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Journal of Chromatography A, 1068 (2005) 279–287
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

New approach to linear gradient elution used for optimisation in reversed-phase liquid chromatography

P. Nikitas *, A. Pappa-Louisi

Laboratory of Physical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

Received 10 March 2004; received in revised form 27 December 2004; accepted 2 February 2005

Abstract

A new mathematical treatment concerning the gradient elution in reversed-phase liquid chromatography when the volume fraction φ of an organic modifier in the water–organic mobile phase varies linearly with time is presented. The experimental $\ln k$ versus φ curve, where k is the retention factor under isocratic conditions in a binary mobile phase, is subdivided into a finite number of linear portions and the solute gradient retention time t_R is calculated by means of an analytical expression arising from the fundamental equation of gradient elution. The validity of the proposed analytical expression and the methodology followed for the calculation of t_R was tested using eight catechol-related solutes with mobile phases modified by methanol or acetonitrile. It was found that in all cases the accuracy of the predicted gradient retention times is very satisfactory because it is the same with the accuracy of the retention times predicted under isocratic conditions. Finally, the above method for estimating gradient retention times was used in an optimisation algorithm, which determines the best variation pattern of φ that leads to the optimum separation of a mixture of solutes at different values of the total elution time.

Keywords: Gradient elution; Optimisation techniques; Liquid chromatography

1. Introduction

Gradient elution is in principle a powerful method that enhanced considerably the separation and peak detection capabilities of many branches of chromatography [1,2]. It is based on programmed separation modes. Thus in reversed-phase liquid chromatography (RP-HPLC) the useful program modes are the mobile phase composition, the flow rate and the column temperature. From these modes the most important is the mobile phase composition and thus the characteristic feature of gradient elution is the programmed change in mobile phase composition. Up to now linear gradients are the most widely used, because they can be described by simple theoretical relationships. However, when gradient elution is used in HPLC linear gradients do not necessarily result in a simple and explicit expression of the retention time in terms of the gradient mode characteristics. This is possible only if $\ln k$ varies linearly with φ, where k is the isocratic retention factor and φ is the volume fraction of the organic modifier in the water–organic mobile phase.

The combination of linear gradient with a linear dependence of $\ln k$ upon φ is called linear solvent strength gradient [2–12]. This approach constitutes the base of DryLab®, the most widely published HPLC simulation package to date [11,12]. An alternative optimisation package, PREOPT, has been proposed by Cela et al. and it is based on the approximation of any linear, curved or composite gradient by a stepwise profile [13–19]. In the present paper we suggest an optimisation technique based on the property that every non-linear $\ln k$ versus φ curve can be subdivided into a finite number of linear portions. This approach is used to develop an analytical expression for the solute retention time under gradient conditions. Our final target is to use this new analytical expression for linear gradient elution in an optimisation algorithm, which will determine the best variation pattern of φ that will lead to the optimum separation of a mixture of solutes.

* Corresponding author. Tel.: +30 2310 997773; fax: +30 2310 997709.
E-mail address: nikitas@chem.auth.gr (P. Nikitas).

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doi:10.1016/j.chroma.2005.02.004

2. Fundamental equations

The fundamental equation for gradient elution may be expressed as

$$\int_0^{t_R-t_0} \frac{dt}{t_\varphi - t_0} = \int_0^{t_R-t_0} \frac{dt}{t_0 k_\varphi} = 1 \quad (1)$$

where t_R is the solute gradient elution time, $t_\varphi \equiv t_{R(\varphi)}$ is the isocratic retention time when the mobile phase composition is constant and equal to φ, t_0 is the column hold up time and $k_\varphi = (t_\varphi - t_0)/t_0$ is the solute retention factor which corresponds to a constant mobile phase composition equal to φ. The derivation of Eq. (1) has been given in a series of publications by Snyder and coworkers [20–25], whereas the origin of this equation may be found in the work carried out by Freiling [26,27] and Drake [28]. An alternative derivation of Eq. (1) is given in [1].

In the linear gradient elution φ varies linearly with time t. Thus in general we have

$$\varphi = \begin{cases} \varphi_{in}, & \text{when } t \le t_{in} \\ \varphi_{in} + \frac{Bt}{t_0}, & \text{when } t > t_{in} \end{cases} \quad (2)$$

It is seen that the φ versus t profile described by Eq. (2) consists of two parts: an initial isocratic part with $\varphi = \varphi_{in}$ for $t \le t_{in}$, and a second part where φ increases or decreases linearly with time for $t > t_{in}$. Note that t_{in} is a programmable time duration, the extent of which determines the initial isocratic part and depends on the optimum separation conditions selected for each sample. Note also that a certain time, the dwell time, t_D, is needed for a certain change in the mixer to reach the beginning of the chromatographic column. This means that the first change in φ reaches the beginning of the column at a time equal to $t_D + t_{in}$. If we take into account the first isocratic part, then the basic equation in gradient elution may be written as [29]

$$\int_0^{t_R-t_0-t_{in}-t_D} \frac{dt}{t_0 k_\varphi} = 1 - \frac{t_D + t_{in}}{t_0 k_{\varphi_{in}}} \quad (3)$$

