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# Optimisation of gradient elution in normal-phase high-performance liquid chromatography

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**Abstract**

An optimisation approach for linear gradient elution in normal-phase chromatography was suggested. The approach is based on predictive calculations of the retention and of the resolution of the individual pairs of compounds in the sample mixture. Isocratic or gradient-elution retention data, acquired in a few initial runs under different conditions, are employed to determine the parameters of retention equations describing the dependence of the retention factor, *k*, on the composition of the mobile phase. Unlike earlier procedures, a more complex, three-parameter retention equation can be used as the basis of predictive calculations, if necessary. The approach allows one to use either maximised minimum resolution in the sample mixture or the minimum time necessary to achieve the required resolution as the optimisation criterion. It accounts for the contribution of the initial isocratic elution step induced by the gradient dwell volume, so that it is not necessary to delay the injection with respect to the start of the gradient. Simultaneous optimisation of the gradient slope and of the gradient range is performed, with the gradient volume (time), plate number and other variables as adjustable parameters. The approach is illustrated by examples of gradient-elution separation of phenylurea herbicides on a silica gel and on a bonded nitrile column, with binary gradients of 2-propanol in *n*-heptane and of dioxane in *n*-heptane. Dried solvents and the temperature (controlled to $\pm 0.1^{\circ}C$) were used to improve the reproducibility of the retention data. © 1998 Elsevier Science B.V.

*Keywords:* Optimisation; Gradient elution; Pesticides; Phenylureas

## 1. Introduction

Chromatography in reversed-phase systems is the most popular mode used in the contemporary practice of liquid chromatography. However, columns packed with polar adsorbents often show better separation selectivities than alkylsilica columns for various positional isomers of moderately polar compounds [1,2] or for oligomers containing repeat polar groups [3].

During gradient-elution chromatography in normal-phase systems, the concentration of one or more polar solvent(s) in a non-polar solvent is increased. A disadvantage of this technique with respect to reversed-phase gradient elution is the possible preferential adsorption of the more polar solvent(s) on the surface of the polar adsorbent, which may lead to important deviations of the actual gradient profile from the pre-set mobile phase composition program.

Reproducibility of gradient-elution retention data in normal phase systems with mobile phases composed of two organic solvents, a polar and a non-polar one, depends on a number of experimental factors that should be controlled. To obtain reproducible results, it is necessary to keep a constant adsorbent activity [4]. It is very important to work at a constant temperature and water content in the mobile phase. This can be achieved with dehydrated solvents that are kept dry over activated molecular sieves and filtered just before the use. Furthermore, in predictive calculation of the gradient retention data in normal-phase gradient-elution chromatog-

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raphy, it is very important to account for the isocratic pre-elution before the start of the gradient, which is caused by the mobile phase of initial composition that is contained in the gradient dwell volume of the instrument [5].

In reversed-phase gradient-elution chromatography, the DryLab computer simulation approach is probably the most widespread approach for optimisation of the operation parameters [6,7]. Here, the retention data from two initial gradient runs are used to adjust the steepness and the range of the gradient and, if necessary, other working parameters. Simplex optimisation may also be used for this purpose [8]. An overlapping resolution mapping scheme has been used for optimisation of iso-selective multi-solvent gradients [9].

We have developed a scheme for the simultaneous optimisation of the gradient steepness and initial composition at the start of gradient elution in reversed-phase gradient-elution chromatography [10]. As this scheme is generally applicable in various chromatographic systems, we have recently adapted this approach to normal-phase chromatography. In this work, these results are presented and illustrated by examples of normal-phase separation of some phenylurea herbicides.

## 2. Theoretical

### *2.1. Description of retention in normal-phase systems*

The retention in normal-phase systems, as a function of the composition of two-component (binary) mobile phases, can be described using theoretical models of adsorption. The first model of retention in adsorption chromatography was developed by Snyder in the 1960s [4,11,12]. Adsorption was understood to be a competition 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 [13,14]. Soczewinski [15] and Soczewinski and Golkiewicz [16] suggested a similar model of retention, assuming that adsorption took place in a monomolecular layer on a heterogeneous surface of adsorbent and that there was cancellation of the solute–solvent interactions in the mobile and stationary phases. With some simplification, both models lead to an identical equation describing the retention factor, $k$, as a function of the concentration of the stronger (more polar) solvent, $\varphi$, in binary mobile phases comprising two solvents of different polarities [11,16,17].

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

$k_0$ and $m$ are experimental constants, $k_0$ being the capacity factor in pure strong solvent. This equation has become known as the Snyder–Soczewinski model equation [12].

Based on the original Snyder concept of adsorption as a competitive phenomenon, but with less simplification than in the derivation of Eq. (1), another retention equation was derived [18,19].

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

Here again, $a$, $b$ and $m$ are experimental constants that depend on the solute and on the chromatographic system, ($a = 1/(k_a)^m$, where $k_a$ is the retention factor in a 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) [17].

