Journal of Chromatography, 552 (1991) 113–135
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2211

Development of a rational optimisation procedure for the automated sample clean-up with column switching in pesticide residue analysis

E. A. HOGENDOORN*, R. HOOGERBRUGGE, C. E. GOEWIE[a] and P. VAN ZOONEN
National Institute of Public Health and Environmental Protection (RIVM), P.O. Box 1, 3720 BA Bilthoven (The Netherlands)
and
P. J. SCHOENMAKERS
Philips Research Laboratories, P.O. Box 80 000, 5600 JA Eindhoven (The Netherlands)

ABSTRACT

Column-switching procedures usually involve at least one step gradient elution over the first column. In order to find optimal conditions for column switching in a rational way a computer program has been developed which calculates retention times and peak volumes for elution under step gradient conditions. Based on experimentally determined relations for retention *versus* mobile phase composition, the program calculates iteratively the displacement of analytes and sample interferences on the first column. Corrections for the distortion of solvent front and peak position of the solutes during step-gradient elution are included. The prediction of the peak volume is based on a diffusion model. The performance of the simulation procedure is evaluated with the application of a practical residue analytical method, the simultaneous determination of procymidone and iprodione in fennel. In this case, the program was shown to accurately predict retention times and peak volumes of a major fennel interference and the compounds of interest eluted under stepwise gradient conditions. Optimal conditions for reversed-phase liquid chromatography column switching are more rapidly found by the present procedure in comparison to trial and error optimisation.

INTRODUCTION

For several years, reversed-phase liquid chromatography (RPLC) with column switching has been applied routinely for the automated clean-up of crop extracts for pesticide residue analysis [1–3]. Besides automation, the most important advantages of this approach are improvement of the sensitivity by introduction of larger sample volumes under controlled band-broadening conditions and improvement of the

[a] Present address: DHV Raadgevend Ingenieursbureau B.V., P.O. Box 85, 3800 AB Amersfoort, The Netherlands.

0021-9673/91/$03.50

selectivity by transferring accurate volumes containing only the analytes of interest to the second column.

The principal events occurring in column switching are schematically shown in Fig. 1. After the injection of an aliquot of uncleaned sample extract (event I) on the first column (C-1), a clean-up (event II) is performed with a certain volume of M-1, the mobile phase of C-1. During this event (II) the more polar sample interferences (S1), usually abundant in concentrated extracts, are removed largely from C-1. Clean-up takes place until the first analyte starts to elute from C-1. After this, in event III of Fig. 1, C-1 is switched temporarily on-line with the second separation column (C-2). With a certain volume of M-2 the analytes (A) are transferred from C-1 to C-2. Finally the analytes (A1 and A2) are separated on C-2 (event IV) and simultaneously (event V) C-1 will be washed with a strong eluent for the removal of the apolar sample interferences (S2) and reconditioned with a volume M-1 prior to the next injection.

Most crucial in the development of column-switching procedures is the choice of the eluotropic strength of the clean-up solvent (M-1). A low eluotropic strength allows the injection of a large volume of sample without significant band-broadening and provides a high potential for the removal of early eluting interferences. However, a higher eluotropic strength will speed up the clean-up process and, more important,

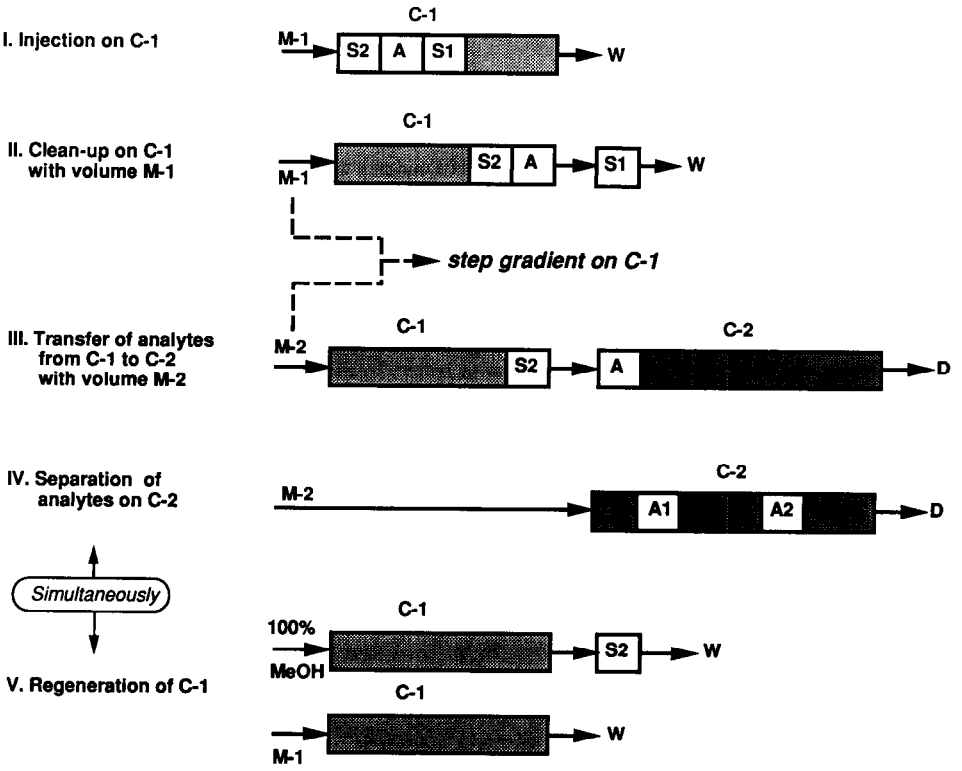


Fig. 1. Principal events in an on-line clean-up with RPLC column switching. C-1 = first C_{18} separation column; C-2 = second C_{18} separation column; M-1 = clean-up eluent of C-1; M-2 = mobile phase of C-2; S1 and S2 = sample interferences; A = analytes; W = waste; D = detection (for further explanation see Introduction).

will decrease the peak volumes of the analytes which is favourable for the transfer of small fractions to the second separation column (C-2). The separation of analytes will mainly take place on C-2 (see Fig. 1), which means that the mobile phase composition M-2 in a column-switching procedure is usually fixed in order to obtain a good separation.

So far, in our applications [1–3] a clean-up solvent (M-1), containing 5–20% of organic modifier, was found by "trial-and-error" to yield a sufficient clean-up performance between the analytes (A) and the early-eluting sample interferences (S1). However, similar to an off-line procedure [4], the residue determination of some fungicides in fennel [5] required more clean-up steps. After considerable trial-and-error effort an optimal clean-up was found using a three-step gradient elution on the first column (C-1). This application illustrates the complexity of finding appropriate on-line clean-up conditions to be used in RPLC column switching. In order to simplify this procedure we investigated the chromatographic processes involved in column switching. This type of elution can be regarded as a step-gradient elution, in which the mobile phase composition on C-1 is changed discontinuously in such a way that the eluotropic strength of each subsequent step increases.

Mathematical equations based on the observed retention volume (V_r) of a solute in an isocratic elution have been described [6–9] for the calculation of the optimal composition and volume of the mobile phases used in step-gradient elution LC. On the basis of a linear relationship between the isocratic capacity factor of a solute and the volume fraction of the stronger eluent component, general equations were derived predicting the retention for step-gradient elution in thin-layer chromatography (TLC) [10–12]. Using TLC as a pilot technique, a simple graphical model was developed for the optimisation of step-gradient elution in high-performance liquid chromatography (HPLC) [13,14]. This model was then further evaluated, providing equations which allow the search for an optimum gradient program by means of calculation [15] or by simulation [16] with a computer program.

This paper describes the development of a computer program which accurately predicts retention times and peak volumes of analytes eluting under step-gradient conditions as applied in RPLC column-switching procedures. The determination of procymidone and iprodione residues in fennel extracts was used as a model system for the development of the proposed procedure.