3. A new strategy for optimisation using linear gradient elution

3.1. Retention prediction

In order to obtain an analytical expression for t_R from Eq. (3), we may proceed as follows. Consider that the retention behaviour of a solute has been studied in the φ region from φ_0 to φ_m and the dependence of $\ln k$ upon φ, i.e. the function $\ln k = f(\varphi)$, has been determined. The $\ln k$ versus φ curve is in general not linear but it can be always subdivided into m linear portions. This means that the region $[\varphi_0, \varphi_m]$ can be divided into m portions, $[\varphi_i, \varphi_{i+1}]$, $i = 0, 1, 2 \ldots, m-1$, in which we have

$$\ln k_i = \ln k_{0i} - b_{i\varphi} \quad (4)$$

where

$$b_i = \frac{-[f(\varphi_{i+1}) - f(\varphi_i)]}{(\varphi_{i+1} - \varphi_i)} \quad \text{and} \quad \ln k_{0i} = f(\varphi_i) + b_i \varphi_i \quad (5)$$

Now it can be shown (Appendix A) that Eq. (3) is reduced to

$$t_R = t_D + t_{in} + t_0 + \frac{C t_0}{B b_{i+n}} \quad (6)$$

provided that $B > 0$. Here, the symbols C, i and n are explained in Appendix A. If $B < 0$, a rarely used gradient profile since it results to longer separation times in comparison to isocratic elution, then Eq. (6) may also be used after a proper re-evaluation of the dependence $\ln k = f(\varphi)$ using the symbol φ for the volume fraction of water in the mobile phase.

Note that in the proposed approach the calculated $\ln k$ versus φ curve and not the experimental one is subdivided into m linear portions. Therefore, the number m of the linear portions may be arbitrarily high and it is in no case related to the number of the experimental $\ln k$ versus φ data, which are usually very limited. Note also that from a mathematical point of view the multi-linear segment curve defined from Eq. (4) becomes identical to the calculated $\ln k$ versus φ curve in the limit $m \to \infty$, which means that in this limit Eqs. (6) and (3) give identical values for t_R. Therefore, a basic prerequisite for the application of Eq. (6) is to find out a reasonably small number for m so that the values of t_R calculated from Eq. (6) converge satisfactorily to those calculated from the basic Eq. (3), i.e. their absolute difference to be lower than a preset value, say 0.5%. As shown in the Section 6, for the systems we study a value $m = 10$ can yield differences between t_R values calculated from Eqs. (6) and (3) lower than 0.5% even if the $\ln k$ versus φ curve exhibits strong curvature.

The calculation of t_R from the basic Eq. (3), i.e. the computation of the root of Eq. (3), was attained by means of Newton's method. At each iteration of this method the integral of Eq. (3) was calculated using Simpson's rule. The number of terms in the sum of Simpson's rule was held constant and equal to 501. The number of iterations was not held constant. The iterations were terminated when two successive approximations of the root differed less than 0.01 min. Newton's method needs an initial estimate of the root to start the iterations and for this purpose we used as initial estimator the retention time obtained from Eq. (6).

3.2. Optimisation algorithm

In the present paper, Eq. (6) was used not only for prediction of the gradient retention time but also in an optimisation technique, which determines the best variation pattern of φ, i.e. the best values of t_{in}, φ_{in} and B, that leads to the optimum

separation of a mixture of solutes. The proposed optimisation technique involves the following steps:

(a) Starting with $t_{in} = 0$ we search for the best pair (φ_{in}, B) selected from preset sets of φ_{in} and B values. In particular, the gradient retention times of all solutes are calculated by means of Eq. (6) at a certain pair of φ_{in} and B values. Then 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 and the maximum t_R, $t_{R,max}$, are selected. Thus we obtain two tables where Δt_R and $t_{R,max}$ are calculated at certain preset φ_{in}, B values. It is evident that the best pair of φ_{in}, B values is that which corresponds to the maximum Δt_R provided that $t_{R,max}$ is smaller than a preset value corresponding to the maximum elution time.
(b) The best value of φ_{in} is kept constant and the previous step is repeated with t_{in} in place of φ_{in}.
(c) Steps (a) and (b) are repeated successively several times until the best values of t_{in}, φ_{in} and B are determined. Then these values are used in Eq. (6) for the calculation of the gradient retention times of all solutes, which correspond to their optimum separation.

Precautions that should be taken into account for a successful application of the above algorithm are discussed in Section 6.

4. Experimental

The liquid chromatography system 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 (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 of the analytes was performed at 0.8 V versus the Ag/AgCl reference electrode.

Eight catechol-related solutes, dopamine (DA), serotonin (5HT), 3,4-dihydroxy phenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (HIAA), vanillylmandelic acid (VMA), 5-hydroxytryptophol (HTOH), 3,4-dihydroxyphenyl glycol (HPG) and homovanillic acid (HVA) were used to test the theory. The mobile phases were aqueous phosphate buffers (pH 2.5) modified either with methanol or acetonitrile. Their total ionic strength was held constant at $I = 0.02$ M. All chemicals were used as received from commercial sources. Note that the isocratic behavior of the above analytes has been studied in [30].