A theoretical description of linear binary gradient elution in normal-phase systems was presented by Jandera and Churáček [19–21]. In these gradients, the concentration of a polar solvent, $\varphi$, increases as the volume of eluate, $V$, increases:

$$\varphi = A + BV \tag{3}$$

Here, $A$ is the initial concentration of the strongly polar organic solvent in the mobile phase and $B$ is the slope (steepness) of the gradient in concentration units per ml of the eluate. If the retention in a normal-phase system can be described by the two-parameter retention equation, Eq. (1), the retention volume, $V_R$, of a sample compound in gradient-elution chromatography can be calculated as [20]:

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

On the other hand, if the three-parameter retention equation (Eq. (2)) is necessary to describe adequately the retention in a given normal-phase system, a slightly more complex equation should be used to

calculate the retention volumes in gradient elution [5]:

$$V_R = \frac{1}{bB}[bB(m+1)V_0 + (a+Ab)^{(m+1)}]^{\frac{1}{m+1}} - \frac{a+Ab}{bB} + V_0 \quad (5)$$

The single-step gradient equations, Eqs. (4) and (5), can be used if the volume between the gradient former and the column (the so-called ''gradient dwell volume''), $V_D$ is so low that it can be neglected or if the injection is delayed with respect to the start of the gradient, to compensate for the dwell volume. Unfortunately, this is often not the case and, with some instruments, the gradient dwell volume can be quite significant (even a few ml). At the start of the gradient, this volume of the instrument is filled with the mobile phase corresponding to the initial gradient conditions and, consequently, the ''dwell volume'' of the mobile phase should flow through the column before the start of the gradient profile arrives at the top of the column. Hence, the expected gradient elution is delayed and some sample solutes, especially weakly retained ones, migrate a certain distance along the column during this unintended initial isocratic step, which contributes to the elution volume. On the other hand, the part of the column length available for the gradient elution is shorter than expected. The gradient dwell volume may differ from one instrument to another and may cause difficulties if an HPLC method developed with one gradient instrument is being transferred to another one. To avoid these problems and to make precise prediction of the gradient elution data by calculation possible, the gradient dwell volume should be accounted for in method development and the instrumental gradient delay should be corrected for in calculations, as follows [5]:

If the gradient dwell volume, $V_D$, cannot be neglected and the elution occurs in two steps: First, isocratic and the second, gradient, the situation is equivalent to elution with two columns in series, where the first is eluted in the isocratic mode by the dwell volume of the mobile phase containing the strong solvent, of concentration $A$ (the starting concentration in gradient elution), and the second is eluted in the gradient mode. The contribution of the first part (column) to the total retention volume of the solute is equal to $V_D$. The part of the column through which the solute has migrated at the end of the first step, i.e., at the time when it is taken by the front of the gradient, has a dead (hold-up) volume, $V_{01}$ corresponding to the proportional part of the total column dead volume, $V_0{:}V_{01}/V_0 = V_D/[V_0(1+k_1)]$, where $k_1$ is the retention factor in the mobile phase of initial composition. Then, $V_{01} = V_D/(1+k_1)$ and the second part (column), which remains available for the gradient elution step has a dead volume $V_{02} = V_0 - V_D/(1+k_1)$. The final retention volume comprises: (1) The contribution of the gradient step to the net retention volume, $V'_{R2}$, which can be calculated from Eq. (4) or Eq. (5) after subtracting $V_0$ and using $V_{02}$ instead of $V_0$ and (2) the contribution of the gradient dwell volume, $V'_{R1} = V_D - V_{01} = V_D/[1+(k_1)^{-1}]$:

$$V_R = V'_{R1} + V'_{R2} + V_0 = V_D - V_{01} + V'_{R2} + V_0 = \frac{V_D}{1+\frac{1}{k_1}} + V'_{R2} + V_0 \quad (6)$$

### 2.2. Predictive optimisation of binary gradient-elution chromatography

Predictive optimisation of gradient elution is based on the calculations of elution volumes of sample solutes and is used to optimise simultaneously two parameters of the gradient, the steepness, $B$, and the initial concentration of the polar solvent at the start of the gradient, $A$ (Eq. (3)).

Appropriate selection of the concentration of the strong eluting component in the mobile phase at the start of the gradient, $A$, is not only important because of possible effects on the reproducibility and precision of the prediction of the elution data, but its influence on the resolution and on the time of analysis is equally as important as that of the gradient steepness, $B$. Furthermore, the effect of the preferential adsorption of polar organic solvents on the retention behaviour is suppressed if gradients are started at 3% or more of the organic solvent [5]. Therefore, we suggest the following strategy, where the gradient parameters $A$ and $B$ are optimised simultaneously [5]:

With a pre-set final concentration of the polar

solvent, $\varphi_G$, that should be achieved at $V=V_G$, the slope $B$ of the gradient is a function of the initial concentration $A$:

$$B = \frac{(\varphi_G - A)}{V_G} \quad (7)$$

The setting of $V_G$ does not significantly affect the results, if $V_G$ is large enough. Then, the elution volume, $V_R$, can be calculated as a function of a single parameter $A$, e.g., for systems described by Eq. (1):

$$V_R = \frac{V_G}{\varphi_G - A}\left[\frac{(m+1)(\varphi_G - A)k_0 V_0}{V_G} + A^{(m+1)}\right]^{\frac{1}{m+1}} - \frac{AV_G}{\varphi_G - A} + V_0 \quad (8)$$

The differences between the $V_R$ of compounds with adjacent peaks or the resolution, $R_s=[V_R(2)-V_R(1)/w_g$, can be plotted versus $A$ in the form of a ''window diagram'', to select the optimum value of $A$ ($w_g$ is the bandwidth in gradient-elution chromatography, calculated for isocratic conditions at the composition of the mobile phase at the time of elution of the band maximum using the isocratic number of theoretical plates of the column). As the pre-set value of $V_G$ limits the analysis time, two different strategies may be used to achieve optimum separation.

(1) The ''window diagram'' may be used to search for the largest value of $A$ at which the desired resolution (e.g., $R_S=1.5$) is achieved for all compounds in the sample mixture. This approach, in most cases, automatically minimises the time of the analysis, as the retention volumes decrease with increasing $A$.

(2) With the ''maximised minimum resolution'' optimisation criterion, the value of $A$ that yields the maximum value of $R_S$ for the ''critical'' pair of compounds showing the worst (minimum) resolution in the sample mixture is determined. This maximised $R_S$ is often lower than the desired resolution and, in such a case, the pre-set gradient volume or the column plate number can be increased to further improve resolution in the mixture. A similar approach can be used to diminish the ''maximised minimum resolution'' if it is excessively large and to decrease the analysis time.