METHOD DEVELOPMENT

General strategy

An important problem in developing an accurate model for the prediction of solute retention under step-gradient conditions is caused by the practical execution of this type of gradient as illustrated in Fig. 2. Compared to the profile (A) of an ideal step gradient, two noticeable deviations always occur in the resulting elution profile (B) obtained from a solvent-delivery system. When executing a step gradient, it takes some time before the first change in solvent composition reaches the top of the column. This first deviation is called the delay time (t_d) of the gradient and is caused by the connective tubing. The second deviation is the time needed to accomplish a new mobile phase composition at the head of the column. This effect is caused by the volume of the mixing chamber and is related to the time constant (τ) of the mixing process. Both t_d

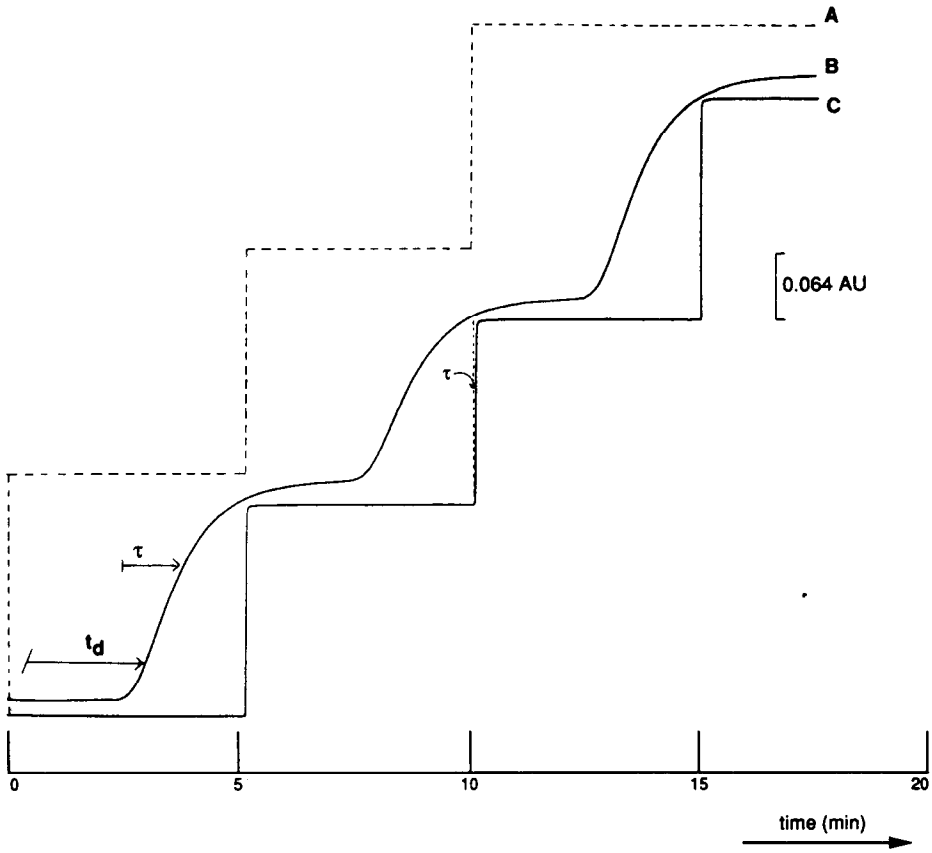


Fig. 2. Different gradient profiles of the same three-step-gradient-elution program, for which each step, executed at time intervals of 0, 5 and 10 min, corresponds with an increase of 20% solvent A (0.1% acetone in water) in solvent B (pure water). A = ideal step-gradient profile; B = step-gradient profile with a binary gradient pump system; C = profile with the gradient pump in combination with a valve-switching system (see Fig. 3) and a stop-flow time of 5 min. B and C are recorded without a column and with UV detection at 254 nm.

and τ of a step gradient obtained from a binary pump system are indicated in Fig. 2.

As was outlined in several studies [17–19], the most important deviation factors for retention prediction in gradient elution are the mixing effect and the delay time of the gradient. For linear gradient elution these effects are negligible if the steepness of the gradient is kept small [17–19].

In a column-switching procedure (see Fig. 1), however, the steepness of the gradient on C-1 during a step gradient elution is almost maximal (see Fig. 2) and therefore the effects of mixing and gradient delay will play a dominant role in the chromatographic process. The resulting disagreements between practical and simulated values for retention in step-gradient elution have been ascribed to these effects [16].

The development of our model will be explained in the next three sections.

Firstly, the theory of step-gradient elution in relation to the transport of a solute under column-switching conditions will be discussed. The following section will explain the principles of the proposed model of chromatogram simulation to be used for the prediction of retention times and peak volumes of analytes eluting under step-gradient conditions. Finally, the different steps of the simulation procedure are summarized in the last section.

Prediction of retention in step-gradient elution

Isocratic retention data. A first step in predicting retention times for solutes eluting under gradient conditions is to describe the capacity factor, k, for each solute as a function of the mobile phase composition. A simple linear relation often used for the description of retention in RPLC [20,21] is:

$$\log k = \log k_w - S\varphi \tag{1}$$

where k_w is the (extrapolated) capacity factor in pure water, φ is the volume fraction of the organic modifier and S is a constant depending on the organic modifier used. This linear equation appeared often not valid in the extreme regions of φ [21], normally used in column-switching procedures, and therefore a quadratic ln k *vs.* φ relationship was preferred according to ref. 21:

$$\ln k = a\varphi^2 + b\varphi + c \tag{2}$$

where the coefficients a, b and c are experimentally determined constants.

An important parameter in the prediction of the retention times in column-switching experiments is the length, L, of the first column (C-1). Separation on C-1 of A from S1 (see Fig. 1) must be completed by the time the compounds have travelled the length of the column. Based on the general equations used in RPLC [22] for the description of the retention time of a solute (t_r) and the unretained compound (t_0), an equation can easily be derived which describes in isocratic elution the distance (ΔL in mm) travelled by a solute during a time Δt as:

$$\Delta L = [L/(1 + k)]\Delta t/t_0 \tag{3}$$

Using eqns. 2 and 3, the retention time of a solute occurring under ideal stepwise gradient elution conditions (see Fig. 2) can be calculated. The solute elutes when the sum of the different travelled distances ($\Sigma\Delta L$), obtained with different isocratic elutions, equals the total length (L) of the first column (C-1).

Correction terms for retention prediction. For an accurate prediction of retention in step-gradient elution two corrections must be made to eqn. 3. Correction for the most obvious deviation, the delay time (t_d), is simply carried out be measuring its value and subtracting it from the time used in the step gradient.

Due to the delay of the next solvent front, a correction must also be made for the position of the solute in the column. Normally in column switching, using two pumps and a high-pressure switching valve, a one-step-gradient elution with two eluents (M-1 and M-2) on C-1 is applied, yielding a profile of the solvent front which corresponds closely to the ideal profile. This is also illustrated in Fig. 2, showing the profile (C) of

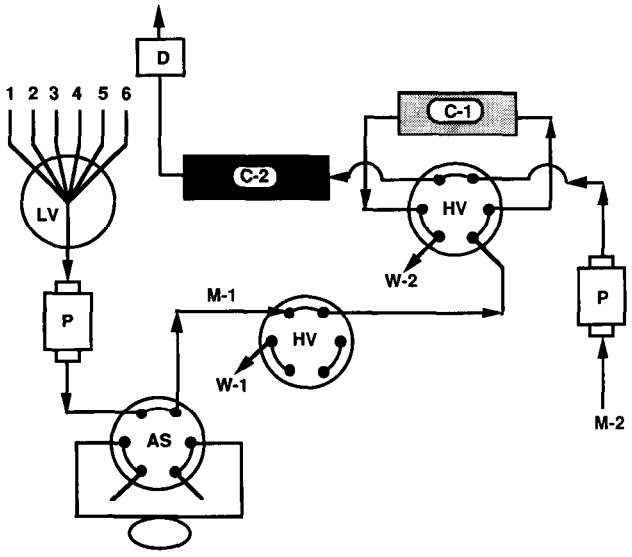


Fig. 3. LC system for the execution of step-gradient elution and column switching. AS = autosampler; LV = six-way low-pressure valve for the selection of different clean-up eluents. HV-1 and HV-2 = six port high-pressure valves; C-1 = first C_{18} separation column; C-2 = second C_{18} separation column; W-1 = waste during a stop-flow on C-1 for the performance of an abrupt solvent change; W-2 = waste during elution (clean-up) on C-1; P = LC-pump; D = UV detector; all flows are set at 1 ml/min (for further details: see Theory, Experimental and Results and Discussion).

a three-step gradient obtained with the LC column-switching system presented in Fig. 3. In this figure, the combined use of a solvent-selection valve (LV) and a high-pressure valve (HV-1) regulates an abrupt change in solvent composition by means of a temporarily "stopped flow" on the first column (C-1).

