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Optimisation technique for stepwise gradient elution in reversed-phase liquid chromatography

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Abstract

An optimisation technique of reversed-phase liquid chromatographic separations based on gradient elution with a stepwise variation pattern of the volume fraction φ of the organic modifier in the water-organic mobile phase is presented. It uses a non-linear least-squares programme with a Monte-Carlo search for initial estimates in order to determine the best variation pattern that leads to the optimum separation of a mixture of solutes. The validity of the above methodology was tested by separating eight catechol-related solutes with mobile phases modified by methanol or acetonitrile and variation patterns of two, three or four steps in the φ values. It was found in all cases a very satisfactory accuracy of the predicted gradient elution times, which is of the same order with the accuracy of the retention times predicted under isocratic or linear gradient conditions. In addition, it was shown that the proposed optimisation technique is both effective and flexible but well-shaped chromatograms are obtained under electrochemical detection only if steps with increasing φ are used and the change in φ is programmed to occur at the intermediate of the predicted peaks.

Keywords: Stepwise gradient elution; Optimisation techniques

1. Introduction

Gradient elution in reversed-phase liquid chromatography (HPLC) is based on the programmed change in mobile phase composition, flow rate and column temperature, with the most important mode the change in mobile phase composition [1,2]. It is a powerful separation technique, which though has the drawback that only limiting cases of gradient modes can be described by simple theoretical relationships. For example, this is attained in the so-called linear solvent strength gradient, where linear gradients are combined with linear plots of $\ln k$ versus φ, where k is the retention factor and φ is the volume fraction of the organic modifier in the water-organic mobile phase [2–8].

In a subsequent paper [9] we attempted to overcome the above problem by subdividing a non-linear $\ln k$ versus φ curve into a finite number of linear portions. In the present investigation we use a stepwise variation pattern for φ. Such a pattern results in a sum of isocratic elutions and therefore it leads to simple analytical expressions for the retention time. The theory of stepwise gradient elution in liquid chromatography was developed mainly by Jandera and Churacek more than two decades ago [1,10–14] and one of its most interesting applications is the approximation of an arbitrary gradient by a stepwise profile suggested by Cela et al. [15–22]. Despite this, stepwise gradient elution has not attracted much attention. A possible reason is that stepwise gradients cannot give satisfactory separations without the use of a proper optimisation algorithm. Such algorithms have been published [7,15,17] but if they are not available, the final stepwise profile is proposed empirically and it is either very simple [23] or the various steps are combined with linear gradients [24–27]. In the present paper first we present an alternative derivation of the fundamental equations and conditions of the stepwise gradient elution based on kinetic arguments and next we attempt to develop a simple and effective programmed optimisation algorithm for the best separation of a mixture of solutes.

2. Theory

Consider a stepwise profile of φ with time t formed in the mixer, like the one shown in Fig. 1. For simplicity, the first

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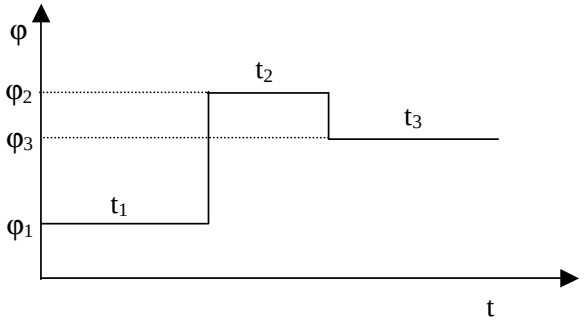

Fig. 1. Schematic three-step variation pattern of φ formed in the mixer.

value of φ, φ_1, that is sent from the mixer to the column for a time period equal to t_1 is considered to be the first step. The nth step reaches the beginning A of the chromatographic column at $t = t_D + t_1 + t_2 + \cdots + t_{n-1}$, where t_D is the dwell time, i.e. the time needed for a certain change in the mixer to reach also the beginning of the column. This step needs more time, say $t = t_D + t_1 + t_2 + \cdots + t_{n-1} + t^*_{n-1}$, to meet the analyte inside the column. Suppose that this event takes place at a distance $s = s_n$ from A. Similarly, the next step reaches the beginning A of the column at $t = t_D + t_1 + \cdots + t_{n-1} + t_n$ and meets the analyte at $t = t_D + t_1 + \cdots + t_{n-1} + t_n + t^*_n$ at a distance $s = s_{n+1}$ from A. We observe that the analyte is under the effect of the nth step, where the mobile phase composition is equal to $\varphi = \varphi_n$, for a time period equal to $t_n + t^*_n - t^*_{n-1}$. Therefore, the distance L_n inside the column that the analyte travels under the influence of the nth step is given by:

$$L_n = s_{n+1} - s_n = v_{\varphi_n}(t_n + t^*_n - t^*_{n-1}) = \frac{L(t_n + t^*_n - t^*_{n-1})}{v_{\varphi_n}} \quad (1)$$

