ is often small enough so that the quadratic term in the Eq. (22) can be neglected, at least over a limited repeat unit range. In this case, the constants, m, a ($\log k_0$) in Eqs. (2) and (4) are directly proportional to n:[39,40]

$$m = m_0 + m_1 n \tag{23a}$$

$$a = \log k_0 = a_0 + a_1 n \tag{23b}$$

These relationships can be introduced into the Eqs. (3), (5), or (7) to describe the dependence of the gradient elution volumes on the number of monomer units, n. Table 3 shows a few examples of the experimental constants of the Eqs. (23a) and (23b) for various oligomers (O) in reversed-phase and in

Table 3A. Constants of Eqs. (23a) and (23b) for Various Oligomers (O): Reversed-Phase Systems

O	Repeat Unit	S	a_0	a_1	m_0	m_1
PS	$C_6H_5-CH-CH_2-$	D	2.49	0.77	3.12	0.83
OEG	$-CH_2-CH_2-O-$	M	−1.1	0.36	0.61	0.6
OEG	$-CH_2-CH_2-O-$	P	−0.9	0.34	−1.4	3.26
OEP	$-CH_2-CH_2-O$	P	2.69	0	3.89	0
OEA	$-CH_2-CH_2-O-$	M	7.37	0	7.49	0
OEA	$-CH_2-CH_2-O-$	A	4.1	0	3.9	−0.1

O–oligomer series: PS–polystyrenes, OEG–oligoethylene glycols, OEP–oligoethyleneglycol nonylphenyl ethers, OEA–oligoethyleneglycol hexadecyl ethers; column: Separon SGX C18, binary mobile phases containing various organic solvents; S: dioxane–D, methanol–M, propan-2-ol–P, acetonitrile–A in water.

Table 3B. Constants of Eqs. (23a) and (23b) for Various Oligomers (O): Normal-Phase Systems

O	Repeat Unit	C, S	a_0	a_1	m_0	m_1
PS	$C_6H_5-CH-CH_2-$	1, T	−1.35	0	0.5	0
PS	$C_6H_5-CH-CH_2-$	1, D	−0.92	0	0.3	0.11
OEP	$-CH_2-CH_2-O-$	1, P	−1.47	0.4	1	0.1
OEP	$-CH_2-CH_2-O-$	1, E	−2.46	0.3	3.3	−0.3
OEP	$-CH_2-CH_2-O-$	2, P	−1.81	0	0.9	0.14
OEP	$-CH_2-CH_2-O-$	3, P	−1.6	0.2	0.6	0
OEP	$-CH_2-CH_2-O-$	4, P	−1.78	0.2	1.3	0
OEA	$-CH_2-CH_2-O-$	4, P	−1.07	0.2	0.15	0.1

O–oligomer series: PS–polystyrenes, OEP–oligoethyleneglycol nonylphenl ethers, OEA–oligoethyleneglycol hexadecyl ethers; C–columns: (1) Separon SGX (silica), (2) Separon SGX Nitrile, (3) Silasorb Diol and (4) Separon SGX Amine, binary mobile phases containing various polar solvents; S: tetrahydrofuran–T, dioxane–D, 2-propanol–P, ethanol–E in hexane.

normal-phase systems. The negative value of m_1 means that the retention decreases in the order of increasing number of monomer oxyethylene units in acetonitrile–water mobile phases on a C18 column.[41,42] Such behavior, which has been called by some authors "liquid exclusion-adsorption chromatography",[43,44] can be explained by negative adsorption energy of an oxyethylene unit, rather than by entropic effects caused by steric exclusion of these units.[40] The constants $a_1 = 0$ and $m_1 = 0$ indicate co-elution of oligoethyleneglycol nonylphenylethers in reversed-phase systems with propan-2-ol–water mobile phases and of oligoethyleneglycol alkylethers in methanol–water mobile phases over a wide range of binary mobile phase compositions. This behavior is often characterized as "liquid chromatography under critical conditions",[45] which can be explained by the compensation of the adsorption energy of the mobile phase and of the repeat monomer units, even in the systems where size-exclusion effects are only of minor importance.[40]