Assuming unretained migration of the solvent front without distortion, two equations were derived (see Appendix I) for more accurate prediction of retention in column-switching experiments. For a one-step-gradient elution the retention is given by the equation:

$$t_r = t_0(1 + k_2) + (t_d + t_1)[(k_1 - k_2)/k_1] \quad (4)$$

and the equation for the retention obtained with a two-step gradient corresponds to

$$t_r = t_0(1 + k_3) + t_d[(k_1 - k_3)/k_1] + [t_1(k_1 - k_2)k_3]/k_1k_2 + t_2[(k_2 - k_3)/k_2] \quad (5)$$

In these equations, k_1, k_2 and k_3 are the capacity factors of the solute at the three different mobile phase compositions.

Design of the model for the prediction of retention and peak volume

Our model, developed for the prediction of retention and peak volume, is based on the diffusion occurring during the transport of a solute in tubing and column. In this model, schematically presented in Fig. 4, the migration of a solute is proposed to

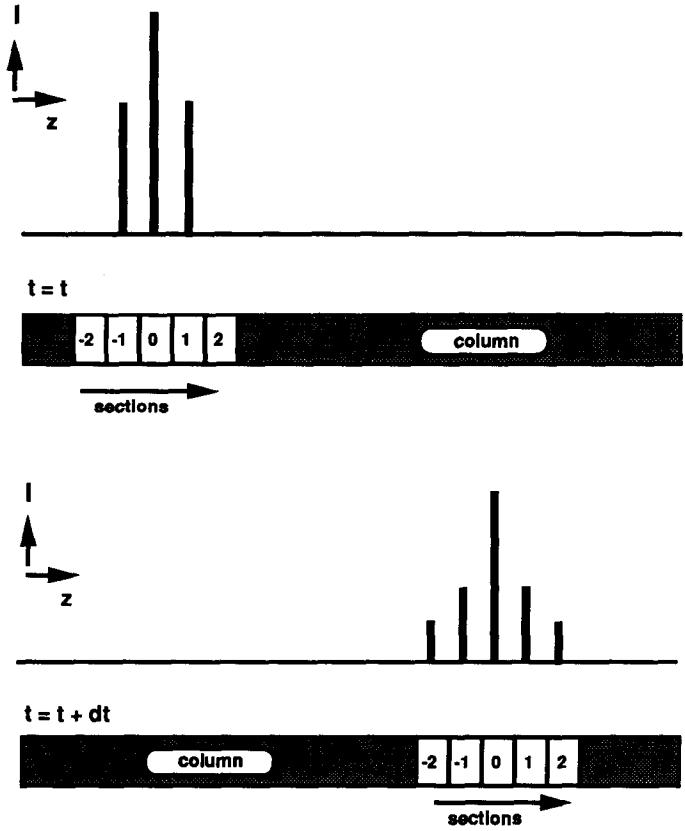


Fig. 4. Schematic representation of the migration (z) of a solute with an intensity I in a number (n) of sections migrating through a column or tubing by means of a diffusion process. For further explanation, see text.

be arranged by means of a number (n) of small interconnected sections (s) moving through the chromatographic system. The position ($n = 0$) of the central section (s_0) as a function of time corresponds with the actual place of the solute in the column (or tubing); the intensity in s_0 and the surrounding sections is determined by diffusion. The schematic presentation of Fig. 4 shows the position of section n, $z_n(t)$, and the intensity in section n, $I_n(t)$, of a solute during migration in five sections at the time intervals t and $t + \Delta t$. The cumulative displacement of a solute in the sections during a certain time interval, Δt, is given by its current place (z_n) plus the travelled distance (Δz) during Δt, which can be expressed by the relation

$$z_n(t + \Delta t) = z_n(t) + \Delta z \tag{6}$$

In this equation a distance (Δz) travelled in the tubing corresponds to

$$\Delta z = u_{tub}\Delta t \tag{7}$$

and a distance (Δz) travelled in the column is equal to

$$\Delta z = u_{col}\Delta t/(1 + k) \tag{8}$$

in which u_{tub} and u_{col} stand for the linear velocity of the unretained compound in tubing or column, respectively.

At the start of an elution, a solute will have a maximum intensity (concentration) in section s_0. After a time (Δt), the new intensity $I_n(t + \Delta t)$, of the solute will be divided over a number of sections at both sides of the maximum intensity in section zero (see Fig. 4). Due to the open connection between the sections the new intensity of a solute in each section is not only determined by the transport of solute *to* the two adjacent sections but also by the transport *from* the adjacent sections. The change in intensity of a solute in a section during the migration can be seen as a diffusion process described for each step (Δt) by the equation

$$I_n(t + \Delta t) = I_n(t) + D\{[I_{n+1}(t) + I_{n-1}(t) - 2I_n(t)]\Delta t/dp_n^2\} \tag{9}$$

in which D is a diffusion coefficient and dp the average distance between two sections corresponding to

$$dp_n = [z_{n+1}(t) - z_{n-1}(t)]/2 \tag{10}$$

Using the general relationship between H, the height of one theoretical plate of a column, and the diffusion coefficient D derived by Giddings [23] as

$$H = 2D/Lv \tag{11}$$

in which v is the migration velocity (mm/s) of a solute, the diffusion coefficient (D) of eqn. 9 can be expressed as

$$D = Hu_{col}/[2(1 + k)] \tag{12}$$

Applying (step-) gradient elution, the exact mobile phase composition (φ) during elution must be known in order to predict accurate chromatographic values. The calculation of φ as a function of z and t, including (i) a correction for solvent front distortion (mixing effect), (ii) the position of the solute in the column and (iii) the delay time (t_d) can be described by the equation

$$\varphi(t,z) = \varphi_0 + \Delta\varphi\{1 - \exp[-(t-[t_s + t_d + z/u_{col}])/\tau]\} \tag{13}$$

in which φ_0 represents the value of φ at the start of the step gradient, $\Delta\varphi$ the size (increase of φ) of a step and t_s the time of solvent change (switching time).

The value of the time constant, τ, can be determined graphically from the step gradient profile as illustrated in profile B of Fig. 2. Knowing the relation between k and φ (eqn. 2) the changing k value during step gradient elution can be calculated.

Final computer-simulation procedure

For the prediction of retention times and peak volumes of solutes eluting under step-gradient conditions a computer program has been written in Lightspeed Pascal. This program performs an iterative calculation, using eqn. 3 for transport, eqns. 9 and 12 for diffusion and eqn. 13 for the distortion of the solvent front.

Before the calculation takes place, the H value of the column, the delay time (t_d) and the time constant (τ) of the gradient, and the coefficients a, b and c (eqn. 2) of the solutes are introduced in the program by means of an input file. The latter parameters must be determined experimentally from a minimum of three isocratic experiments.

A visual simulated chromatogram is obtained by transferring the data to a graphic computer program which prints the peak intensities (I) against the elution times.

In order to speed up the procedure, two selection criteria are added to the computer program. The first one is the application of eqns. 4 and 5. These equations rapidly provide the retention times of the compounds eluting under a selected one- or two-step gradient. If the retention difference between adjacent peaks (Δt_r) is promising, the simulation is started to provide complete chromatographic information.

The second time-saving criterion is the simultaneous calculation of the resolution, $R_s = \Delta t_R/[2(\sigma_1 + \sigma_2)]$, between successive peak pairs in the simulated chromatogram. Depending on the desired resolution, a decision can be made on whether a graphical output of the chromatogram is made. The scheme of the final rational optimisation procedure is given in Fig. 5.

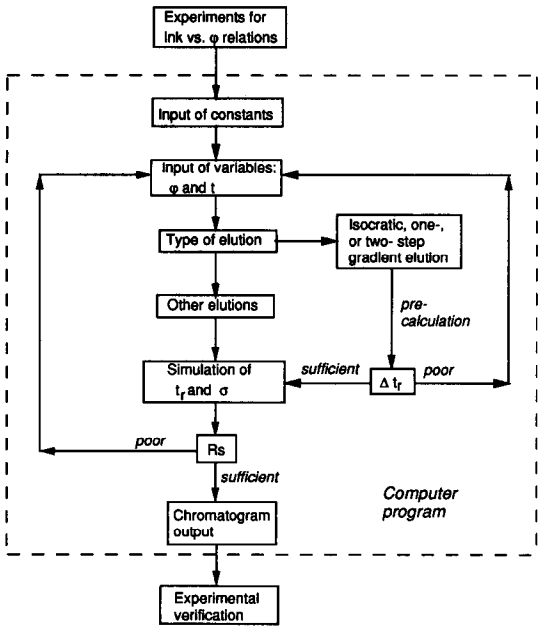


Fig. 5. Scheme of the rational optimisation procedure.