because the velocity, v_{φ_n}, of the analyte under isocratic conditions ($\varphi = \varphi_n$) is equal to $v_{\varphi_n} = L/t_{\varphi_n}$, where L is the length of the chromatographic column and t_{φ_n} is the isocratic retention time when $\varphi = \varphi_n$. In addition, the mobile phase travels a distance equal to s_n at a time equal to t^*_{n-1} and this distance becomes s_{n+1} when $t = t^*_n$. Therefore, we have:

$$L_n = s_{n+1} - s_n = v_0(t^*_n - t^*_{n-1}) = \frac{L(t^*_n - t^*_{n-1})}{t_0} \quad (2)$$

where $v_0 = L/t_0$ is the velocity of the mobile phase inside the column, t_0 being the column dead time. If the two expressions for $L_n = s_{n+1} - s_n$ given by Eqs. (1) and (2) are equated, we obtain:

$$t^*_n = t^*_{n-1} + \frac{t_0 t_n}{t_{\varphi_n} - t_0} = t^*_{n-1} + \frac{t_n}{k_{\varphi_n}} \quad (3)$$

where $k_{\varphi_n} = (t_{\varphi_n} - t_0)/t_0$ is the retention factor. The calculation of t^*_n, $n = 2, 3, \ldots, p$ by means of Eq. (3) presumes the knowledge of t^*_1, i.e. the time needed for the second step with mobile phase composition $\varphi = \varphi_2$ to meet the analyte inside the column. It can be easily shown that:

$$t^*_1 = t_0 \frac{t_1 + t_D}{t_{\varphi_1} - t_0} = \frac{t_1 + t_D}{k_{\varphi_1}} \quad (4)$$

Indeed, when the first change in φ (second step) reaches the beginning of column at time equal to $t_D + t_1$, the distance travelled by the analyte inside the column with constant velocity $v_{\varphi_1} = L/t_{\varphi_1}$ is given by $s_D = v_{\varphi_1}(t_D + t_1)$. If s_2 is the distance from the beginning of the column up to the point where the second step meets the analyte, then we have: $s_2 = v_0 t^*_1$ and $s_2 - s_D = v_{\varphi_1} t^*_1$, from which we readily obtain Eq. (4).

Eqs. (3) and (4) lead straightforwardly to the calculation of the distance L_n inside the column that the analyte travels under the influence of the nth step. Thus for L_1 we have $L_1 = v_{\varphi_1}(t_D + t_1 + t^*_1) = L(t_D + t_1 + t^*_1)/t_{\varphi_1}$, which, in combination with Eq. (4), yields:

$$L_1 = \frac{L(t_1 + t_D)}{t_{\varphi_1} - t_0} = \frac{L(t_1 + t_D)}{t_0 k_{\varphi_1}} \quad (5)$$

For L_n we have from Eq. (1) that $L_n = v_{\varphi_n}(t_n + t^*_n - t^*_{n-1})$ and eventually:

$$L_n = \frac{L t_n}{t_{\varphi_n} - t_0} = \frac{L t_n}{t_0 k_{\varphi_n}}, \quad n = 2, 3, \ldots, p \quad (6)$$

Note that Eqs. (5) and (6) are valid if the solute is not eluted during the first or the nth step, respectively. If this prerequisite does not hold, then we have:

$$L_{1,\text{final}} = L \quad (7)$$

and

$$L_{n,\text{final}} = \frac{L(t_R - t_D - t_1 - t_2 - \cdots - t_{n-1} - t^*_{n-1})}{t_{\varphi_n}} \quad (8)$$

where $L_{n,\text{final}}$ denotes the distance inside the column that the analyte is under the influence of the nth step and during this step it is eluted. Eq. (8) arises from the fact that L/t_{φ_n} is the velocity of the analyte during the nth step and the quantity within the brackets is the time of the analyte movement under the influence of this step, because t_R is the elution time, i.e. the time needed for the analyte to pass from the column, and $t = t_D + t_1 + t_2 + \cdots + t_{n-1} + t^*_{n-1}$ is the time needed for the nth step to meet the analyte inside the column. Therefore, $t_R - t = t_R - t_D - t_1 - t_2 - \cdots - t_{n-1} - t^*_{n-1}$ is the time that the analyte is under the effect of the nth step.

At this point it is worth noting that if we take the sum $L_1 + L_2 + L_3 + \cdots + L_n = L$ using Eqs. (5) and (6) and pass to the limit $n \to \infty$, then we readily obtain the fundamental equation of gradient elution valid when φ varies continuously with time after an elapsed time equal to t_1 [9]:

$$\int_0^{t_R - t_0 - t_1 - t_D} \frac{dt}{t_0 k_\varphi} = 1 - \frac{t_D + t_1}{t_0 k_{\varphi_1}} \quad (9)$$