In both optimisation approaches, with the optimum value of $A$ having been established, $B$ can be calculated for the pre-set gradient volume, $V_G$, and the final concentration, $\varphi_G$, from Eq. (7).

In addition to the gradient volume and to the column plate number, the gradient shape can be adjusted. A gradient curvature parameter can be used in the optimisation calculations [19–21]. It is also possible to adapt the calculation procedure to optimise linear segmented gradients, if necessary. The optimised conditions can be transferred between various instruments and columns. The gradient dwell volume, the column dead volume and the plate number should be known and the product, $V_0 \cdot B$ should be kept constant.

The optimisation can be performed using a spreadsheet program.

INPUT:

1. The parameters of the retention equation for each sample solute determined from at least two–three isocratic or gradient experimental runs.
2. The column plate number, $N$, and dead volume, $V_0$, the gradient dwell volume, $V_D$, the gradient volume, $V_G$, or the gradient time, $t_G$, and the flow-rate, $F_m$ (and the gradient curvature parameter, if necessary).

OUTPUT:

1. Diagrams of the retention volumes, $V_R$, and of the resolution, $R_S$, for all sample components as a function of the concentration of solvent $b$ in the mobile phase (isocratic), to check if gradient elution is necessary.
2. Diagrams of the retention volumes and of the resolution for all sample components as a function of the concentration of solvent $b$ at the start of the gradient, $A$, with $V_G$, $N$, $V_0$ ($F_m$, $V_D$) as adjustable parameters.
3. Optimum $A$ is determined from the diagram $R_S - A$ for maximised minimum resolution of solutes or for a required resolution and minimum time (volume) of separation. The concentration at the end of the gradient, $c_g$, is determined from the diagram $V_R - A$ (from the $V_R$ of the last compound).
4. With optimised conditions, chromatogram can be calculated and plotted, if required.

5. If the optimised separation is not satisfactory, $V_G$ or other parameters ($N$, $V_0$) can be varied to find the optimum chromatographic conditions. $V_G$ should be varied also if the calculated retention volumes of more strongly retained compounds indicate that they elute after the end of the gradient ($V_R > V_G + V_0 + V_D$),
6. If further refinement of the separation is required, the use of curved or segmented gradients can be attempted.

## 3. Experimental

An HP 1090M liquid chromatograph equipped with a UV diode-array detector, operated at 230 nm, an automatic sample injector, a 3DR solvent delivery system, a 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 (150×3.3 mm I.D.), packed with silica gel Separon SGX, 7.5 μm ($V_0$ = 0.905 ml), and Separon SGX Nitrile, 7.5 μm ($V_0$ = 0.95 ml), were obtained from Tessek (Prague, Czech Republic). The flow-rate of the mobile phases was kept at 1 ml/min and the temperature was maintained at 40°C in all experiments.

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

The columns were first equilibrated with the mobile phase and then the retention volumes, $V_R$, of the sample compounds were measured under isocratic conditions in mobile phases with different

Table 1
Parameters *a*, *b* and *m* of Eq. (2) for the phenylurea herbicides in the test sample mixtures

| Sample solute | Mobile phase | | | | | |
|---|---|---|---|---|---|---|
| | 2-Propanol–*n*-heptane | | | Dioxane–*n*-heptane | | |
| | $a \cdot 10^3$ | *b* | *m* | $a \cdot 10^3$ | *b* | *m* |
| *Separon SGX silica gel column* | | | | | | |
| Neburon | 0 | 5.793 | 1.296 | 97.08 | 3.027 | 2.239 |
| Chlorobromuron | 0.64 | 2.870 | 0.747 | 156.7 | 2.674 | 2.039 |
| 3-Chloro-4-methylphenylmethylurea | 10.68 | 3.089 | 1.749 | 31.81 | 1.364 | 2.596 |
| Desphenuron | 14.82 | 2.173 | 1.466 | 120.6 | 1.130 | 3.144 |
| Isoproturon | 15.17 | 2.726 | 1.777 | 45.19 | 1.678 | 2.228 |
| Diuron | 214.3 | 2.222 | 2.943 | 60.68 | 1.615 | 2.367 |
| Metoxuron | 48.46 | 1.720 | 1.979 | 148.3 | 1.26 | 3.244 |
| Deschlorometoxuron | 33.23 | 1.583 | 1.838 | 69.12 | 1.165 | 2.552 |
| *Separon SGX Nitrile bonded column* | | | | | | |
| 3-Chloro-4-methylphenylmethylurea | 14.51 | 8.086 | 1.162 | 177.6 | 3.381 | 3.024 |
| Linuron | 156.3 | 4.371 | 0.969 | 183.5 | 3.531 | 1.479 |
| Neburon | 80.96 | 5.235 | 1.280 | 80.56 | 2.925 | 1.776 |
| Fluometuron | 35.24 | 3.884 | 1.352 | 113.3 | 2.072 | 2.146 |
| Diuron | 42.64 | 3.688 | 1.431 | 84.34 | 1.969 | 2.063 |
| Chlortoluron | 59.95 | 3.445 | 1.497 | 87.69 | 1.748 | 2.064 |
| Desphenuron | 3.37 | 3.435 | 1.461 | 103.5 | 1.302 | 2.846 |
| Phenuron | 69.18 | 2.577 | 1.514 | 127.3 | 1.411 | 2.439 |

concentrations of 2-propanol or of dioxane in *n*-heptane. The parameters of the retention equations, Eqs. (1) and (2), were determined from the isocratic retention factors, $k=(V_R/V_0-1)$, as described previously [22]. In gradient-elution experiments, a 5-min reversed gradient and a 5-min equilibration time were used after the end of each experiment to re-equilibrate the column. The column dead (hold-up) volume, $V_0$, was determined using trichloroethylene as the marker.