EXPERIMENTAL

Reagents

The fungicides iprodione, 1-isopropylcarbamoyl-3-(3′,5′-dichlorophenyl)hydantoin, and procymidone, N-(3′,5′-dichlorophenyl)1,2-dimethylcyclopropane dicarboximide), both with a purity > 99%, were obtained from Dr. S. Ehrenstorfer (Promochem, Wesel, Germany). Analytical-grade dichloromethane was bought from Merck (Darmstadt, Germany) and methanol and acetonitrile, both HPLC grade, were purchased from Baker (Deventer, The Netherlands). Potassium bromide (KBr) and anhydrous sodium sulphate (Na_2SO_4) were bought from Merck. Demineralised water was purified in a Milli-Q (Millipore, Bedford, MA, U.S.A.) system to obtain LC-grade water for use in eluents and standard solutions. The solvent compositions (v/v) of the isocratic mobile phases were prepared by accurately weighing the necessary volumes of water and organic modifier. Stock standard solutions of the fungicides were prepared in acetonitrile and for the LC analyses dilutions were made in the mobile phase. RPLC test mixtures, containing uracil and fluoranthene for the measurement of the retention of an unretained compound and the efficiency (N) of a column, respectively, were obtained from Chrompack (Middelburg, The Netherlands).

Equipment

The LC instrumentation consisted of the following components: an ASPI 232-401 autosampler (Gilson, Villiers-le-Bel, France) equipped with three programmable high-pressure valves (Type 7010, Rheodyne, Cotati, CA, U.S.A.) and one programmable six-way solvent-selection valve (Type 5011, Rheodyne); two isocratic pumps: one Model 9208 from Kipp Analytica (Delft, The Netherlands) and one Model 400 from Kratos (Ramsey, NJ, U.S.A.); one binary gradient pump Model 250 from Perkin-Elmer (Norwalk, CT, U.S.A.) with a helium degassing system from Perkin-Elmer equipped with air tight bottle connections from Omnifit (Cambridge, U.K.) for delivering mobile phases to the pumps under light pressure (60 p.s.i.).

The flow valve scheme for executing a stepwise gradient elution with a solvent-selection valve in combination with column switching is depicted in Fig. 3. All flow-rates were set at 1 ml/min.

A 50 × 3 mm I.D. column, packed with ChromSpher C_{18}, 5 μm-particles, from Chrompack, was used as a first column (C-1) in combination with a 10 × 2 mm I.D. guard column packed with 40-μm pellicular C_{18} material. A 100 × 4.6 mm I.D. column, packed with MicroSpher C_{18}, 3-μm particles, from Chrompack, was used as a second separation column (C-2). Detection (UV at 229 nm) was performed with a LC-95 UV detector (Perkin-Elmer) equipped with a Kipp recorder.

A Macintosh McII (Apple, Cupertino, CA, U.S.A.) personal computer was used for calculation and simulation.

Preparation of fennel extracts

A 50-g aliquot of an homogenised blank fennel sample was weighed into a cup of a Warring blender, together with 200 ml of dichloromethane and 100 g of sodium sulphate. After blending for 3 min the mixture was transferred into a tube and centrifuged for 5 min at 7000 g. A 100-ml volume of the dichloromethane phase was dried over sodium sulphate and concentrated to 5 ml in a Kuderna Danish apparatus.

For experiments 200 μl of extract, corresponding with 1 g of fennel, were pipetted into a calibrated tube and made to dryness with a gentle stream of nitrogen. Prior to the LC analysis, the residue was dissolved in 100 μl of acetonitrile and brought to a volume of 1 ml with water (blank fennel extract) or with a suitable standard solution of procymidon and iprodione in water (spiked fennel solution).

RESULTS AND DISCUSSION

Application of the computer-simulation program

The application of the optimisation procedure is schematically presented in Fig. 5. The program does not (yet) find optimal separation conditions automatically. At this stage it should be applied by an analyst with a basic knowledge of RPLC. The optimal separation conditions must be found step by step with the application of different elution programs in a rational way. An important tool in selecting suitable gradient-elution programs is the graphical interpretation of the resulting ln k *vs.* φ plots. The first step is to enter, by means of an input file, into the computer program the values of the parameters which will serve as constants for a particular application. These are the a, b and c coefficients of the quadratic ln k *vs.* φ relations of the compounds to be separated and the constants of the LC system. The relevant parameters are the injection volume, the gradient delay time (t_d), the time constant (τ) for the distortion of the solvent front, the flow and the efficiency of the column (H).

The second step is to enter the variables for an elution program. The program asks for the number of steps and the length (min) and fraction of modifier (φ) of each step.

After introducing the variables and constants, the program calculates first rapidly the retention times of the solutes. Based on the resulting differences in retention (Δt_r) the analyst must decide to proceed with a simulation or to introduce new step-gradient elution conditions (φ and t values).

After the simulation, the analyst must decide again, based on the calculated resolutions (R_s), whether other elution conditions must be tried out to improve the separation. This decision depends mainly on the type of application and the concentration of the interferences (S1 or S2, see Fig. 1). In pesticides residue analysis an obtained partial preseparation on a short first column (C-1) is normally sufficient for the final separation on the highly efficient second separation column (C-2).

If the resolution seems appropriate, the data of the calculated intensities and peak volumes are converted into a chromatogram rendering the exact volumes for clean-up and transfer to be used in column-switching procedure (see Fig. 1).

The simulation time is mainly determined by the number of compounds and by the step size of the iterative calculation procedure. Using an iteration step of 0.004 min, the simulated chromatograms of the three compounds used in this application were calculated within 2 min. The graphical display of a chromatogram containing three solutes takes about 5 min.

Retention behaviour of iprodione, procymidone and fennel

The earlier described automated clean-up procedure of fennel extracts for the determination of iprodione and procymidone [5] was performed with a three-step gradient elution on a 15 × 3.2 mm I.D. C_{18} precolumn. However, in this study, it

appeared that the efficiency (N) of this precolumn and of other small columns tested with a variety of C_{18} materials decreased very rapidly on injection of fennel extracts. Therefore this type of columns were not suitable for the purpose of this study. Acceptable results in terms of stability were obtained with 50 × 3 mm I.D. C_{18} columns, in combination with small disposable guard columns directly connected to the separation column (see Experimental). When replacing the low-cost guard column every 2 days while analysing fennel extracts, the efficiency (N of the column) remained constant (1750 ± 250) for more than 7 days.

The first step of the proposed procedure was the determination of the retention behaviour of the compounds by establishing their ln k *vs.* φ relations. In the fennel extract, one major interference was found to elute closely to the two fungicides. The retention of this fennel interference, denoted as "fennel", and the retention of the two fungicides iprodione and procymidone was measured in five different mobile phases providing the ln k *vs.* φ plots presented in Fig. 6. The crossing of the ln k *vs.* φ lines illustrates that this is an interesting clean-up (separation) problem. As explained in the introduction (see Fig. 1), clean-up in an on-line column-switching procedure is normally focussed on the removal of the first eluting sample interferences (S1). However, in this application the situation is reversed. The clean-up eluent (M-1) must prevent the elution of the fennel interference (S2 in Fig. 1) from C-1 during the transfer of the analytes to the second column (C-2). In the next sections the computer program will be applied to establish optimal clean-up conditions for this problem.

Prediction of retention and peak volume under isocratic conditions

The plots of Fig. 6 suggest that the clean-up can be performed with the use of only one clean-up eluent (M-1) consisting of a high percentage of methanol (>60%). This solvent is also appropriate for the mobile phase (M-2) of the second column. To confirm this, the performance of the computer program was first tested for isocratic elutions. In order to compare the simulated chromatograms with experimental ones, fennel extracts were spiked with approximately 50 ppm of iprodione and procymidone,

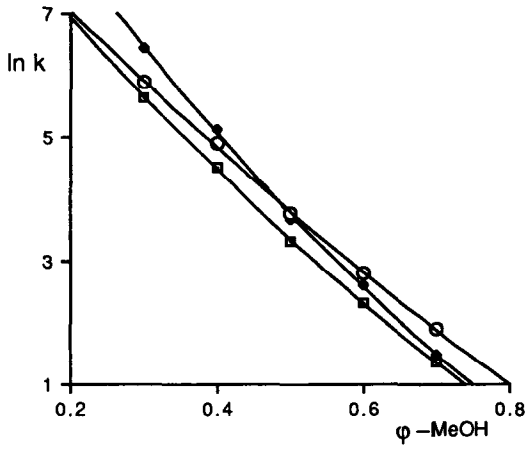


Fig. 6. Plots of ln k *vs.* volume fraction of organic modifier (φ) in water for procymidone (□), iprodione (◆) and fennel (○) on a 50 × 3 mm I.D. C_{18} column (C-1). MeOH = Methanol.

rendering an equal UV response at 229 nm for the compounds involved.