Eqs. (5)–(8) are the fundamental equations of the stepwise gradient elution, because they can be straightforwardly used

for the calculation of the gradient elution time, t_R, of an analyte which is under the influence of a certain stepwise variation pattern of the mobile phase composition. Consider a p-step variation pattern. The conditions that the analyte would be eluted during the nth step, where $n < p$, may be expressed as $(L_1+L_2+\cdots+L_{n-1})/L < 1$ and $(L_1+L_2+\cdots+L_n)/L \geq 1$, which are transformed into:

$$\frac{t_1+t_D}{t_0 k_{\varphi_1}} + \frac{t_2}{t_0 k_{\varphi_2}} + \cdots + \frac{t_{n-1}}{t_0 k_{\varphi_{n-1}}} < 1 \quad \text{and}$$
$$\frac{t_1+t_D}{t_0 k_{\varphi_1}} + \frac{t_2}{t_0 k_{\varphi_2}} + \cdots + \frac{t_n}{t_0 k_{\varphi_n}} \geq 1 \tag{10}$$

Now from the relationship $L_1 + L_2 + \cdots + L_{n,\text{final}} = L$ we obtain

$$t_R = (t_D + t_1)\frac{k_{\varphi_1} - k_{\varphi_n}}{k_{\varphi_1}} + t_2\frac{k_{\varphi_2} - k_{\varphi_n}}{k_{\varphi_2}} + \cdots + t_{(n-1)}\frac{k_{\varphi_{(n-1)}} - k_{\varphi_n}}{k_{\varphi_{(n-1)}}} + t_0(1 + k_{\varphi_n}) \tag{11}$$

If $(L_1 + L_2 + \cdots + L_{p-1})/L < 1$, the analyte is eluted during the last step and from $L_1 + L_2 + \cdots + L_{p,\text{final}} = L$ we obtain that t_R is given by Eq. (11) provided that subscript n is replaced by p.

3. An optimisation technique

When a certain stepwise variation pattern of φ versus t is formed in the mixer, the gradient retention time t_R can be easily calculated from Eq. (11), provided that the dependence of k upon φ is known. However, the determination of the best variation pattern of φ versus t that leads to the optimum separation of a mixture of solutes necessarily needs an optimisation technique. The optimisation technique we propose is based on the non-linear least squares algorithm developed in [28] and involves the following steps.

(1) We define the number of steps, say p, the maximum elusion time, $t_{R,max}$, and ranges within which the values of $\varphi_1, \varphi_2, \ldots, \varphi_p, t_1, t_2, \ldots, t_{p-1}$ vary.
(2) From these ranges a certain variation pattern of φ versus t, i.e. a certain set $\{\varphi_1, \varphi_2, \ldots, \varphi_p, t_1, t_2, \ldots, t_{p-1}\}$, is selected using random numbers and the values of t_R of all solutes are calculated by means of Eq. (11).
(3) The differences $\delta t_R = |t_R(\text{solute } i) - t_R(\text{solute } j)|$ are calculated for all possible values of i, j, and the minimum value of δt_R, δt_m, and the maximum t_R, t_{max}, are selected. If $t_{max} \leq t_{R,max}$, then δt_m and the set $\{\varphi_1, \varphi_2, \ldots, \varphi_p, t_1, t_2, \ldots, t_p\}$ are stored.
(4) Steps 2 and 3 are repeated m times and the maximum of the stored δt_m values, $\delta t_{m,max}$, is determined. In addition, the set $\{\varphi_1, \varphi_2, \ldots, \varphi_p, t_1, t_2, \ldots, t_p\}$ that corresponds to $\delta t_{m,max}$ is selected. To clarify these points consider that m is only 2, which means that we select using random numbers two sets $\{\varphi_1, \varphi_2, \ldots, \varphi_p, t_1, t_2, \ldots, t_{p-1}\}$. Then we calculate the corresponding chromatograms and the value δt_m that corresponds to each chromatogram. The maximum of these two values of δt_m is the quantity $\delta t_{m,max}$, which is determined during step 4 under the prerequisite that t_{max} of the chromatogram that corresponds to $\delta t_{m,max}$ is less than $t_{R,max}$.
(5) The components of the set $\{\varphi_1, \varphi_2, \ldots, \varphi_p, t_1, t_2, \ldots, t_p\}$ found in the previous step are used as initial estimates in a non-linear least squares routine, which determines the values of φ_i, t_i that give the maximum δt_m value under the constraint that $t_{max} \leq t_{R,max}$. It is evident that these values of φ_i, t_i correspond to a potentially good variation pattern of φ versus t. This variation pattern and its corresponding value of $\delta t_{m,max}$ are stored.
(6) Steps 2–5 are repeated q times and the best variation pattern of φ versus t is selected on the basis of the $\delta t_{m,max}$ values.

It is seen that the target of the optimisation is the maximization of δt_m, i.e. the maximization of the minimum peak pair distance, $|t_R(\text{solute } i) - t_R(\text{solute } j)|$. This maximization is performed by means of a non-linear least squares routine, which uses a Monte-Carlo search for initial estimates. The objective function δt_m may be replaced by $\delta t_m/t_{max}$. In our case both these two functions gave similar results. A better objective function might be the minimum peak pair resolution, $R_s = 2\delta t_m/(w_i + w_j)$, where w_i, w_j are the widths of peaks i, j, the distance of which is equal to δt_m. The incorporation of R_s in our algorithm is straightforward provided that the dependence of w_i, w_j upon φ is known from the isocratic study of the analytes. However, in most of the cases the use of R_s leads to an unnecessary increase of the computations because if we adopt steps with increasing φ, as suggested in the present study, the dependence of peak's widths upon elution time is small (see Figs. 4–7) and therefore R_s is expected to give similar results with the functions δt_m and $\delta t_m/t_{max}$.