The necessity for using gradient elution in IC separations of polymers can be derived directly from the Eqs. (23a) and (23b), whose constants a and m regularly increase with increasing number of repeat monomer units, because each monomer unit provides a constant contribution to $\log k$ (at least over a limited monomer unit range).[40] Hence, a and m may have very large values for higher polymers. For example, the constants, m, in reversed-phase systems are in between 2–4.5 for toluene to decylbenzene with $M_r = 92$–218 (Fig. 8),[39] but the constants, m, are in the range 25–70 for polybutylacrylates and polystyrenes with M_r in between 10,000 and 20,000 (Fig. 9).[46]

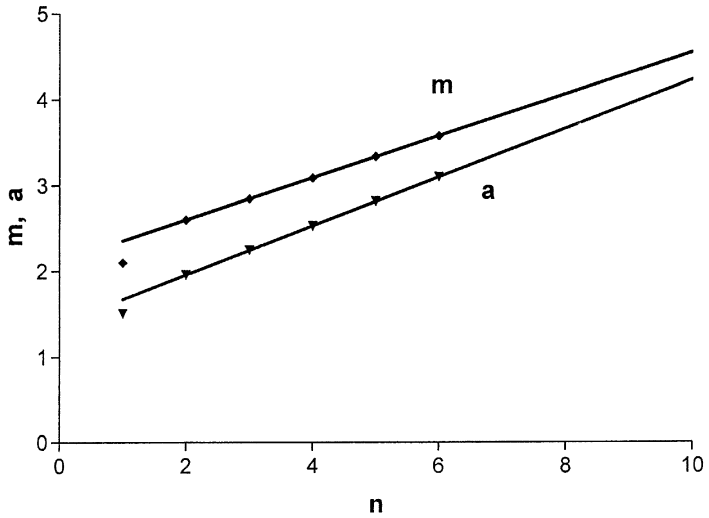

Figure 8. Dependencies of the constants a and m of Eq. (2) on the number of carbon atoms, n, in the alkyl chains of alkylbenzenes. Column: Lichrospher 60RP-select B, 5 μm, 125 mm × 4 mm I.D., mobile phases acetonitrile–water, 40°C.

Because $\log k$ are directly proportional to the product $m\varphi$ [in reversed-phase systems, Eq. (2)], or to the product $m\log\varphi$ [in normal-phase systems, Eq. (4)], a small change in the concentration of the strong solvent B, φ, causes a much more significant change in retention, k, of large molecules than in the retention of small molecules, and often increasing the concentration of B by even a few tenths of % may cause transition from "full retention" to "full elution". Consequently, only a narrow composition range of the mobile phase is available for the elution of large molecules. For example, from the data in Table 3, it can be predicted that a polystyrene sample with molecular mass 10,000 (with approximately 100 repeat units) has $k = 2$ on a C18 column in 86.9% dioxane in water (best elution conditions), but $k = 300$ in 85% dioxane (very strong retention) and $k = 0.3$ (very low retention) in 88% dioxane. This means that such macromolecular samples are either fully retained or fully non-retained almost over the whole composition range of the mobile phases, except for a very narrow composition interval, which is often difficult to find out and employ reproducibly for isocratic polymer separations. The elution range is even more limited for higher molar mass samples. Consequently, the application of gradient elution is a pre-requisite in order to utilize the narrow mobile phase "composition window" available for elution of the individual species in the polymer samples.[40]