The chromatographic results for simulated and experimental isocratic elutions on a 50 × 3 mm I.D. column (C-1) are listed in Table I. It must be mentioned that in this study still the same column is used for the comparison between experiment and prediction meaning that the calculated values are based on the input of the measured values for retention.

The separations between procymidone and iprodione and between iprodione and fennel, mentioned in Table I, are characterised by the resolution (R_s). The obtained resolutions at 40% methanol are disceptive due to the elution of fennel between procymidone and iprodione at this mobile phase composition. The results of Table I show that sufficient resolution ($R_s = 2$) between fungicides and fennel can be obtained at $\varphi = 0.6$ and also that the resolution increases with increasing eluotropic strength. This is also demonstrated in Fig. 7, which shows the simulated and corresponding experimental chromatograms of two different isocratic elutions with φ values of 0.65 (A) and 0.60 (B), respectively.

The good agreement between the simulated and experimental chromatograms emphasises the usefulness of an accurate chromatogram simulation in selecting clean-up conditions. For example, Fig. 7 illustrates that with a transfer fraction of 2.6 ml of 60% methanol or 1.6 ml of 65% methanol from the first column (C-1) to the second column (C-2), only the fungicides will be transported to C-2 and the fennel (S2) will be retained on C-1. Of course, the first 1 ml of 65% or the 1.5 ml of 60% methanol of the chromatogram, containing the early-eluting interferences (S1), will be sent to waste in a column-switching procedure. Based on these results it can be concluded that an appropriate separation can be achieved with an isocratic heart-cutting procedure, using a mobile phase in the range of 60 to 70% methanol.

TABLE I

COMPARISON OF SIMULATED (sim.) AND EXPERIMENTAL (exp.) VALUES FOR DIFFERENT ISOCRATIC ELUTIONS ON C-1

σ = Peak volume at 0.6 of the peak height.

φ (Methanol)		Procymidone		Iprodione		Fennel		R_s	
		t_r (min)	σ (min)	t_r (min)	σ (min)	t_r (min)	σ (min)	Procymidone/ iprodione	Iprodione/ fennel
0.4	exp.	14.9	0.36	27.4	0.65	22.1	0.43	4.6	2.5
	sim.	14.9	0.38	26.5	0.69	22.1	0.57	3.8	1.8
0.5	exp.	4.51	0.11	6.81	0.17	6.85	0.16	4.1	0.1
	sim.	4.59	0.12	6.84	0.17	7.06	0.18	3.9	0.3
0.6	exp.	1.85	0.045	2.32	0.062	2.85	0.063	2.2	2.1
	sim.	1.82	0.045	2.28	0.057	2.79	0.069	2.2	2.0
0.7	exp.	0.94	0.031	1.02	0.031	1.38	0.035	0.7	2.7
	sim.	0.94	0.025	1.02	0.026	1.39	0.035	0.7	3.1

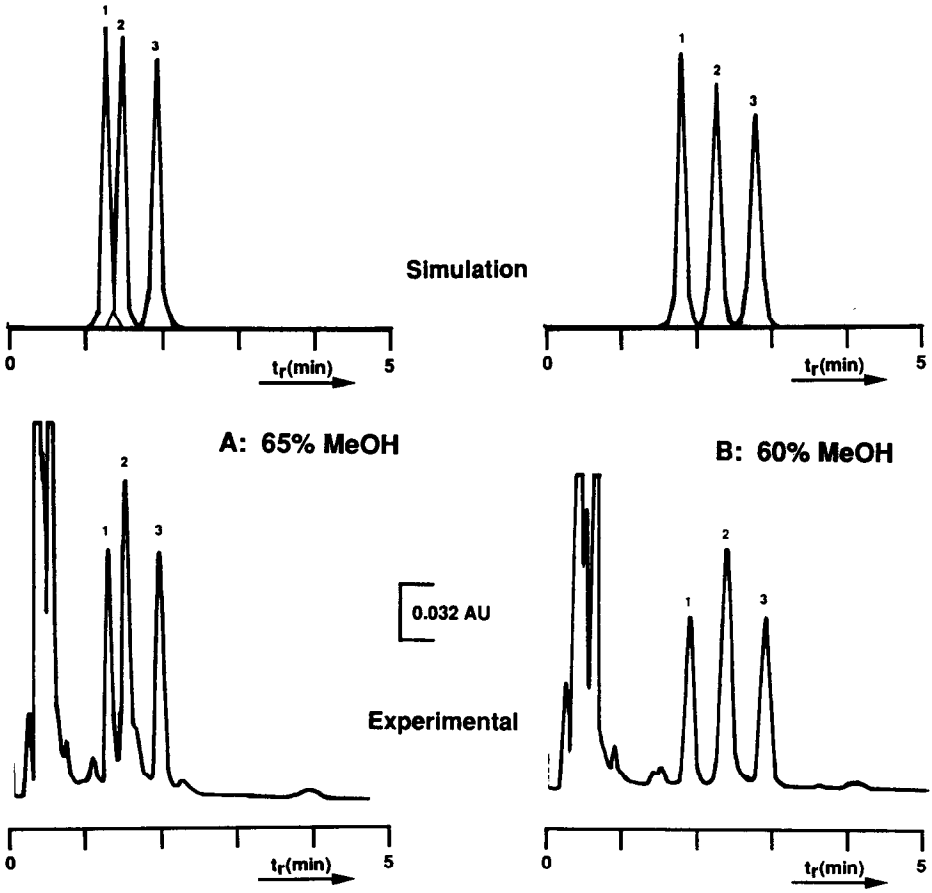


Fig. 7. Comparison of simulated and experimental isocratic chromatograms on C-1: A, 65% methanol and B, 60% methanol as the mobile phase. Injection of a 50-μl fennel extract (0.1 g/ml) containing 50 ppm procymidone (1), 65 ppm iprodione (2) and a fennel interference (3); UV detection at 229 nm; flow-rate, 1 ml/min.

Prediction of retention and peak volume in step-gradient elution

The computer program used to simulate step-gradient conditions was first tested for a number of different one-step gradient elutions. The experimental values were obtained under column-switching conditions with the gradient profile C of Fig. 2, which has a delay time (t_d) of 0.18 min and time constant (τ) of 0.03 min.

The results of nine different one-step-gradient elutions are listed in Table II. These data show a good agreement between prediction and experiment of chromatographic values for the various one-step-gradient elutions. The deviations of the retention times are less than 2% and the deviations of the peak volumes are generally below 10%. Only in the last two gradients ($\Delta\varphi > 0.35$), three deviations of approximately 25% in peak volume were observed.

Two examples, illustrating the good match of simulated and experimental

TABLE II

COMPARISON OF SIMULATED AND EXPERIMENTAL VALUES FOR ONE-STEP-GRADIENT ELUTION

exp. = Experimental with switching valves (Fig. 3); sim. = computer simulation with profile C of Fig. 2; calc. = calculated with eqn. 4.