4. Experimental

The liquid chromatography system used for the gradient measurements is the same with that used in another paper of this series of publications [9]. Thus it consisted of a Shimadzu LC-10AD pump, equipped with a low pressure gradient system (FCV-10AL), a C_{18} column [250 mm × 4 mm MZ-Analysentechnik column (5 μm Inertsil ODS-3)] thermostatted by a CTO-10AS Shimadzu column oven at 25 °C, and a Gilson electrochemical detector (model 141) equipped with a glassy carbon electrode. The detection was performed at 0.8 V versus the Ag/AgCl reference electrode. The mobile phases were aqueous phosphate buffers (pH 2.5) modified either with methanol or acetonitrile. The eight catechol-related analytes were: dopamine (DA), serotonin (5HT), 3,4-dihydroxy phenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (HIAA), vanil-

lylmandelic acid (VMA), 5-hydroxytryptophol (HTOH), 3,4-dihydroxyphenyl glycol (HPG) and homovanillic acid (HVA).

Isocratic $\ln k$ versus φ data of the above analytes were taken from [29]. The data obtained in the presence of acetonitrile were recalculated using a constant t_0 value equal to 1.717 min, which corresponds to the average value of t_0 [29].

5. Data analysis

The value of t_D needed for the calculation of t_R from Eq. (11) can be obtained from the application of a two-step gradient with $t_1 = 0$. Then Eq. (11) yields:

$$t_R = t_D \frac{k_{\varphi_1} - k_{\varphi_2}}{k_{\varphi_1}} + t_0(1 + k_{\varphi_2}) \quad (12)$$

and therefore

$$t_D = k_{\varphi_1} \frac{t_R - t_0 - t_0 k_{\varphi_2}}{k_{\varphi_1} - k_{\varphi_2}} = (t_{\varphi_1} - t_0) \frac{t_R - t_{\varphi_2}}{t_{\varphi_1} - t_{\varphi_2}} \quad (13)$$

where t_{φ_1} and t_{φ_2} are the isocratic elution times in mobile phases with $\varphi = \varphi_1$ and $\varphi = \varphi_2$, respectively.

In order to determine t_D by means of Eq. (13) we used two solutes, HIAA and VMA, in both modifiers applying several steps in the variation of φ. From these values we obtained that in our experimental system $t_D = 4.6 \pm 0.2$ min [9].

The isocratic dependence of k upon φ was calculated from:

$$\ln k = a - \frac{c\varphi}{1 + b\varphi} + d\varphi \quad (14)$$

using for a, b, c and d the values of Table 2 in [9]. Note that Eq. (14) can be used as four- and as three-parameter equation. In the latter case d was set equal to zero.

The technique followed for finding the optimum isocratic separation of a mixture of analytes is described in [9]. For the stepwise gradient elution, a suitable macro has been written that realises at Microsoft Excel spreadsheets the optimisation techniques described in Section 3. The arrangement of the spreadsheet we used for a three-step gradient was as follows. The labels "φ_1=", "φ_2=", "φ_3=", "t_1=", "t_2=", "$\delta t_{m,max}$=" are deposited in cells A2–A7 and the lower and the higher limits of the set $\{\varphi_1, \varphi_2, \varphi_3, t_1, t_2\}$ are inserted in cells B2–B6 and C2–C6, respectively. The macro using random numbers selects from these ranges a certain set of values and calculates the values of t_R of all solutes by means of Eq. (11), the objective function δt_m, and finally selects

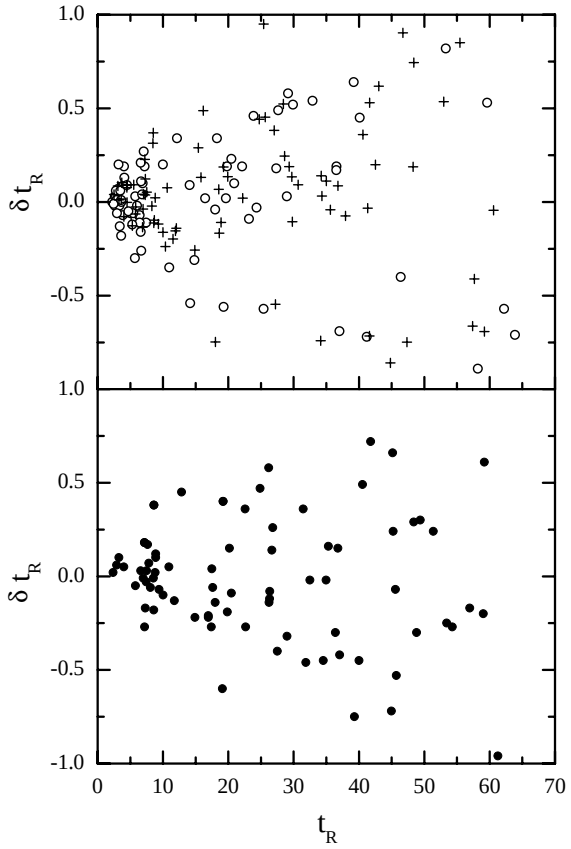

Fig. 2. Differences between experimental and predicted retention times under various stepwise (●), isocratic (○) and linear gradient (+) variation patterns of φ in aqueous mobile phases modified with methanol. Data (○) and (+) were taken from [9].