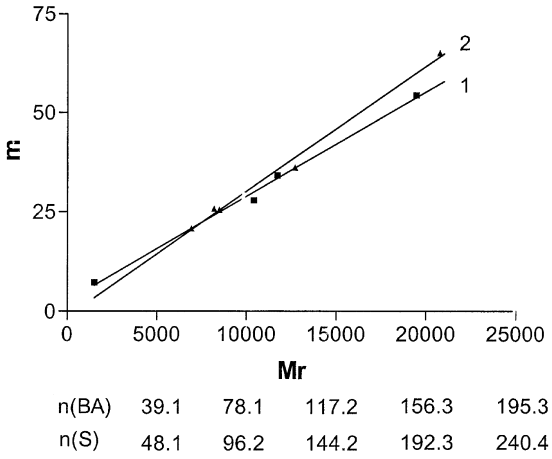

Figure 9. Dependencies of the constants a and m of Eq. (2) on the molecular weight, Mr, corresponding to the number of carbon atoms, n, of polybutylacrylates (1) and polystyrenes (2) on a Symmetry C18, 5 μm (100 Å), 150 mm × 3.9 mm I.D., column in tetrahydrofurane–water mobile phases, 20°C.

Further, the gradient profile should be adjusted with great care, and relatively shallow gradients should be preferred to obtain good resolution of macromolecular compounds. It should be noted, that the width of the mobile phase "composition window" depends on the value of the coefficient m_1, which is a measure of the effect of the solvent, B, on the repeat unit selectivity. This means that correct selection of the type of the solvent, B, is essential for successful separation.

Similar rules apply, also, in normal-phase chromatography on polar adsorbents. Relatively steep gradients with a wide concentration range can be used for separations of low-molecular oligomers, such as linear gradient from 0 to 90% propan-2-ol in heptane for the separation of oligoethyleneglycol nonylphenyl ethers with 0–25 oxyethylene units ($M_r = 220$–1320), shown in Fig. 10.[47] On the other hand, shallow gradients are necessary for normal-phase separations of polymers, such as for polystyrenes with $M_r = 35{,}000$–$470{,}000$ on two silica gel columns in series using a gradient from 47% to 50% dioxane in hexane in 15 min, shown in Fig. 11. Here, the elution volumes of narrow-distribution polystyrene standards with molecular masses 110,000 and 470,000 differ by only 7 mL, which precludes the separation of the individual high-molecular polymer species according to the number of monomer units. The polystyrenes with $M_r < 20{,}000$ are not retained under these conditions and elute from the column at the size-exclusion volume. In this

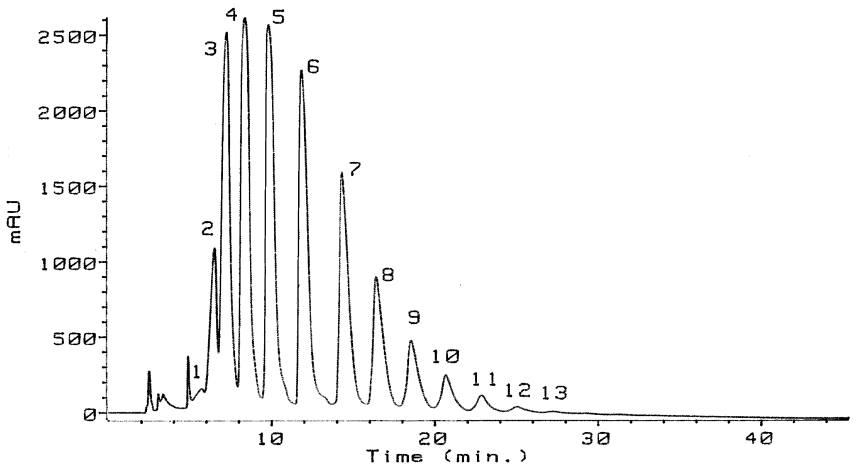

Figure 10. Normal-phase gradient elution separation of an oligoethylene glycol nonylphenyl ether sample (Serdox NNP 4) with 1–13 oxyethylene units. Column: Separon SGX Amine (5 μm, 200 × 4 mm I.D.). Linear gradient 0–45% propan-2-ol in heptane in 30 min, 1 mL/min, UV detection, 230 nm.

chromatographic system, possible size-exclusion effects play only minor roles in the separation.