All optimisation calculations and modelling of chromatograms were performed in the form of a spreadsheet using the Quattro Pro 5.0 table editor.

## 4. Results and discussion

The optimisation approach is illustrated by the example of separation of a mixture of eight phenylurea herbicides on a silica gel column using elution with binary gradients of 2-propanol or of dioxane in *n*-heptane. The parameters of Eq. (2), found by non-linear regression of the experimental retention data as a function of the concentration of either 2-propanol or dioxane in *n*-heptane, are listed in Table 1. These parameters were used in predictive optimisation calculations using Eqs. (5) and (6), with *B* expressed from Eq. (7).

### *4.1. Optimisation of gradient elution with binary gradients of 2-propanol in n-heptane*

First, the isocratic elution of the sample mixture was optimised using a diagram of the calculated resolution of the adjacent pairs of peaks as a function of the concentration of 2-propanol in the mobile phase. The diagram predicts that optimum resolution with the shortest retention time will be achieved in a mobile phase containing 19% 2-propanol in *n*-heptane. With this mobile phase, the last compound is eluted in approximately 8 min (Fig. 1).

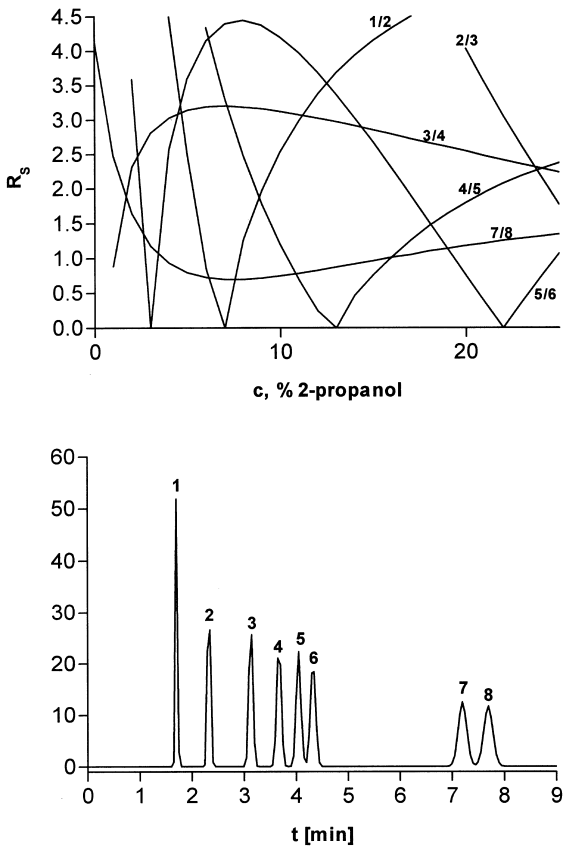


Fig. 1. Resolution diagram for the isocratic separation of eight herbicides (Table 1) on a silica gel column as a function of the concentration of 2-propanol in *n*-heptane as the mobile phase and the separation under optimised conditions with 19% 2-propanol. Column plate number, $N=5000$.

Fig. 2 shows the overlapping resolution map for gradient elution of this sample mixture using linear gradients of 2-propanol in *n*-hexane, with the gradient volume, $V_G$, and the initial concentration of 2-propanol, *A*, as the *x*-axis and *y*-axis coordinates, respectively. The contour plots in these coordinates correspond to the resolution $R_S=1$ for the individual pairs of compounds and the regions where this or higher resolution is predicted for all sample compounds are in the area below the curve for the pair 4–5 and above the curve for the pair 5–6 in the left upper corner and is limited by the curves for pairs 5–4, 8–7 and 2–1 in the rest of the diagram. It should be noted that reversal in the elution order from 6–5–4 to 4–5–6 is observed between the gradient volumes of 9 and 14 ml.

From the diagram in Fig. 2, detailed information about the analysis time or about the actual resolution of the individual pairs of compounds cannot be directly obtained and determination of the optimum conditions of separation is difficult. For this purpose, simultaneous optimisation of the gradient steepness

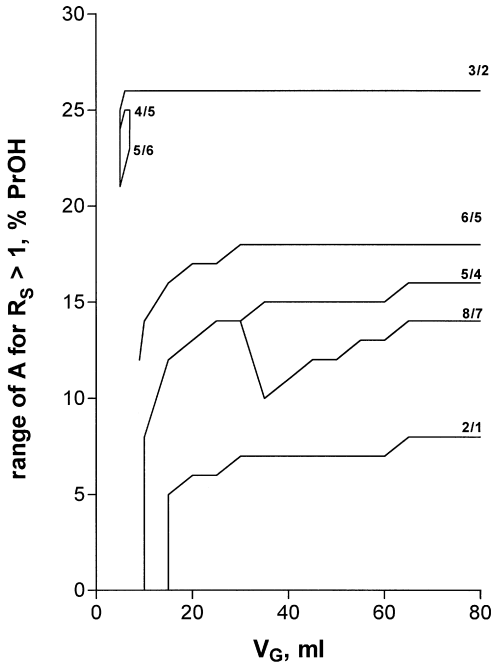


Fig. 2. Contour plots for $R_S = 1$ for the adjacent peaks of eight herbicides (Table 1) in gradient elution with gradients of 2-propanol in *n*-heptane on a silica gel column. *A* is the initial concentration of 2-propanol at the start of the gradient and $V_G$ is the gradient volume (Eq. 7) from *A* to $c_G = 0.5$ (%, v/v·$10^{-2}$). Minimum resolution $R_S \geq 1$ in the sample mixture is obtained in the range limited by the curves 4/5 and 5/6 (low $V_G$) and by the curves 8/7 and 2/1 (higher $V_G$).