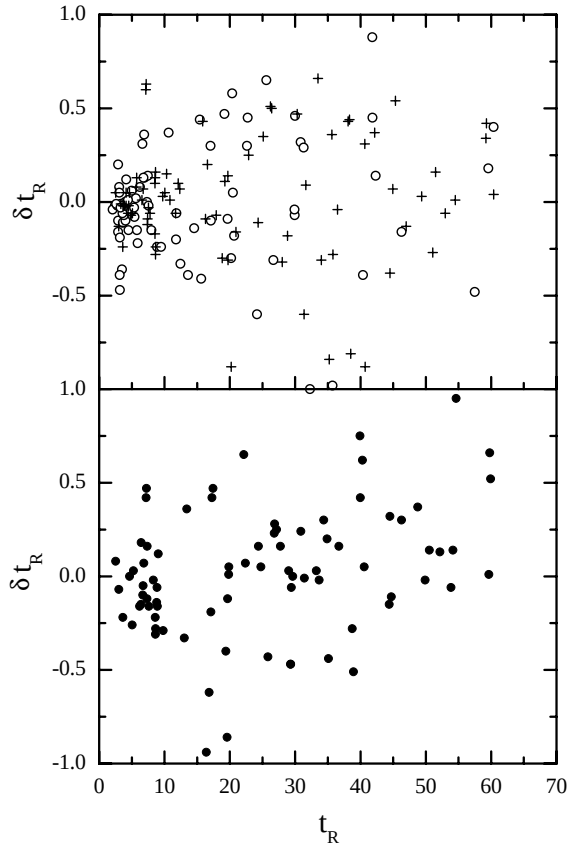

Fig. 3. Differences between experimental and predicted retention times under various stepwise (●), isocratic (○) and linear gradient (+) variation patterns of φ in aqueous mobile phases modified with acetonitrile. Data (○) and (+) were taken from [9].

t_{max}. If $t_{max} \leq t_{R,max}$, then δt_m and the set $\{\varphi_1, \varphi_2, \varphi_3, t_1, t_2\}$ are stored. This procedure is repeated ~1000 times and the maximum of the stored δt_m values and the corresponding $\{\varphi_1, \varphi_2, \varphi_3, t_1, t_2\}$ set are selected. The macro deposits this set in cells D2–D6. The label "δt_m=‘’ is deposited in cell C13 and one row below the following labels are deposited in cells A14–I14: "$\ln k(\varphi_1)$", "$\ln k(\varphi_2)$", "$\ln k(\varphi_3)$", "c_1", "c_2", "t_a", "t_b", "t_c" and "t_R". In cells A15–A22 the values of $\ln k_{\varphi_1}$ are calculated for all analytes of the mixture by means of Eq. (14) using the value of φ_1 in cell D2 and the values of parameters *a*, *b*, *c* and *d* of Eq. (14), which are deposited in cells G30:J37. The same procedure is repeated for $\ln k_{\varphi_2}$ and $\ln k_{\varphi_3}$ in cells B15–B22 and C15–C22 using for φ the values φ_2 and φ_3 in cells D3 and D4, respectively. In the next two columns, D15–D22 and E15–E22 the quantities $c_1 = (t_1 + t_D)/t_0 k_{\varphi_1}$ and $c_2 = (t_1 + t_D)/t_0 k_{\varphi_1} + t_2/t_0 k_{\varphi 2}$ are calculated, respectively, using t_1 and t_2 values from cells D5 and D6. Note that these quantities appear in the left-hand side of inequalities 10. In the next columns the following quantities are computed: $t_a = t_0(1 + k_{\varphi_1})$ in F15–F22 provided that $c_1 \geq 1$, $t_b = (t_D + t_1)(k_{\varphi_1} - k_{\varphi_2})/k_{\varphi_1} + t_0(1 + k_{\varphi_1})$ in G15 to G22 when $c_2 \geq 1$, and $t_c = (t_D + t_1)(k_{\varphi_1} - k_{\varphi_3})/k_{\varphi_1} + t_2(k_{\varphi_2} - k_{\varphi_3})/k_{\varphi_2} + t_0(1 + k_{\varphi 3})$ in H15–H22. Now the gradient time t_R is calculated in cells I15–I22 from the minimum value of t_a, t_b and t_c. For the determination of δt_m the differences $\delta t_R = |t_R(\text{solute } i) - t_R(\text{solute } j)|$ are calculated in cells A30–A57, since there are $8!/(6!2!) = 28$ values of δt_R, and the minimum of these values, which is the objective quantity δt_m, appears in D13. This cell is the target cell of Solver, which runs through the macro to maximise δt_m by changing cells D2–D6. The solution of Solver appears in cells E2–E6 and the maximum δt_m is deposited in cell E7. The whole procedure is repeated 50–100 times and each solution with the maximum δt_m is stored in subsequent columns starting from E2–E7. Now the determination of the best variation pattern that leads to the optimum separation of a mixture of solutes as well as the examination of a certain solution of Solver is straightforward. However, for the proper function of the macro certain constraints should be imposed to Solver. For example, t_1, t_2 cannot take negative values and φ_i cannot exceed 1 or be negative.