5. Data analysis

5.1. Determination of t_D

The value of t_D needed for the calculation of t_R from Eqs. (3) and (6) can be obtained as follows. If we consider a step increase in φ from $\varphi = \varphi_{in}$ to φ_1 at $t = 0$, i.e. using $t_{in} = 0$, then Eq. (3) yields

$$\frac{t_R - t_0 - t_D}{t_0 k_1} + \frac{t_D}{t_0 k_{in}} = 1 \tag{7}$$

and therefore

$$t_D = k_{in}\frac{t_R - t_0 - t_0 k_1}{k_{in} - k_1} = (t_{\varphi_{in}} - t_0)\frac{t_R - t_{\varphi_1}}{t_{\varphi_{in}} - t_{\varphi_1}} \tag{8}$$

where $t_{\varphi_{in}}$ and t_{φ_1} are the isocratic elution times when $\varphi = \varphi_{in}$ and $\varphi = \varphi_1$, respectively. In order to determine t_D by means of Eq. (8) we used two solutes, HIAA and VMA, in both modifiers applying several steps in the variation of φ. The steps used in the present investigation and the calculated values of t_D are shown in Table 1. From these values we obtain that in our experimental system we have $t_D = 4.6 \pm 0.2$ min.

Alternatively t_D can be obtained by a single step gradient where the concentration of methanol jumps from $\varphi = 1$ to $\varphi = 0$. Recording the absorbance curve by a UV detector (Shimadzu SPD-10A) working at 203 nm, we determined again the above value for $t_D = 4.6$ min.

5.2. Description of the isocratic elution

The application of Eq. (3) as well as the calculation of t_R from Eq. (6) require the functional dependence of k upon φ

Table 1
Experimental data for determination of t_D

Modifier	Solute	φ_{in}	φ_1	$t_{\varphi_{in}}$	t_{φ_1}	t_R	t_0	t_D
MeOH	HIAA	0.14	0.3	26.167	7.352	10.743	1.844	4.384
MeOH	HIAA	0.1	0.2	41.112	14.503	17.605	1.844	4.578
MeOH	HIAA	0.2	0.3	14.503	7.352	10.009	1.844	4.704
MeOH	VMA	0.02	0.14	15.124	5.687	8.886	1.844	4.502
MeOH	VMA	0.05	0.1	10.851	7.221	9.145	1.844	4.774
MeOH	VMA	0	0.05	19.922	10.851	13.250	1.844	4.781
ACN	HIAA	0.1	0.15	16.020	8.612	11.103	1.717	4.809
ACN	HIAA	0.06	0.2	39.013	6.182	10.052	1.717	4.396
ACN	HIAA	0.1	0.2	16.020	6.182	9.442	1.717	4.740
ACN	VMA	0	0.03	19.922	10.393	12.805	1.717	4.608
ACN	VMA	0.02	0.06	12.528	7.132	9.518	1.717	4.780
ACN	VMA	0	0.06	19.922	7.132	10.256	1.717	4.447

under isocratic conditions. For this reason we used the experimental data $\ln k$ versus φ of the eight catecholimines published in [30]. However, we should point out the following. The present treatment of the gradient elution requires the hold-up time, t_0, to be constant and independent of the mobile phase composition. This prerequisite is valid for mobile phases modified by methanol, where $t_0 = 1.844$ min, but not for mobile phases modified by acetonitrile, where t_0 varies from 1.52 to 1.83 min [30]. For this reason the $\ln k$ data taken from [30] for water–acetonitrile solutions were recalculated using a constant t_0 value equal to 1.717 min, which corresponds to the average value of t_0 for mobile phases modified by acetonitrile [30].

The functional dependence of k upon φ under isocratic conditions is succeeded by fitting the original and the recalculated $\ln k$ versus φ data to a certain equation. In order to examine the effect that this primary fitting procedure has on the predicted retention times under isocratic and gradient conditions, we used for fitting two-, three- and four-parameter equations. In particular, the basic equation used was the following empirical expression of $\ln k$

$$\ln k = a - \frac{c\varphi}{1 + b\varphi} + d\varphi \quad (9)$$

where a, b, c and d are adjustable parameters. It is a four-parameter equation and becomes a three-parameter equation if we put $d = 0$ and a two-parameter if $c = 0$. The analysis of data was carried out at Microsoft Excel spreadsheets using Solver for all fittings. The minimized quantity was the sum of squares of residuals, SSR. From this quantity, the standard error of fit, σ, was calculated by means of the relationship $\mathrm{SSR} = \sigma^2(N - p)$, where N is the number of fitted data points and p (=2, 3 or 4) is the number of the adjustable parameters. For the linear fitting of Eq. (9) with $c = 0$, we have examined two cases: (a) we fitted to Eq. (9) with $c = 0$ the complete data set in the φ region from 0.1 to 0.5 for MeOH and 0.06 to 0.3 for ACN, and (b) we have used only two data points from the linear region to calculate parameters a and d of this equation. The points selected were at $\varphi = 0.2$ and 0.4 for MeOH and $\varphi = 0.1$ and 0.2 for ACN.