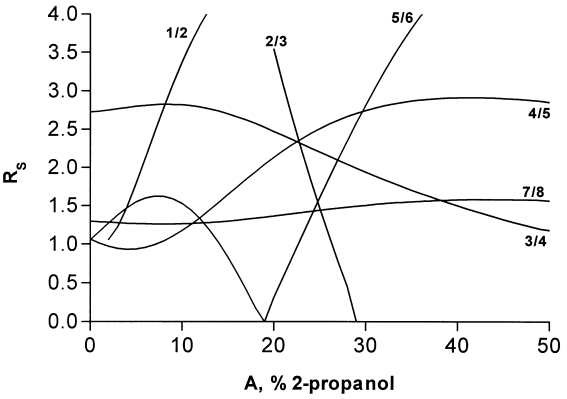


Fig. 3. Resolution diagram for the gradient-elution separation of eight herbicides (Table 1) on a silica gel column as a function of the initial concentration of 2-propanol in *n*-heptane at the start of the gradient, *A*, with optimum gradient volume $V_G = 10$ ml. Column plate number, $N = 5000$.

and of the gradient range at a given gradient volume (Eq. (7)) is more convenient.

A few pre-set values of $V_G$ were tested and $V_G = 10$ ml was found to be the best choice. With this value, the diagram of resolution for the individual pairs of compounds as a function of the initial concentration of 2-propanol at the start of the gradient, *A*, revealed two maxima of minimum $R_S$ at 12 and 25% 2-propanol (Fig. 3). Fig. 4 shows the chromatograms obtained for the two optimised conditions. Chromatogram A does not represent significant improve-

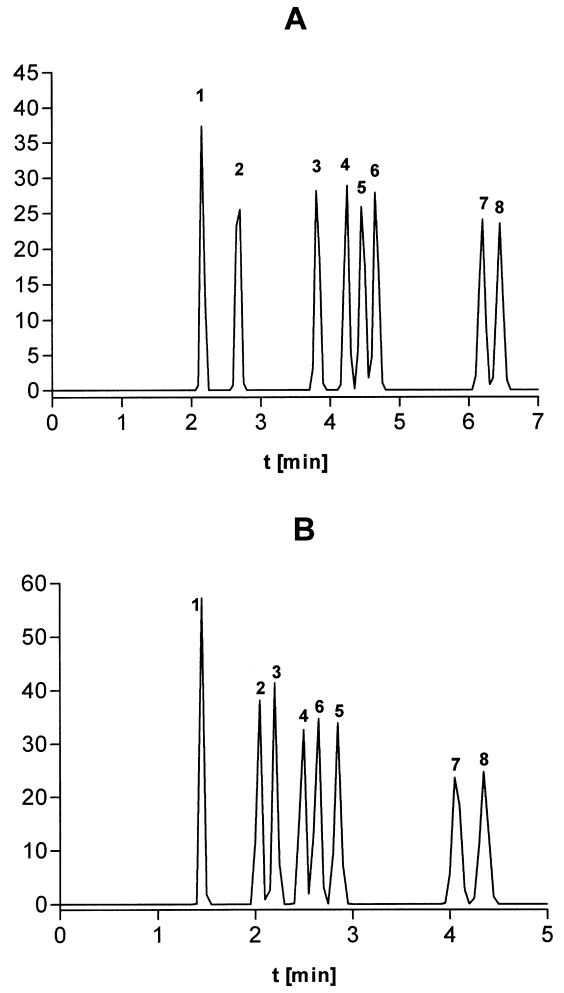


Fig. 4. Separation of eight herbicides (Table 1) under optimised gradient-elution conditions (maxima of the minimum resolution in Fig. 3) with gradients from 12 to 38.6% 2-propanol in *n*-heptane in 7 min (A) and from 25 to 37.5% 2-propanol in *n*-heptane in 5 min (B). Column plate number, $N = 5000$; flow-rate, 1 ml/min.

ment with respect to the isocratic separation in Fig. 1. With the gradient starting at 25% 2-propanol, the order of elution of compounds 5 and 6 is changed and the time of separation is reduced from 8 to 4.5 min (at a flow-rate of 1 ml/min). From a practical point of view, even this gain in analysis time probably will not be worth using gradient elution, taking into account the time necessary for re-equilibration of the column after the end of the gradient (5–10 min).

Fig. 5 illustrates the effect of the pre-set gradient volume on the optimum value of the initial concentration of 2-propanol and on the elution volume of the last eluted compound (8) using two different optimisation criteria: (1) Minimum analysis time necessary to achieve $R_S \geq 1$ and (2) maximized minimum resolution for the ''critical'' pair of compounds. Selection of the optimisation criterion did not significantly affect the results and, for gradient volumes in the range 15–60 ml, the elution volume