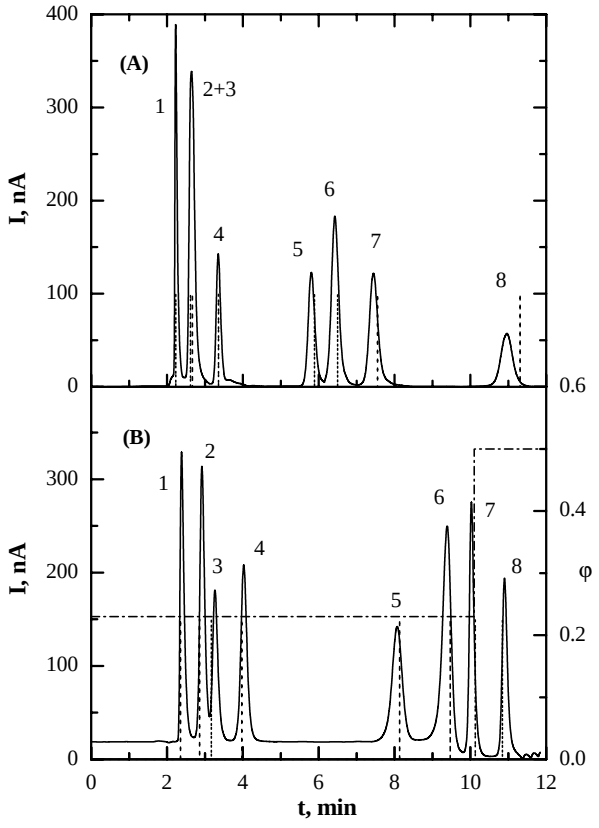

Fig. 4. Electrochemical detection chromatograms of an eight-component mixture composed of (1) DA; (2) HPG; (3) 5HT; (4) VMA; (5) DOPAC; (6) HTOH; (7) HIIA; and (8) HVA. They are recorded in an aqueous mobile phase modified with methanol under (A) isocratic conditions using $\varphi = 0.29$ and (B) using two steps in φ:$\varphi_1 = 0.23$, $\varphi_2 = 0.50$, and $t_1 = 3.5$ min, which correspond to the optimum separation of the mixture when $t_{R,max} = 12$ min. The dotted vertical lines indicate the predicted retention times by means of Eq. (14) (A) and Eqs. (11) and (14) (B), whereas the dash-dotted line shows the variation pattern of φ when it reaches the electrochemical detector.

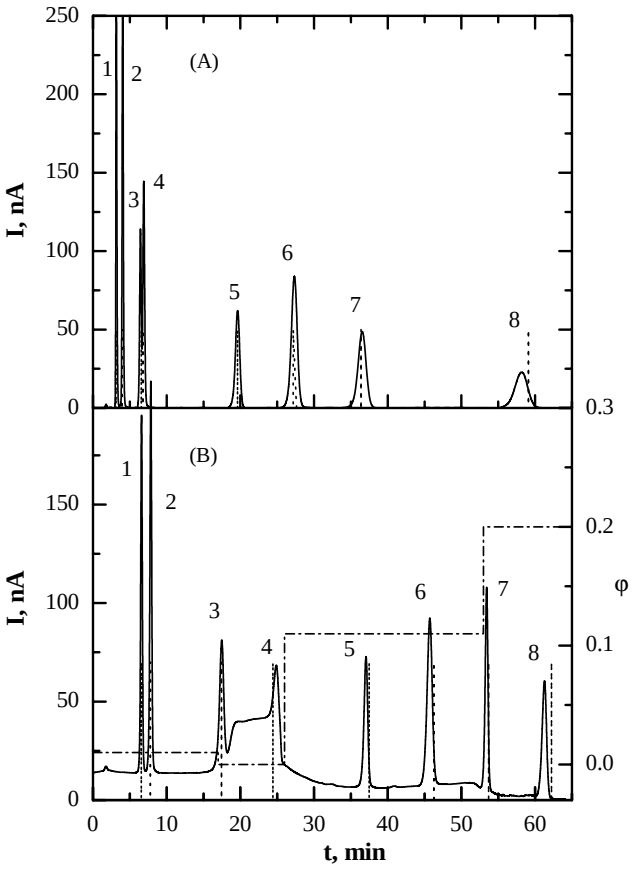

Fig. 5. As in Fig. 4 but for (A) $\varphi = 0.11$ and (B) using a four-steps variation pattern, $\varphi_1 = 0.01$, $\varphi_2 = 0$, $\varphi_3 = 0.11$, $\varphi_4 = 0.2$, $t_1 = 10$, $t_2 = 9$, and $t_3 = 27$ min, when $t_{R,max} = 65$ min.