The results obtained from the fitting procedures when Eq. (9) is treated as a three- and four-parameter equation are shown in Table 2. This Table shows also the t-ratio values of parameter d calculated from $t\text{-ratio} = d/\sigma_d$, where d is the absolute value of parameter d and σ_d its standard error obtained from the curvature matrix method [31]. Taking into account that parameter d is statistically significant only if its t-ratio is greater than 2 [32], we readily conclude that parameter d is necessary for the description of the isocratic data in mobile phases modified by methanol, whereas half of the systems in water–ACN solutions can be described by using $d = 0$ in Eq. (9). The same results arise from the application of the F-test [32]. However, at this point we should clarify that these results concern strictly the isocratic behaviour of the solutes we study. Under gradient conditions, the choice of the proper relationship between $\ln k$ and φ is governed by the necessity of this relationship to reproduce the experimental data as accurately as possible within the maximum gradient

Table 2
Fitted parameters of Eq. (9)

Parameter	DA	HPG	5HT	VMA	DOPAC	HTOH	HIAA	HVA
Methanol–water								
a	1.115	1.324	2.625	2.289	3.805	4.423	4.808	5.353
b	2.545	5.375	1.425	5.689	7.442	6.269	6.324	7.380
c	20.644	14.201	22.506	13.769	15.366	18.296	18.547	19.744
d	2.618	−2.009	4.105	−3.367	−5.542	−5.567	−6.139	−6.531
σ	0.027	0.011	0.088	0.010	0.014	0.012	0.014	0.017
t-ratio*	2.2	9.4	0.5	20.3	29.0	29.4	28.1	28.4
a	1.140	1.294	2.647	2.244	3.729	4.349	4.729	5.259
b	4.113	2.782	2.315	2.036	1.527	1.758	1.644	1.648
c	20.187	13.695	19.716	13.645	15.003	18.226	18.743	18.936
d	–	–	–	–	–	–	–	–
σ	0.041	0.031	0.083	0.043	0.067	0.068	0.071	0.084
Acetonitrile–water								
a	1.081	1.436	2.794	2.355	3.869	4.495	4.878	5.408
b	0.167	31.159	10.357	4.170	6.249	8.151	7.514	8.240
c	19.416	39.369	53.469	32.689	36.481	42.499	43.303	48.044
d	–	−6.477	–	4.555	−1.797	−2.963	−2.893	−3.646
σ	0.164	0.132	0.114	0.037	0.031	0.020	0.027	0.045
t-ratio*	–	4.2	–	1.5	1.2	2.8	4.2	1.9
a	1.081	1.325	2.794	2.380	3.857	4.470	4.855	5.377
b	0.167	3.919	10.357	7.264	5.097	5.752	5.492	5.633
c	19.416	20.984	53.469	32.374	36.417	41.190	42.447	46.417
d	–	–	–	–	–	–	–	–
σ	0.164	0.182	0.114	0.049	0.031	0.038	0.038	0.055

* The t-ratio values concern parameter d.

time. It is evident that if this time is short enough, the dependence of $\ln k$ upon φ is likely to be linear. In this case the proposed optimisation technique is reduced to that based on the linear solvent strength gradient. However, in cases that the gradient time does not correspond to linear $\ln k$ versus φ plots, the use of three- or even four-parameter equations, like Eq. (9), is necessary.

Finally, the value of φ that corresponds to the optimum isocratic separation of a mixture of analytes was determined by the maximum value of $\Delta t_R = |t_R(\text{solute } i) - t_R(\text{solute } j)|$ at a certain preset maximum elution time, $t_{R,max}$. That is, Δt_R and $t_{R,max}$ were calculated by means of Eq. (9) and recorded as a function of φ. The maximum Δt_R at a certain $t_{R,max}$ determined the value of φ for optimum isocratic separation when the total elution time was less than $t_{R,max}$.

5.3. Analysis of data for gradient elution

For the application of the optimisation technique described above and in general for the application of the various equations developed above for the calculation of t_R, three macros have been written. The first macro is used to examine whether a certain subdivision of the $\ln k$ versus φ experimental isocratic curves into m linear portions is indeed effective. The second macro realises at a spreadsheet the first step of the proposed optimisation technique. This macro also calculates the predicted t_R values of a set of solutes at certain B, t_{in} and φ_{in} values. Finally, the third macro realises at a spreadsheet the second step, step (b), of the optimisation technique. On a PC Intel Pentium 4 computer the calculation of t_R from Eq. (6) followed by the numerical computation of t_R from Eq. (3) lasts less than 2 s.

6. Results and discussion

Two types of experiments have been carried out to verify the theory. The first type was directed to the verification of the two equations for gradient elution, i.e. Eqs. (3) and (6), since deviations from the ideal behaviour cannot be excluded [33–36], whereas the target of the second type of experiments was the evaluation of the effectiveness of the adopted optimisation technique. For the first type of experiments we recorded electrochemical detection (ED) chromatograms of a mixture of eight catecholamines under gradient elution involved arbitrary B, t_{in} and φ_{in} values. In contrast, for the second type of experiments we first obtained the optimum B, t_{in} and φ_{in} values for separation of the mixture of the eight catecholamines and using these values we recorded the ED chromatograms. Note that the optimum values of B, t_{in} and φ_{in} are closely related to the preset maximum elution time, $t_{R,max}$. In the present investigation we examined three $t_{R,max}$ values. In particular, we recorded 10 chromatograms per modifier; 7 corresponding to the first category of experiments and 3 to the second one with $t_{R,max}$ = 12, 45 and 65 min, respectively. In addition, for comparison we recorded other 10 chromatograms per modifier under isocratic conditions; 7 using arbitrary φ values and 3 corresponding to the optimum separation of the mixture when $t_{R,max}$ = 12, 45 and 65 min.