Elution programme			Procymidone		Iprodione		Fennel	
Time (min)	φ (Methanol)		t_r (min)	σ (min)	t_r (min)	σ (min)	t_r (min)	σ (min)
4	0.50	exp.	4.49	0.042	5.19	0.055	5.38	0.065
∞	0.60	sim.	4.41	0.047	5.12	0.057	5.37	0.070
		calc.	4.46	–	5.14	–	5.38	–
1.5	0.60	exp.	1.89	0.038	2.19	0.036	2.51	0.047
∞	0.65	sim.	1.81	0.036	2.11	0.035	2.47	0.047
		calc.	1.81	–	2.09	–	2.44	–
5	0.40	exp.	6.05	0.032	6.39	0.040	6.68	0.045
∞	0.65	sim.	6.07	0.031	6.41	0.036	6.71	0.048
		calc.	6.08	–	6.4	–	6.69	–
2	0.40	exp.	3.25	0.033	3.58	0.042	3.88	0.038
∞	0.65	sim.	3.31	0.031	3.57	0.036	3.95	0.048
		calc.	3.29	–	3.54	–	3.92	–
5	0.40	exp.	6.38	0.045	7.01	0.055	7.28	0.065
∞	0.60	sim.	6.43	0.045	7.07	0.057	7.37	0.070
		calc.	6.43	–	7.05	–	7.36	–
3	0.40	exp.	4.58	0.045	5.15	0.062	5.54	0.061
∞	0.60	sim.	4.66	0.046	5.23	0.057	5.61	0.070
		calc.	4.65	–	5.2	–	5.59	–
2	0.40	exp.	3.77	0.048	4.38	0.058	4.85	0.062
∞	0.60	sim.	3.78	0.045	4.31	0.057	4.74	0.069
		calc.	3.75	–	4.28	–	4.71	–
5	0.30	exp.	6.31	0.028	6.56	0.042	6.95	0.038
∞	0.65	sim.	6.39	0.028	6.64	0.036	7.02	0.048
		calc.	6.36	–	6.59	–	6.98	–
5	0.30	exp.	6.01	0.021	6.12	0.032	6.42	0.032
∞	0.70	sim.	6.09	0.023	6.21	0.024	6.53	0.034
		calc.	6.06	–	6.16	–	6.49	–

chromatograms, are presented in Fig. 8. The chromatograms in this figure clarify that with the use of a one-step gradient the separation between fungicides and fennel is poor when using clean-up eluents of 50 or 60% methanol (Fig. 8A). Applying a one-step-gradient elution, baseline resolution between fungicides and fennel can be achieved with 30% methanol (for the removal of S1) and 70% methanol (to retain fennel as S2 on C-1) as clean-up solvents (Fig. 8B).

Finally, some two- and three-step-gradient elutions were investigated. As in a column-switching procedure, the instantaneous solvent change was achieved by stopping the flow on the column until the next solvent composition had reached the top

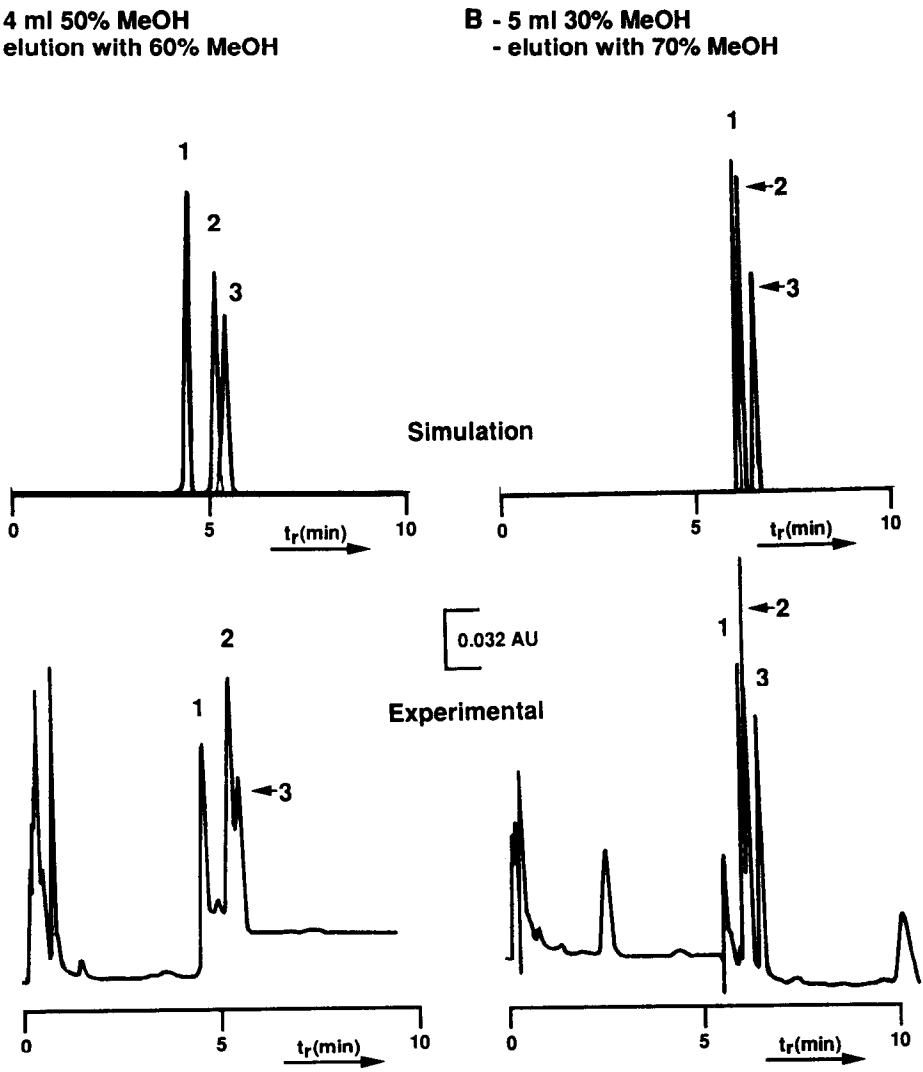


Fig. 8. Comparison of simulated and experimental one-step-gradient-elution chromatograms; conditions as in Fig. 7. For discussion, see text.

(entrance) of the column (see Fig. 3). In order to predict peak volumes under this type of multi-step gradient elution, the contribution to the peak volume (σ) during a "stop-flow" period must be known. For stop-flow experiments with time intervals of 1 to 5 min, using mobile phases of 40, 50 and 60% methanol, no significant contribution to the peak volume ($< 1\%$) of the solutes was measured on the 50 × 3 mm I.D. C_{18} column. This interesting result implies that the increase in peak volume during a stop-flow is small. A diffusion coefficient of 0.001 mm^2/min for each compound was used in the computer program to account for this effect.

The obtained simulated and experimental results of one two-step- and two

different three-step-gradients are summarised in Table III. The similarity between predicted and experimental retention is again satisfactory. The predicted peak volumes are somewhat smaller than the experimental values with deviations in the range 10–30%. The performance of the simulation program is illustrated in Fig. 9, showing a good resemblance between a simulated and an experimental three-step-gradient elution chromatogram.

The data on the last two lines of Tables III include the results of the three-step-

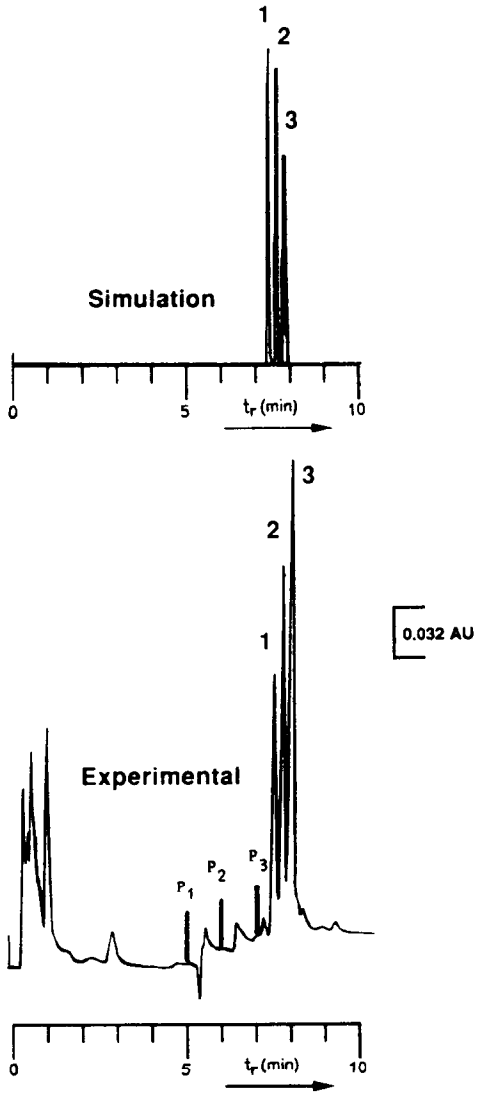


Fig. 9. Comparison of a simulated and experimental three-step-gradient-elution chromatogram: 5 min 30% methanol; 1 min 50% methanol; 1 min 60% methanol; elution with 70% methanol. P_1–P_3: stop-flow points of 2.5 min to obtain the right mobile phase composition (see Fig. 3); further conditions as in Fig. 6. Peaks: 1 = procymidone; 2 = iprodione; 3 = fennel.