The considerations based on the validity of the Eqs. (23a) and (23b) lead—in agreement with numerous experimental results—to the conclusion that there is a certain upper molar mass, depending on the polymer or oligomer type and on the chromatographic system used, which sets practical limits to the possibilities of separation of the individual high-molecular polymer species using interactive chromatography in reversed-phase or in normal-phase LC systems. This can be illustrated using the example of reversed-phase separation of polystyrene samples in dioxane–water mobile phase using the data in Table 3. The polymer with 1000 units (with molar mass approximately 100,000) has $k = 1.890$ in 87.45% dioxane, but the next polymer with 1001 units has $k = 1.892$, so that the repeat unit selectivity characterized by the separation factor α is only 1.001, and a column with 36 million theoretical plates would be required to separate, completely, these two species.[40]

Linear gradients often provide satisfactory separation of oligomers and lower polymers, but non-linear convex gradients can improve peak capacity and band spacing in the chromatograms of macromolecular samples and decrease the analysis time under both reversed-phase and normal-phase conditions.[48]

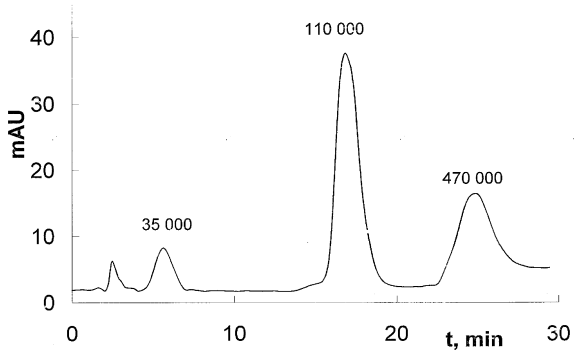

Figure 11. Normal-phase gradient elution separation of polystyrene standard samples with $M_r = 35{,}000$, 110,000 and 470,000, respectively. Two Nova-Pak silica gel columns in series, 4 μm (3.9 × 150 mm I.D. each), gradient 47–50% dioxane in hexane in 15 min, 1 mL/min, 40°C, UV detection at 254 nm.

CONCLUSION

The theory of gradient elution allows predicting the retention and optimizing the resolution both in reversed-phase and in normal-phase systems, using the parameters of the experimentally determined dependencies of sample retention on the mobile phase composition. The effect of changing gradient time and range can be rapidly estimated using simple calculation rules. However, for exact prediction of the retention, it is possible to use sophisticated calculations, taking into account the gradient dwell volume of the instrument and possible effects of the preferential adsorption of strong solvent in the column during the gradient run. To avoid the effect of the adsorption of polar solvents and water from the mobile phase in normal-phase chromatography, dried mobile phases should be used and the gradients should be preferably started at a non-zero concentration of the polar solvent.

To obtain desired results when the flow rate of the mobile phase, or when the column dimensions are changed, the gradient time should be appropriately adjusted. Further, transferring a gradient method to another column type with different ratio of mobile and stationary phase volumes (such as monolithic or superficially porous particle columns) requires re-adjusting the gradient range. To achieve optimum separation in minimum time, both the gradient slope and the initial concentration of the strong solvent at the start of gradient elution should be adjusted simultaneously. A spreadsheet window-diagram-like program can be used for this purpose with common PC table editor software.

The separation of polymeric compounds may be very sensitive to even minor changes in the mobile phase composition. Separation of many oligomers or lower polymers, according to the molar mass distribution with good resolution between the individual species differing in the numbers of repeat monomer units, can be achieved using broad range gradients in normal-phase or in reversed-phase systems. The suitability of a chromatographic system for the separation of oligomers or polymers is determined by the polarity of the repeat monomer units. However, depending on the type of the monomer and of the end groups, there is a certain molecular size that limits the possibility of separation of the species differing by a single one monomer unit. Some separation of higher polymers according to the molar mass distribution can be obtained in normal-phase or in reversed-phase systems, using shallow gradients over a narrow mobile phase composition range, as an alternative to size-exclusion chromatographic separations.

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