In order to carry out the above target we should first determine a reasonably small number for m so that the values of t_R calculated from Eq. (6) practically converge to those obtained from the basic Eq. (3). Extensive calculations have shown that a proper subdivision of the φ range into $m = 10$ linear portions yields differences between t_R values calculated from Eqs. (6) and (3) lower than 0.5% even if the $\ln k$ versus φ curve exhibits strong curvature. Some indicative results of these calculations are shown in Table 3. This table shows the differences between t_R values calculated from these two equations at three different subdivisions of the range [0,0.3] of φ values at acetonitrile–water solutions into linear portions. As expected when the $\ln k$ versus φ curve is effectively subdivided into many narrow linear portions, the predicted t_R values from Eq. (6) are in complete agreement with those calculated from Eq. (3). This observation can lead to a significant reduction of the computation time and effort, since it is much simpler to use Eq. (6) than Eq. (3). Based on these results a proper subdivision of the φ range into $m = 10$ linear

Table 3
Differences between t_R values calculated from Eq. (6) and those obtained from Eq. (3) and the correspondence percentage error when $\varphi_{in} = 0$, $\varphi_{max} = 0.3$, $t_{in} = 0$ and $B = 0.003$, at three different subdivisions of the range [0,0.3] of φ values at acetonitrile–water solutions into linear portions

Solute	t_R	Subdivision I[a]		Subdivision II[b]		Subdivision III[c]	
		δt_R	Percent	δt_R	Percent	δt_R	Percent
1	6.78	0.00	0.00	0.00	0.00	0.00	0.00
2	8.71	−0.03	0.34	−0.10	1.19	−0.13	1.55
3	19.54	−0.05	0.27	−1.30	6.67	−2.71	13.87
4	16.80	−0.02	0.11	−0.34	2.04	−0.71	4.25
5	36.70	−0.09	0.24	−0.87	2.36	−3.99	10.88
6	45.58	−0.14	0.31	−1.09	2.40	−5.51	12.10
7	52.08	−0.16	0.30	−1.13	2.17	−5.15	9.88
8	59.64	−0.24	0.40	−1.17	1.96	−4.64	7.79

The second column shows values of t_R calculated from Eq. (3).
[a] φ_i: 0, 0.01, 0.02, 0.03, 0.05, 0.07, 0.1, 0.15, 0.2, 0.3.
[b] φ_i: 0, 0.05, 0.1, 0.15, 0.2, 0.3.
[c] φ_i: 0, 0.1, 0.2, 0.3.

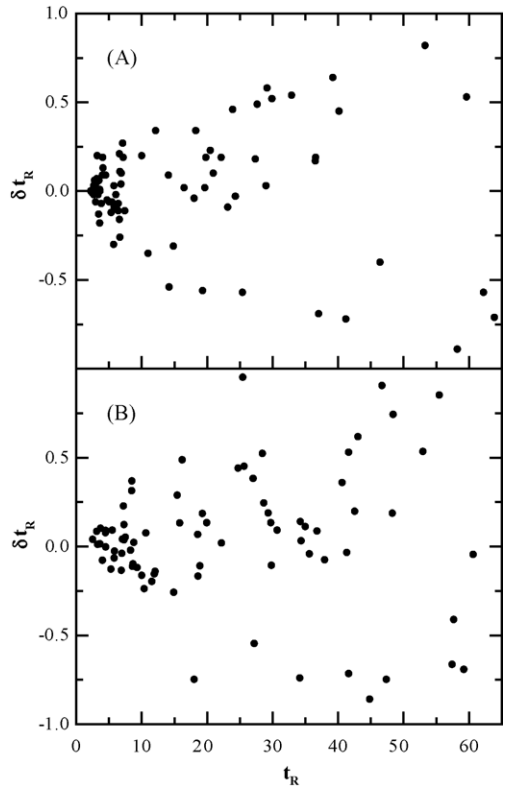

Fig. 1. Differences between experimental and predicted retention times under (A) isocratic elution using various φ values, and (B) various gradient elution schemes in aqueous mobile phases modified with methanol. The predicted retention times were calculated from Eq. (6) and the four-parameter Eq. (9) as described in text.

portions was adopted for all applications of Eq. (6) in this paper.

Fig. 1 shows the differences, δt_R, between experimental and predicted from Eq. (6) retention times under (A) isocratic elution using various φ values and (B) various gradient elution schemes in aqueous mobile phases modified with methanol when the four-parameter Eq. (9) is used for the prediction. The behaviour in aqueous mobile phases modified with acetonitrile is precisely the same. It is seen that the maximum deviation of the predicted retention times from the experimental ones is always less than 1 min and that these deviations obtained under gradient conditions is of the same order with those obtained isocratically. The same result arises also from the absolute mean values of the differences between experimental and predicted retention times listed in Table 4. Therefore, Eq. (6) and consequently Eq. (3) describe absolutely satisfactorily the gradient elution and consequently they can be used in optimisation procedures as that adopted in the present study. Note also that the accurate prediction of gradient retention by these equations shows that non-ideal effects, such as column equilibration during the change in the mobile phase composition, dependence of t_0 upon φ, distortion of gradient profile, etc., have a small or even negligible contribution.