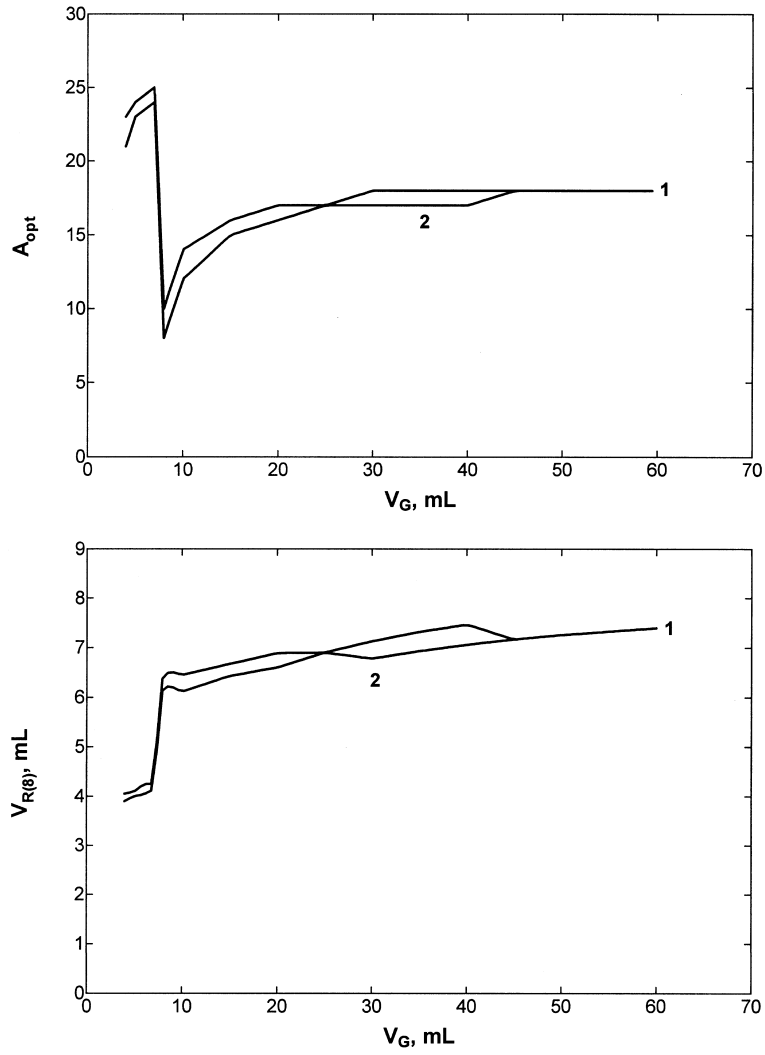


Fig. 5. Plots of optimised initial concentration of 2-propanol at the start of the gradient ($A_{opt}$, %, v/v) and of elution volume ($V_{R(8)}$) of the last eluted herbicide (deschlorometoxuron; Table 1) as a function of the pre-set gradient volume, $V_G$. Conditions were optimised to give the minimum analysis time with $R_S \geq 1$ (1) and for maximized minimum resolution in the sample mixture (2).

of the last eluted compound was within the limits 6.5 to 7.5 ml, which is not a very significant difference. (It should be noted that $V_G$ does not mean the real volume of the mobile phase from the start to the end of the gradient, as the elution can be stopped after the elution of the last eluted compound).

### *4.2. Optimisation of gradient elution with binary gradients of dioxane in n-heptane*

A similar approach to that used for optimisation of gradient elution with 2-propanol was applied to the separation of eight herbicides on a silica gel column with a linear gradient of dioxane in *n*-heptane. The window diagram of the resolution of the individual pairs of compounds under isocratic conditions (Fig. 6) shows that the shortest analysis time with a minimum $R_S > 1$ is achieved in a mobile phase containing 13% dioxane. Under these conditions, the separation takes approximately 60 min. The long time required is dictated by the necessity of achieving adequate resolution of four pairs of compounds with similar retention times (Fig. 6).

With the pre-set gradient volume of 30 ml, the diagram showing the dependence of the resolution of the individual pairs of compounds on the initial concentration of dioxane at the start of the gradient predicts that the minimum time necessary to achieve a resolution of $R_S \geq 1$ for all sample compounds

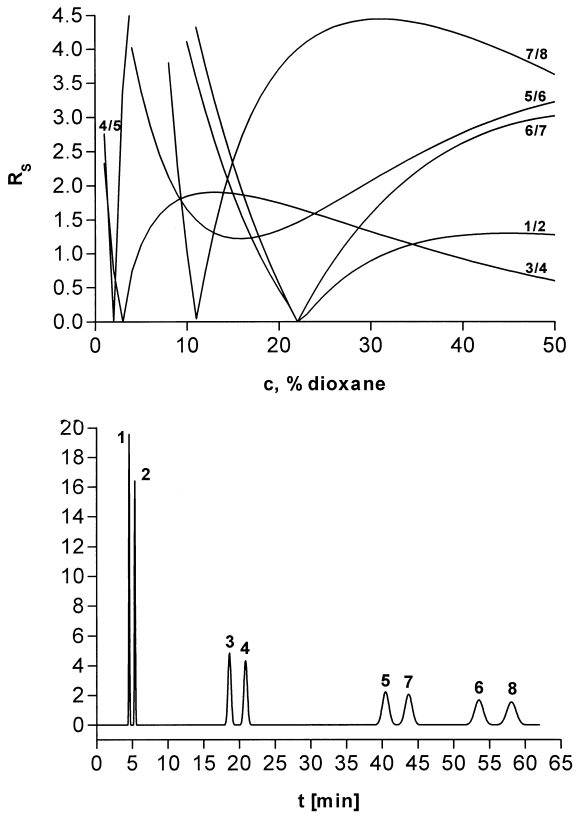


Fig. 6. Resolution diagram for the isocratic separation of eight herbicides (Table 1) on a silica gel column as a function of the concentration of dioxane in *n*-heptane as the mobile phase and the separation under optimised conditions with 13% 2-propanol. Column plate number, $N = 5000$.