TABLE III

COMPARISON OF SIMULATED AND EXPERIMENTAL VALUES FOR TWO- AND THREE-STEP-GRADIENT ELUTIONS

exp. = Experimental with switching valves (Fig. 3); sim. = computer simulation with profile C of Fig. 2; calc. = calculated with eqn. 5; exp.* = experimental with a binary pump system; sim.* = computer simulation with profile B of Fig. 2.

Elution programme			Procymidone		Iprodione		Fennel	
Time (min)	φ (Methanol)		t_r (min)	σ (min)	t_r (min)	σ (min)	t_r (min)	σ (min)
5	0.3	exp.	6.91	0.028	7.15	0.031	7.37	0.029
1.5	0.6	sim.	6.84	0.024	7.06	0.024	7.31	0.034
∞	0.7	calc.	6.9	–	7.09	–	7.32	–
2	0.4	exp.	4.47	0.047	4.71	0.041	4.92	0.047
1	0.5	sim.	4.34	0.024	4.59	0.024	4.81	0.034
1	0.6							
∞	0.7							
5	0.3	exp.	7.51	0.035	7.72	0.038	7.93	0.041
1	0.5	sim.	7.41	0.022	7.63	0.024	7.85	0.034
1	0.6							
∞	0.7	exp.*	8.48	0.045	8.95	0.051	9.15	0.051
		sim.*	8.64	0.035	9.04	0.035	9.28	0.044

gradient, but now executed with a binary pump possessing a gradient profile B as shown in Fig. 2. It is remarkable that also in this situation with a delay time of 2.5 min and a time constant of 1.3 min for the distortion of the solvent front, the predicted values for retention and peak volume correspond very well with the experimental values. Tables II and III also contain the results of the retention predicted with eqns. 4 and 5. The calculated values for the step-gradient elutions are in good agreement with the experimental and simulated values. Using these equations, the retention of solutes for one- and two-step-gradient programs performed with column switching can be determined very quickly.

Determination of procymidone and iprodione in fennel extracts

With the developed computer program two different clean-up procedures with either isocratic elution (heart-cutting) or a step-gradient elution were found to be suitable for the determination of procymidone and iprodione in fennel extracts. To verify the predicted optimal clean-up conditions experimentally, realistic additions of procymidone and iprodione at the 1-ppm level to fennel extracts were made and the spiked samples were analysed with an RPLC column-switching system as described in Fig. 3. In this set-up, a 100 × 4.6 mm I.D. column packed with MicroSpher C_{18}, 3 μm, was used as the second separation column (C-2). In a first experiment the two separation columns, C-1 and C-2, were coupled on-line and a sample (50 μl) of a spiked fennel extract (1 g/ml) was analysed without clean-up using a mobile phase of 70% methanol in water (1 ml/min). The resulting chromatogram, shown in Fig. 10A, seems to illustrate that a clean-up procedure with column switching is not necessary. However, it is not advisable to inject fennel extracts without clean-up onto analytical columns.

Chromatogram B of Fig. 10 demonstrates the result of a selected two-step-gradient elution (result of Fig. 8B), using a clean-up on C-1 with 5 ml of 30% methanol and 0.9 ml of 70% methanol, followed by a transfer of the fungicides containing fraction from C-1 to C-2 with 0.3 ml of 70% methanol.

The last chromatogram (Fig. 10C) shows the predicted isocratic heart-cutting clean-up on C-1 using 70% methanol for M-1 and M-2 (see Table I). An even more "clean" chromatogram is obtained with this eluent using 0.85 ml for the clean-up on C-1 and 0.3 ml for the transfer of the fungicides from C-1 to C-2.

So far, the solvent strength in our column-switching procedures was only varied using one organic modifier. The good separation result with the proposed procedure is in agreement with earlier studies [24,25], indicating that an accurate variation of the solvent strength can result in useful changes in elution order favourable for the separation of compounds with a similar molecular size and chemical nature. It is well known that solvent optimization using different solvents (usually methanol, acetonitrile and tetrahydrofuran) can be very successful for influencing the elution order for maximum resolution of the sample compounds [21,26,27]. In future research we will examine if this powerful option can be built into the prediction program. However, in this study, the same retention behaviour of the involved compounds was observed (similar to Fig. 6) using acetonitrile instead of methanol which made the application of a second modifier not attractive for this study.

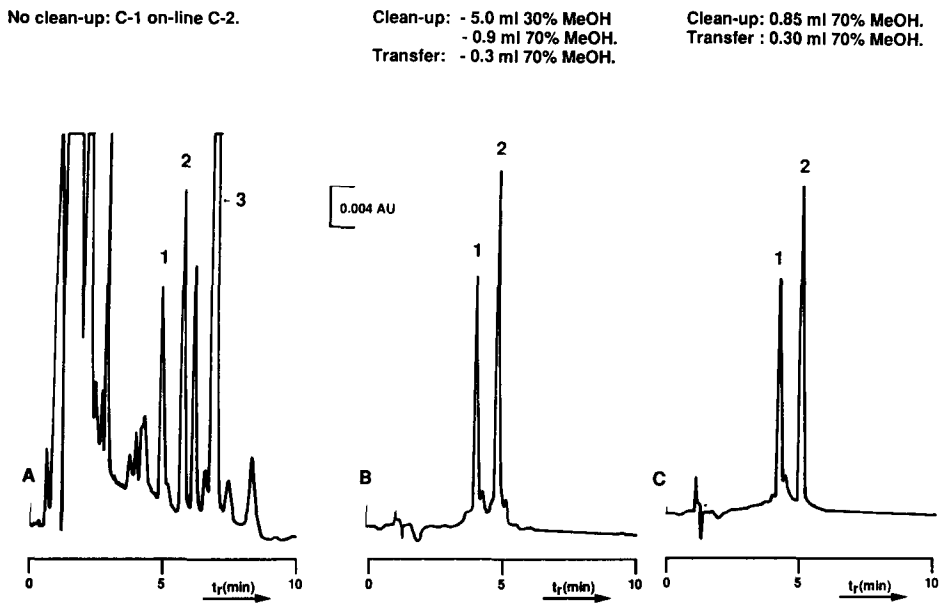


Fig. 10. Final results of the clean-up performance of a fennel (3) extract (1 g/ml) spiked with 1 ppm procymidone (1) and 1.3 ppm iprodione (2). Injection: 50 μl on C-1; UV detection at 229 nm. For further explanation, see text.

CONCLUSIONS

A new approach for the prediction of retention and peak volume of compounds, occurring under step-gradient RPLC conditions has been described. From the ln k *vs.* φ relations of the involved compounds and the plate number (N) of the column, accurate chromatograms are rapidly simulated with a computer program. Compared to trial-and-error optimisation of column-switching procedures this approach yields a significant time saving in finding optimal clean-up conditions with beneficial aspects, such as reduced solvent consumption and extension of the column life.

Good performance of the simulation program has been demonstrated for the residue analysis of procymidone and iprodione in fennel extracts.

The developed procedure, tested in this paper on column switching, is not restricted by the dimensions of the column. Consequently, the computer program is amendable for the optimisation of a separation problem on a high-efficiency column applying step-gradient elution. Using sensitive UV detection, step-gradient elution will provide more stable baselines in comparison to linear gradient elution [28]. This aspect makes this type of elution very suitable for trace-level determination of pesticides which we will examine in future research.

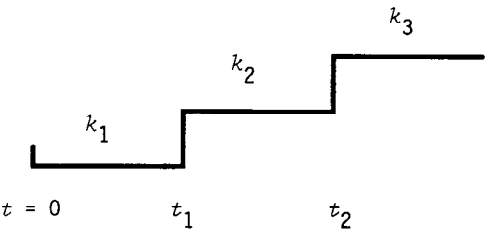
ACKNOWLEDGEMENTS

The technical assistance of Mrs. M. de Lange and Mr. J. Smits is greatly acknowledged.