Table 4
Mean value of the absolute differences, $\langle|\delta t_R|\rangle$, and the corresponding maximum absolute differences, $|\delta t_{R,max}|$, between experimental and predicted retention times

Modifier	p[a]	Isocratic		Gradient, Eq. (6)	
		$\langle\|\delta t_R\|\rangle$	$\|\delta t_{R,max}\|$	$\langle\|\delta t_R\|\rangle$	$\|\delta t_{R,max}\|$
MeOH	4	0.221	0.89	0.257	0.95
MeOH	3	0.495	2.05	0.403	1.95
ACN	4	0.232	1.17	0.230	0.88
ACN	3	0.458	2.23	0.375	1.45

[a] Number of the adjustable parameters used in Eq. (9).

Table 5
Optimum conditions for gradient elution separation

$t_{R,max}$	Methanol–water solutions, $\varphi_{max} = 0.5$			Acetonitrile–water solutions, $\varphi_{max} = 0.3$		
	t_{in}	φ_{in}	B	t_{in}	φ_{in}	B
12	0	0.2	0.08	0	0.1	0.04
45	10	0.02	0.02	10.6	0	0.011
65	11	0	0.0096	12.4	0	0.0044

Having verified the accuracy of Eq. (6), we proceeded to examine the effectiveness of the optimisation technique. The conditions for optimum separation under gradient elution obtained from this technique are given in Table 5. On the basis

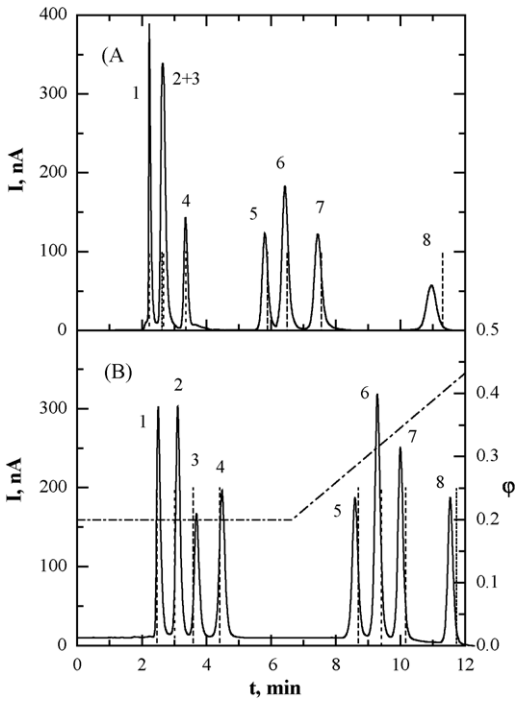

Fig. 2. ED chromatograms of an eight-component mixture composed of (1) DA, (2) HPG, (3) 5HT, (4) VMA, (5) DOPAC, (6) HTOH, (7) HIAA, and (8) HVA. They are recorded under (A) isocratic conditions in an aqueous mobile phase modified with methanol using $\varphi = 0.29$, and (B) gradient conditions using $\varphi_{in} = 0.2$, $\varphi_{max} = 0.5$, $t_{in} = 0$, $B = 0.08$, 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. (9) (A) and Eqs. (6) and (9) (B), whereas the dash-dotted line shows the variation pattern of φ when it reaches the electrochemical detector.

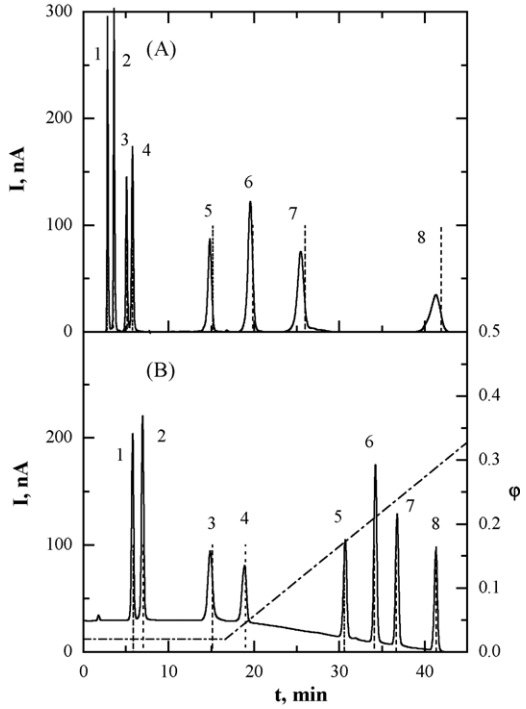

Fig. 3. As in Fig. 2 but for (A) $\varphi = 0.14$ and (B) $\varphi_{in} = 0.02$, $\varphi_{max} = 0.5$, $t_{in} = 10$ min, $B = 0.02$, which correspond to the optimum separation of the mixture when $t_{R,max} = 45$ min.

of these conditions the chromatograms have been recorded, some of which are depicted in Figs. 2–5. For comparison the corresponding chromatograms recorded under optimum isocratic conditions are also included in these figures.