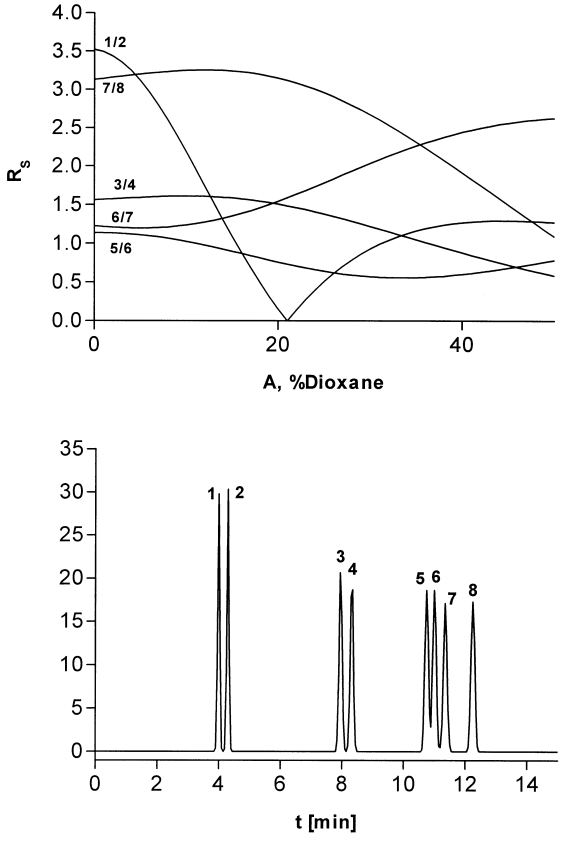


Fig. 7. Resolution diagram for the gradient-elution separation of eight herbicides (Table 1) on a silica gel column as a function of the initial concentration of dioxane in *n*-heptane at the start of the gradient, *A*, with the optimum gradient volume $V_G = 30$ ml and the separation of eight herbicides (Table 1) under optimised gradient-elution conditions (maximum of the minimum resolution) with a gradient from 11 to 41.5% dioxane in *n*-heptane in 14 min. Column plate number, $N = 5000$; flow-rate, 1 ml/min.

would be obtained with a gradient starting at 11% dioxane in *n*-heptane. The elution order with optimised gradient-elution separation (Fig. 7) is the same as in optimised isocratic elution, except for the pair of compounds 6 and 7, (Fig. 6), but the analysis time is decreased from 60 to 12.5 min (at a flow-rate of 1 ml/min).

Fig. 8 shows the effect of the pre-set gradient volume on the optimum value of the initial concentration of dioxane and on the elution volume of the last eluted compound (8) using the two different optimisation criteria described earlier. With the optimisation approach, determining conditions for the minimum analysis time necessary to achieve the required resolution ($R_S \geq 1$), the initial concentration of dioxane and the elution time of the last compound

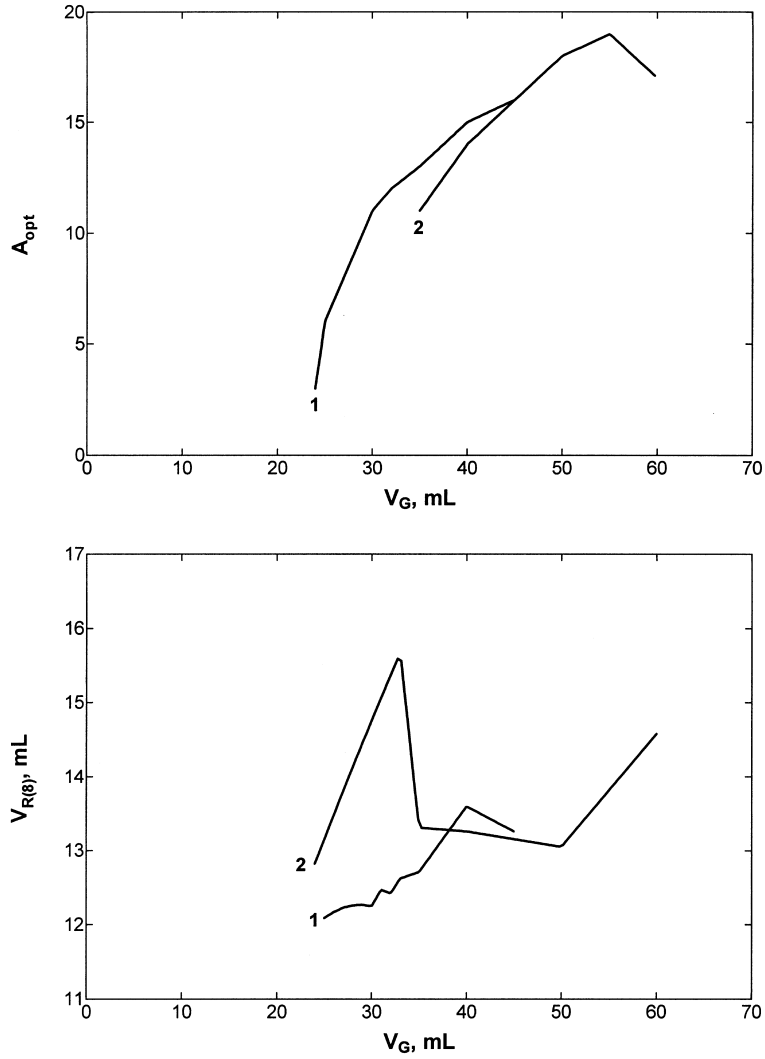


Fig. 8. Plots of the optimised initial concentration of dioxane at the start of the gradient ($A_{opt}$, % v/v) and of elution volume ($V_{R(8)}$) of the last eluted herbicide (deschlorometoxuron, Table 1) as a function of the pre-set gradient volume, $V_G$. Conditions were optimised to give the minimum analysis time with $R_S \geq 1$ (1) and for maximized minimum resolution in the sample mixture (2).

are higher than with maximised minimum resolution for the ''critical'' pair of compounds for gradients starting at 24–35% dioxane, but are practically the same at higher gradient volumes. Here again, selection of the gradient volume does not significantly affect the elution volume of the last eluted compound, which is within the limits of 12 to 14.5 ml, with the first optimisation criterion (curve 1 on Fig. 8).