APPENDIX I

Derivation of equations for the prediction of the retention time of a solute, i, under one- and two-gradient-elution conditions with correction for the place in a column

To derive an equation correcting the position of a solute for the changing solvent fronts used in step-gradient elution, we consider an ideal one- or two-step gradient with solvent fronts migrating through the column without deformation. Schematically the involved steps are as follows:



In this diagram t_1 and t_2 are the switching-time intervals after the start of the elution program at $t = 0$ and k_1, k_2 and k_3 are the corresponding capacity factors of the solute at three different mobile phase compositions. During the first elution with a time t_1, the solute will travel a distance, ΔL, which equals

$$\Delta L = ut_1/(1 + k_1) \tag{A1}$$

where u is the velocity of the unretained compound.

After the first change in composition the solute migrates with k_1 until it is taken over by the next front. The passing takes place at time t_{p1}, when the position of the solute corresponds to that of the front, according to the equation

$$ut_{p1}/(1 + k_1) = u(t_{p1} - t_1 - t_d) \tag{A2}$$

in which t_d is delay time of the step gradient for reaching the top of the column. Rearranging eqn. A2 gives

$$t_{p1} = \{(1 + k_1)/k_1\}(t_1 + t_d) \tag{A3}$$

The travelled distance during the first step with correction for solvent delay is equal to

$$\Delta L_1 = ut_{p1}/(1 + k_1) = (u/k_1)(t_1 + t_d) \tag{A4}$$

For a one-step gradient the solute will elute from the column with the second solvent. The distance to be travelled during this final step corresponds to

$$\Delta L_2 = L - \Delta L_1 \tag{A5}$$

With $L = ut_0$ (t_0 is the retention time of the unretained compound), the retention time of the second step, t_{p2}, is obtained by dividing ΔL_2 by the migration factor $u/(1 + k_2)$ which results in

$$t_{p2} = u[t_0 - (t_1 + t_d)/k_1](1 + k_2)/u \tag{A6}$$

Eqn. A6 can be rearranged into

$$t_{p2} = t_0(1 + k_2) - [(t_1 + t_d)(1 + k_2)/k_1)] \tag{A7}$$

The final retention time, t_r, is obtained by adding the two retentions ($t_{p1} + t_{p2}$) which results in

$$t_r = t_0(1 + k_2) + (t_d + t_1)[(k_1 - k_2)/k_1] \tag{A8}$$

This equation describes the retention time of a solute for one-step gradient elution.

For the prediction of the retention time occurring in a two-step gradient elution, the process continues at eqn. A4, the travelled distance during the first elution with correction for solvent delay. After the executing of the second switch, at time t_2, the second solvent front will pass the solute at time t_{p2} according to the equation

$$(u/k_1)(t_1 + t_d) + u/(1 + k_2)\{t_{p2} - [(1 + k_1)/k_1](t_1 + t_d)\} = u(t_{p2} - t_2 - t_d) \tag{A9}$$

Reorganising eqn. A9 renders

$$t_{p2} = [(t_1 + t_d)(k_2 - k_1)]/k_2k_1 + [(t_2 + t_d)(1 + k_2)]/k_2 \tag{A10}$$

The travelled distance of the solute during time t_{p2} is then

$$\Delta L_2 = \{u(t_{p2} - t_{p1})\}/(1 + k_2) \tag{A11}$$

Substitution of eqns. A3 and A10 in eqn. A11 gives

$$\Delta L_2 = [u/(1 + k_2)]\{[(t_1 + t_d)(k_2 - k_1)]/k_2k_1 + [(t_2 + t_d)(1 + k_2)]/k_2 - [(1 + k_1)/k_1](t_1 + t_d)\} \tag{A12}$$

Rearranging eqn. A12 gives

$$\Delta L_2 = (u/k_2)(t_2 - t_1) \tag{A13}$$

which is the travelled distance during the second elution with correction for the delay of the solvent front.

The sum of the travelled distance is obtained by the addition of eqns. A4 and A13 rendering

$$\Sigma\Delta L = u[t_d/k_1 + t_1(k_2 - k_1)/k_2k_1 + t_2/k_2] \tag{A14}$$

After the two elution steps the solute will be eluted from the column with the third and final elution solvent. The distance to be travelled during this last step corresponds with

$$\Delta L_3 = L - \Sigma\Delta L \tag{A15}$$

With $L = ut_0$, the retention time of the third step, t_{p3}, is found by dividing ΔL_3 by the migration factor $u/(1 + k_3)$ which gives

$$t_{p3} = u[t_0 - t_d/k_1 - t_1(k_2 - k_1)/k_2k_1 - t_2/k_2](1 + k_3)/u \tag{A16}$$

For the final elution time, t_r, the retention time of the first two steps (t_{p2}, eqn. A10) must be added to the retention time of last step (t_{p3}, eqn. A16)

$$t_r = [(t_1 + t_d)(k_2 - k_1)]/k_2k_1 + [(t_2 + t_d)(1 + k_2)]/k_2 + t_0(1 + k_3) - [t_d(1 + k_3)/k_1] - t_1[(k_2 - k_1)(1 + k_3)]/k_2k_1 - t_2(1 + k_3)/k_2 \tag{A17}$$

Reorganising eqn. A17 gives

$$t_r = t_0(1 + k_3) + t_d[(k_1 - k_3)/k_1] + [t_1(k_1 - k_2)k_3]/k_1k_2 + t_2[(k_2 - k_3)/k_2] \tag{A18}$$

which is the predicted retention time of a solute in a two-step-gradient elution.

REFERENCES

1 C. E. Goewie and E. A. Hogendoorn, *J. Chromatogr.*, 404 (1987) 352.
2 C. E. Goewie and E. A. Hogendoorn, *J. Chromatogr.*, 410 (1987) 211.

3 E. A. Hogendoorn and C. E. Goewie, *J. Chromatogr.*, 475 (1989) 432.
4 E. A. Hogendoorn, C. E. Goewie, H. H. van den Broek and P. A. Greve, *Meded. Fac. Landbouwwet., Rijksuniv. Gent*, 49 (1984) 1219.
5 P. A. Greve (Editor), *Analytical methods for Residues of Pesticides in Foodstuffs*, SDU Publishers, The Hague, 5th ed., 1988.
6 P. Jandera and J. Churácek, *J. Chromatogr.*, 170 (1979) 1.
7 P. Jandera and J. Churácek, *J. Chromatogr.*, 91 (1974) 223.
8 M. Borówko, M. Jaroniec, J. Narkiewicz, A. Patrykiejew and W. Rudzinski, *J. Chromatogr.*, 153 (1978) 309.
9 M. Borówko, M. Jaroniec, J. Narkiewicz and A. Patrykiejew, *J. Chromatogr.*, 153 (1978) 321.
10 W. Golkiewicz and M. Jaroniec, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 1 (1978) 245.
11 E. Soczewinski and W. Markowski, *J. Chromatogr.*, 370 (1986) 63.
12 W. Markowski, *J. Chromatogr.*, 485 (1989) 517.
13 W. Golkiewicz, *Chromatographia*, 14 (1981) 411.
14 W. Golkiewicz, *Chromatographia*, 14 (1981) 629.
15 W. Golkiewicz, *Chromatographia*, 21 (1986) 259.
16 W. Markowski and W. Golkiewicz, *Chromatographia*, 25 (1988) 339.
17 B. F. D. Ghrist, B. S. Cooperman and L. R. Snyder, *J. Chromatogr.*, 459 (1988) 1.
18 B. F. D. Ghrist and L. R. Snyder, *J. Chromatogr.*, 459 (1988) 25.
19 B. F. D. Ghrist and L. R. Snyder, *J. Chromatogr.*, 459 (1988) 43.
20 J. W. Dolan, J. R. Grant and L. R. Snyder, *J. Chromatogr.*, 165 (1979) 31.
21 P. J. Schoenmakers, H. A. H. Billiet, R. Tijssen and L. de Galan, *J. Chromatogr.*, 149 (1978) 519.
22 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979.
23 J. C. Giddings, *J. Chem. Phys.*, 31 (1959) 1462.
24 M. A. Quarry, R. L. Grob, L. R. Snyder, W. Dolan and M. P. Rigney, *J. Chromatogr.*, 384 (1987) 163.
25 L. R. Snyder, M. A. Quarry and J. L. Glajch, *Chromatographia*, 24 (1987) 33.
26 S. Sekulic, P. R. Haddad and C. J. Lamberton, *J. Chromatogr.*, 363 (1986) 125.
27 P. J. Schoenmakers and M. Mulholland, *Chromatographia*, 25 (1988) 737.
28 E. A. Hogendoorn and P. van Zoonen, *Meded. Fac. Landbouwwet., Rijksuniv. Gent*, 55 (1990) 1275.