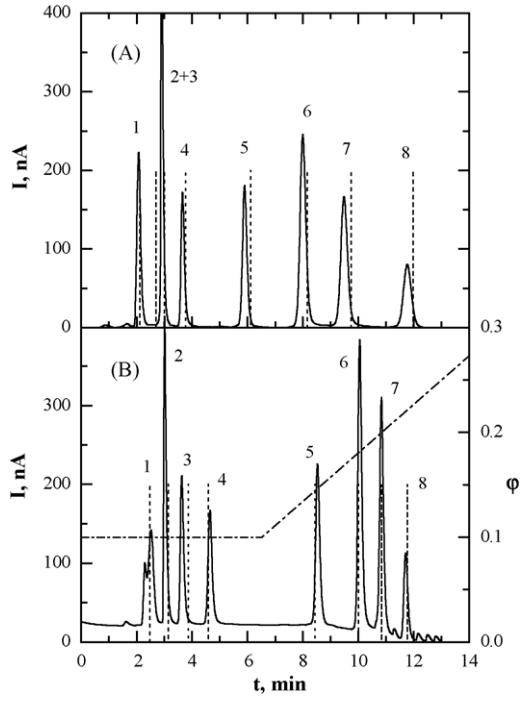

Fig. 4. As in Fig. 2 but for acetonitrile instead of methanol using (A) $\varphi = 0.14$ and (B) $\varphi_{in} = 0.1$, $\varphi_{max} = 0.3$, $t_{in} = 0$, $B = 0.04$.

From the recorded chromatograms we observe that the separation of the constituents of the mixture of catecholamines can be effectively take place at a maximum elution time about 12 min under gradient elution, whereas this is impossible under isocratic elution. Two other features are common in chromatograms recorded under gradient conditions: (a) The chromatographic peaks even at great times are sharp provided that during the elution the concentration of the organic solvent is increased in the mobile phase ($B > 0$), see for example Figs. 3 and 5; (b) the change in the mobile phase composition may change the base line of the chromatograms, especially when they are recorded using an electrochemical detector (Figs. 3 and 4).

Finally, we should point out that the choice of the equation used to represent the isocratic behaviour of a solute plays a significant role in the prediction of the retention time not only under isocratic but also under gradient conditions, because a reliable description of isocratic retention data is a prerequisite for a success use of all equations describing gradient retention data. Table 4 shows that the error in the predicted retention times is increased when we use a three-parameter equation, Eq. (9) with $d = 0$. Note that this error is doubled under isocratic conditions when we use Eq. (9) with $d = 0$. However, the increase in the prediction uncertainty appears at retention times greater than 30 min and for this reason the three-parameter Eq. (9) can be effectively used for optimisation in the systems we study. On the contrary, the two-variable Eq. (9) with $c = 0$, due to the fact that the substances chosen for the present study exhibit $\ln k$ versus φ curves with signif-

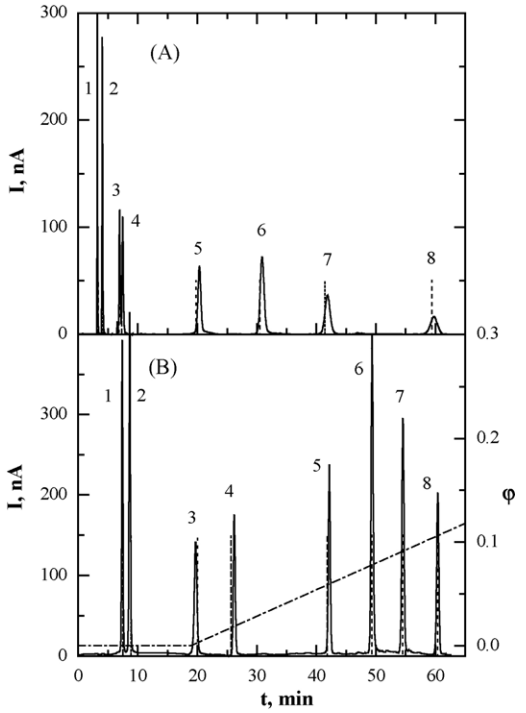

Fig. 5. As in Fig. 2 but for acetonitrile instead of methanol using (A) $\varphi = 0.05$ and (B) $\varphi_{in} = 0$, $\varphi_{max} = 0.3$, $t_{in} = 12.4$ min, $B = 0.0044$, which correspond to the optimum separation of the mixture when $t_{R,max} = 65$ min.

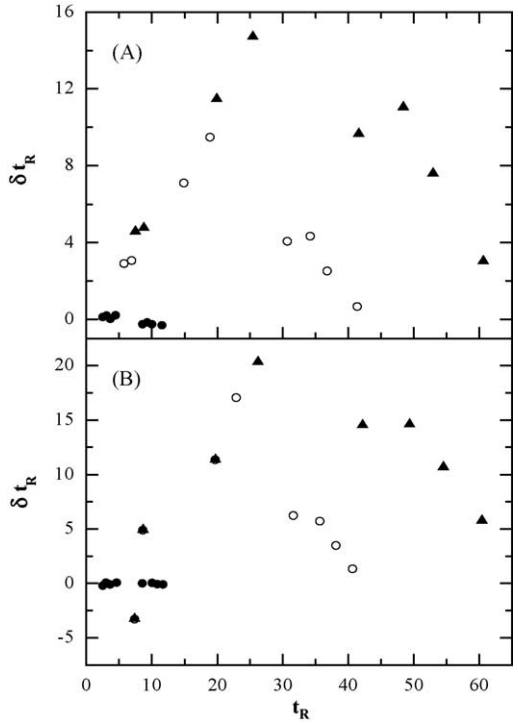

Fig. 6. Differences between experimental and predicted retention times under the optimum conditions of Table 5 in aqueous mobile phases modified with (A) methanol and (B) ACN. Points correspond to $t_{R,max}$ = 12 (●), 45 (○) and 65 (▲) min. For the predicted retention times Eq. (9) with $c = 0$ was fitted to two data points of the linear region, as described in text.