6. Results and discussion

The mixer used in the experimental set up can mix four solvents. For this reason we used two- three- and four-step variation patterns in φ to test the theory. In particular, the validity of the proposed equations and the effectiveness of the optimisation technique were tested by separating eight catechol-related solutes with mobile phases modified by methanol or acetonitrile and variation patterns of two, three or four steps in the φ values. For comparison with our previous work on the linear gradient elution [9] we adopted three preset maximum elution times, $t_{R,max} = 12$, 45 and 65 min, respectively, and we recorded 10 chromatograms per modifier; 7 to test the proposed equations and 3 to test the optimisation technique.

Figs. 2 and 3 show the differences, δt_R, between experimental and predicted retention times under various stepwise variation patterns of φ in aqueous mobile phases modified with methanol and acetonitrile, respectively. These figures include also the corresponding differences under isocratic and linear gradient elution conditions taken from [9]. The predicted stepwise retention times have been calculated from Eq. (11) accounting for inequalities 10 and

Table 1
Mean value of the absolute differences between experimental and predicted retention times

Modifier	*P*-value[a]	Mean value of $\lvert\delta t_R\rvert$		
		Isocratic	Linear gradient	Stepwise gradient
MeOH	4	0.221	0.257	0.252
MeOH	3	0.495	0.399	0.332
ACN	4	0.232	0.230	0.254
ACN	3	0.458	0.369	0.342

[a] Number of the adjustable parameters used in Eq. (14).

using the four-parameter Eq. (14) for the isocratic dependence of k upon φ. It is seen that the maximum deviation of the predicted retention times from the experimental ones is always less than 1 min. Moreover, the deviations between theory and experiment obtained under stepwise gradients are of the same order with those obtained isocratically and under linear gradient elution. The same conclusion arises also from the absolute mean values of the differences between experimental and predicted retention times listed in Table 1. Therefore, the equations developed above describe absolutely satisfactorily the stepwise

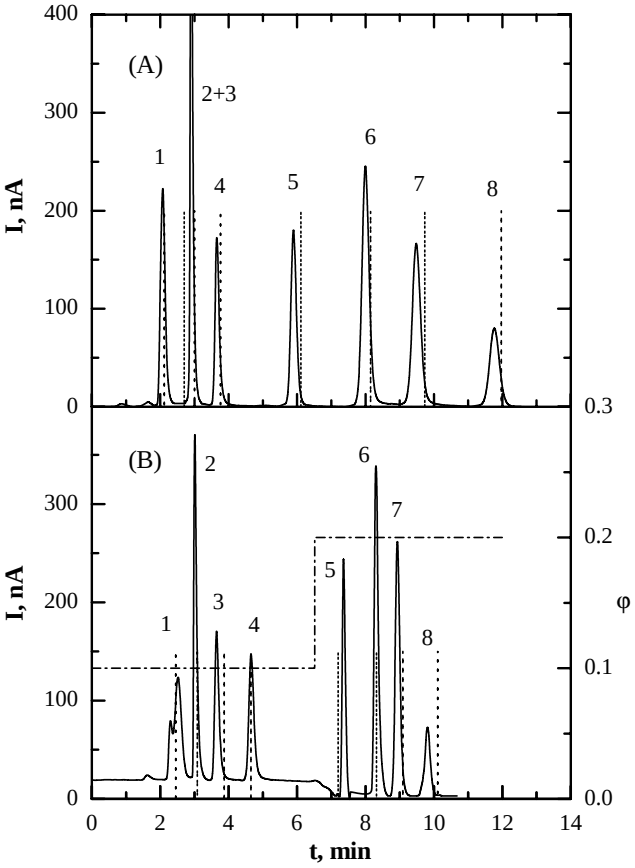

Fig. 6. As in Fig. 4 but for acetonitrile instead of methanol using (A) $\varphi = 0.14$ and (B) a two-steps variation pattern of φ of acetonitrile–water solutions, $\varphi_1 = 0.1$, $\varphi_2 = 0.2$, and $t_1 = 0$ min, when $t_{R,max} = 15$ min.