### *4.3. Optimisation of gradient elution on the nitrile column with binary gradients of 2-propanol and of dioxane in n-heptane*

Figs. 9 and 10 show the results of the optimisation of linear gradients of 2-propanol and of dioxane in *n*-heptane if a Silasorb nitrile column is used instead of the silica gel column. The optimisation approach predicted that 7% 2-propanol and 31% dioxane would be the optimum initial con-

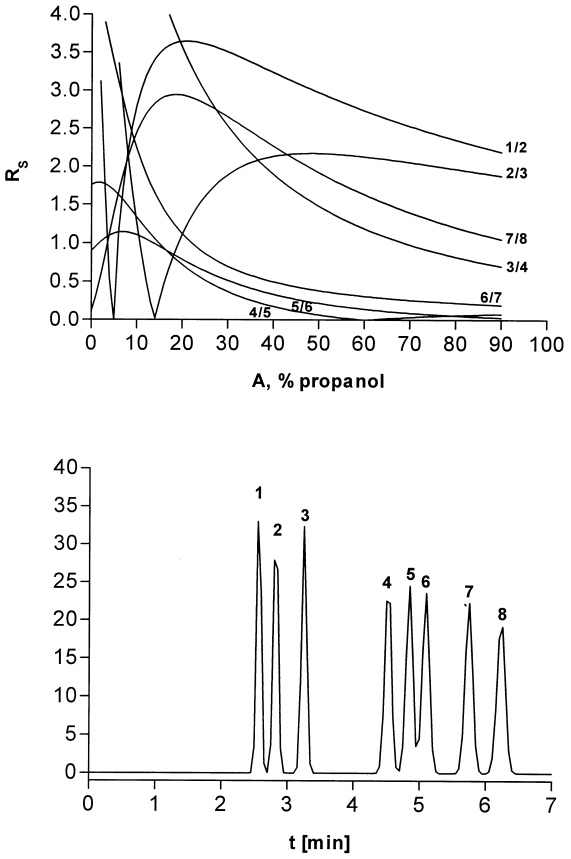


Fig. 9. Resolution diagram for the gradient-elution separation of eight herbicides (Table 1) on a bonded nitrile column as a function of the initial concentration of 2-propanol in *n*-heptane at the start of the gradient, *A*, with optimum gradient volume $V_G = 75$ ml and the separation of eight herbicides (Table 1) under optimised gradient-elution conditions (maximum of the minimum resolution) with a gradient from 7 to 15.8% 2-propanol in *n*-heptane in 7 min. Column plate number, $N = 5000$; flow-rate, 1 ml/min.

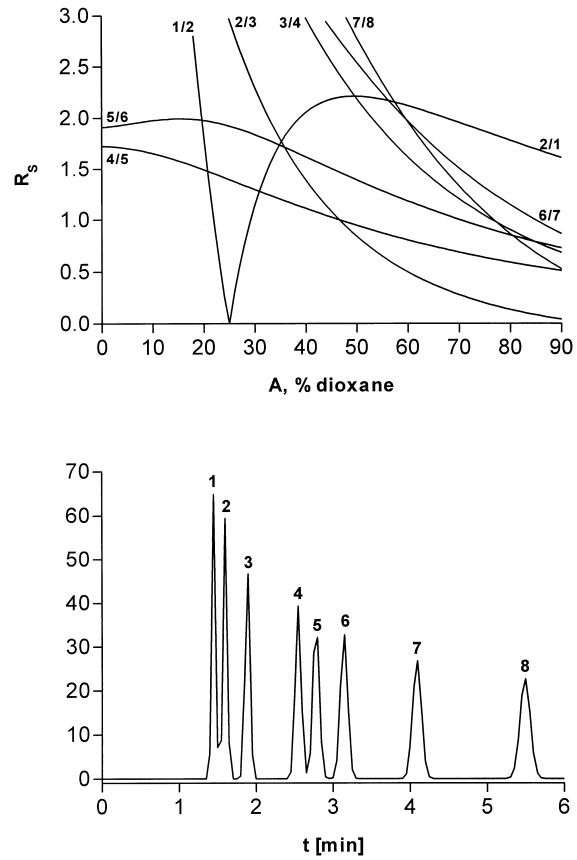


Fig. 10. Resolution diagram for the gradient-elution separation of eight herbicides (Table 1) on a bonded nitrile column as a function of the initial concentration of dioxane in *n*-heptane at the start of the gradient, *A*, with optimum gradient volume $V_G = 25$ ml and the separation of eight herbicides (Table 1) under optimised gradient-elution conditions (maximum of the minimum resolution) with a gradient from 31 to 47.5% dioxane in *n*-heptane in 6 min. Column plate number, $N = 5000$; flow-rate, 1 ml/min.

centrations at the start of the gradient for obtaining $R_S \geq 1$ in the sample mixture with the shortest analysis time. Here, the elution order and the time of analysis are similar with the two binary linear gradients and the main difference is in the critical pair of compounds in the sample mixture, i.e., 5–6 in 2-propanol–*n*-heptane and 1–2 in dioxane–*n*-heptane.

## 5. Conclusions

Precise predictive calculations of elution data are possible in HPLC gradient elution chromatography in normal-phase systems using an instrument with precise metering of the individual solvents, controlled constant temperature and dry solvents, if the gradient dwell volume is taken into account in the calculations for two-step elution with the initial isocratic step. The three-parameter retention equation, Eq. (2), improves the precision of prediction. Simultaneous optimisation of the gradient range and steepness is possible with a pre-set gradient time (volume). Selection of the gradient volume does not significantly affect the results of the optimisation procedure.

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