icant curvature, gives unacceptable results unless the maximum gradient time is small enough. In particular, we found that the gradient predictions by means of this equation are reasonably close to the experimental data only in the case of $t_{R,max}$ = 12 min. This is clearly depicted in Fig. 6 where we show the differences between experimental and predicted retention times under the optimum conditions of Table 5 in aqueous mobile phases modified with (A) methanol and (B) ACN. For the predictions Eq. (9) with $c = 0$ was fitted to two data points from the linear region, but the results are quite similar if we use the complete data set defined in Section 5.2. Note how great these differences are when $t_{R,max}$ = 45 and 65 min, irrespective of the modifier used.

7. Conclusions

To sum up the subdivision of the $\ln k$ versus φ curve of an analyte into a finite number of linear portions has as a consequence the fundamental Eq. (3) to be reduced to Eq. (6), which allows for the analytical calculation of t_R upon the gradient conditions and the isocratic behaviour of an analyte. With the proper subdivision Eq. (6) gives results that practically converge to those of the fundamental equation of gradient elution, Eq. (3), and they are in reasonably good agreement with experimental data. Therefore, Eq. (6) can be directly used in optimisation techniques, like the one adopted in the present paper. This technique was found to work effectively under all circumstances; the test mixture of the eight catecholamines was separated easily to its constituents even if the maximum elution time was set as low as 12 min, whereas this is impossible under isocratic elution.

Appendix A

Consider that the region $[\varphi_0, \varphi_m]$ has been divided into m portions, $[\varphi_i, \varphi_{i+1}]$, $i = 0, 1, 2 \ldots, m-1$, in which we have

$$\ln k_i = \ln k_{0i} - b_{i\varphi} \tag{A1}$$

where $\ln k_{0i}$ and b_i are given by Eq. (5). The calculation of t_R by means of Eq. (3) may be achieved as follows. First we find the interval $[\varphi_i, \varphi_{i+1}]$ in which φ_{in} belongs, i.e. we find the value of i which fulfils the condition $\varphi_i < \varphi_{in} < \varphi_{i+1}$ and establish the correspondence between t and φ_i values through the equation $\varphi_{i+1} = \varphi_{in} + Bt_{i+1}/t_0$. Note that $t_i = 0$.

Let us examine the case that B in Eq. (2) is positive and the solute is eluted before φ reaches its maximum value $\varphi_{fin} = \varphi_m$. Under these conditions Eq. (3) may be written as

$$\int_0^{t_{i+1}} \frac{dt}{t_0 k_{\varphi_i}} + \int_{t_{i+1}}^{t_{i+2}} \frac{dt}{t_0 k_{\varphi_{i+1}}} + \cdots + \int_{t_{i+n}}^{t_g - t_0 - t_D - t_{in}} \frac{dt}{t_0 k_{\varphi_{i+n}}} + I_S = 1 \tag{A2}$$

yielding

$$\sum_{j=i}^{i+n-1} A_j (e^{Bb_j t_{j+1}/t_0} - e^{Bb_j t_j/t_0}) + A_{i+n}(e^{Bb_{i+n}t_f/t_0} - e^{Bb_{i+n}t_{i+n}/t_0}) + I_S = 1 \tag{A3}$$

where, $t_i = 0$, $t_f = t_R - t_0 - t_D - t_{in}$, and A_j and I_S are given by

$$A_j = \frac{e^{b_j \varphi_{in}}}{Bb_j k_{0j}}, \qquad I_S = \frac{t_D + t_{in}}{t_0 k_{\varphi_{in}}} \tag{A4}$$

Note that the maximum number of integrals in Eq. (A2) is equal to $m - i$. Therefore, this is the maximum value of n, n being the smaller integer for which the following inequality is fulfilled

$$a_i + a_{i+1} + \cdots + a_{i+n} + I_S > 1 \tag{A5}$$

where

$$a_{i+j} = A_{i+j}(e^{Bb_{i+j}t_{i+j+1}/t_0} - e^{Bb_{i+j}t_{i+j}/t_0}) \tag{A6}$$

When the solute is eluted before φ reaches its maximum value φ_{fin}, there is always a certain value of $n \le m - i$ such that inequality (A5) is fulfilled. Then from Eq. (A3) we readily obtain that

$$t_R = t_D + t_{in} + t_0 + \frac{Ct_0}{Bb_{i+n}} \tag{A7}$$

where

$$C = \ln\left[\frac{1 - s_{i+n-1} - I_S + A_{i+n}\, e^{Bb_{i+n}t_{i+n}/t_0}}{A_{i+n}}\right] \quad \text{(A8)}$$

and

$$s_{i+n-1} = a_i + a_{i+1} + \cdots + a_{i+n-1} \quad \text{(A9)}$$

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