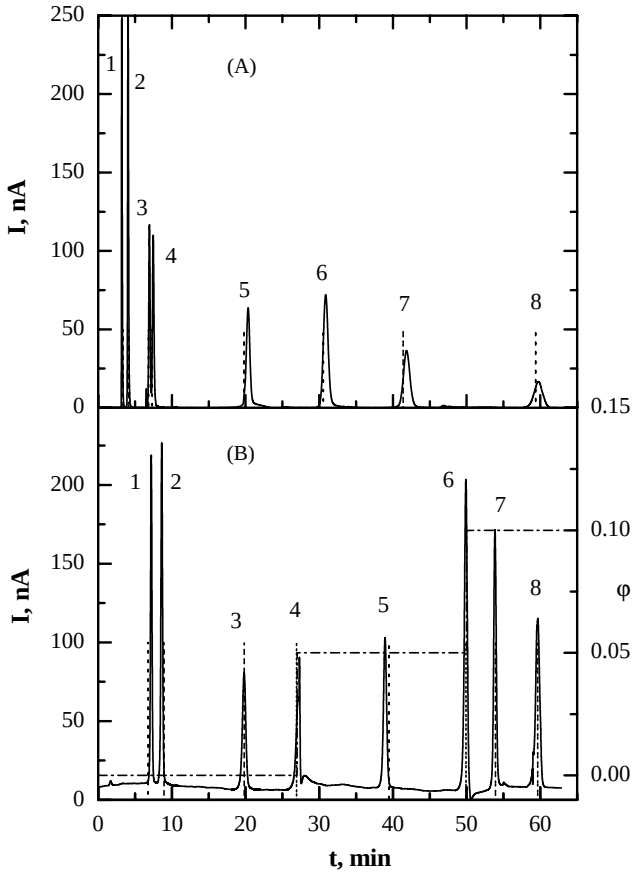

Fig. 7. As in Fig. 4 but for acetonitrile instead of methanol using (A) $\varphi = 0.05$ and (B) a three-steps variation pattern of φ of acetonitrile–water solutions, $\varphi_1 = 0$, $\varphi_2 = 0.05$, $\varphi_3 = 0.1$, $t_1 = 20$, and $t_2 = 23$ min, when $t_{R,max} = 65$ min.

gradient elution. However, as in [9], a basic prerequisite for this is the accurate representation of the isocratic behaviour of the individual solutes. Thus from Table 1 we note that the use of Eq. (14) as a three-parameter equation ($d = 0$) increases the error in the predicted retention times.

In what concerns the performance of the proposed optimisation technique, we found that more than one patterns of φ variation can lead to equally well chromatograms, especially when we adopt variation patterns of three or four steps in the φ values. Selected chromatograms recorded under optimum separation conditions using two, three and four steps are shown in Figs. 4–7, where the dash-dotted lines represent the variation pattern of φ when it reaches the electrochemical detector. We readily conclude that the proposed optimisation technique is both effective and flexible, since we can use several variation schemes of φ in order to achieve the best separation. A comparison with the isocratic elution (shown in the same figures) and the linear gradient elution presented in [9] reveals the following.

The effectiveness of the stepwise gradient elution for separation of the constituents of a mixture is comparable to that of the linear gradient elution. For example, the separation of the mixture of catecholamines can be achieved at a maximum elution time less than 12 min, as in the case of the linear gradient elution. However, as pointed out above, the advantage of the stepwise gradient is that we can find more than one variation patterns of φ that can be used for an optimum separation at a certain maximum elution time, $t_{R,max}$. For example, the separation of the mixture of catecholamines in acetonitrile–water mixtures can be achieved at $t_{R,max} = 12$ min using the following two-steps patterns: ($\varphi_1 = 0.1$, $\varphi_2 = 0.2$, $t_1 = 0$), ($\varphi_1 = 0.09$, $\varphi_2 = 0.2$, $t_1 = 2$) and ($\varphi_1 = 0.09$, $\varphi_2 = 0.17$, $t_1 = 0$). Therefore, the stepwise gradient elution technique is more flexible than that of the linear gradient elution.

However, in order to take well-shaped chromatograms two precautions should be taken into account. First, we should use steps with increasing φ, i.e. $\varphi_1 < \varphi_2 < \varphi_3 < \cdots$, in order to obtain sharp peaks even at great t_R values. Second, and most important, we should have in mind that the change in the mobile phase composition may change the base line of the chromatograms. The same phenomenon has been observed in electrochemical detection chromatograms recorded under linear gradient conditions but here it is more pronounced (Figs. 4–6) and it may distort the shape of chromatographic peaks. Note the increase in the base line when φ decreases (Fig. 5), which may also result in peak distortion. These phenomena enhanced by the electrochemical detection used in the present study have no effect on the shape of the chromatographic peaks if we take the precaution the steps to occur at the intermediate of the peaks, a condition that can be easily checked and taken into consideration by the suggested optimisation technique.

7. Conclusions

The stepwise gradient elution is a powerful variation of the gradient elution. It gives analytical expressions for the gradient retention time t_R that can be used in simple optimisation techniques. The proposed optimisation technique combines good performance and great flexibility, since several variation schemes of φ can be used in order to achieve the best separation of a mixture of solutes. However, in order to record well-shaped chromatograms, especially when an electrochemical detection mode is used, two precautions should be taken into account: (a) we should use steps with increasing φ and (b) the changes in φ should occur at the intermediate of the predicted peaks.

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