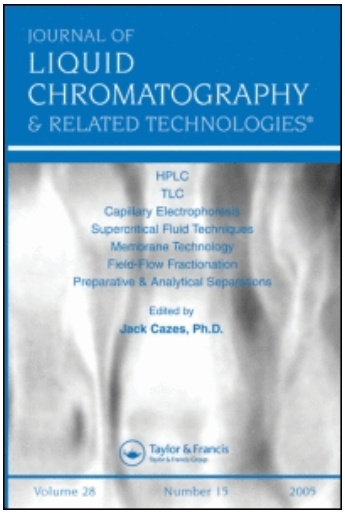

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New Approaches to Linear Gradient Elution Used for Optimization in Reversed-Phase Liquid Chromatography

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New Approaches to Linear Gradient Elution Used for Optimization in Reversed-Phase Liquid Chromatography

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Abstract: The various approaches developed for prediction and optimization in linear gradient elution in reversed-phase liquid chromatography are critically reviewed. These approaches concern both single-mode and multi-mode gradient elution, the latter involving either gradients related exclusively to the mobile phase composition or combined gradients of the mobile phase composition with flow rate and/or column temperature or combined gradients of flow rate and column temperature. The advantages and disadvantages of each method are discussed and special attention is devoted to the factors that affect the quality of the prediction and their impact to the optimisation. Finally, the fitting techniques and optimisation methods adopted in linear gradient elution are presented and the most effective algorithms used for this purpose are indicated and discussed.

Keywords: Linear gradient elution, Liquid chromatography, Optimization

INTRODUCTION

Gradient elution is, in principle, a powerful method that enhances considerably the separation and peak detection of many branches of

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chromatography.[1–6] It is based on programmed separation modes and, therefore, the operation conditions are changed during the elution, according to a pre-set program, so as to achieve adequate resolution for early eluted compounds and acceptable resolution and short elution times of the last eluted compounds. 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.

The profile, i.e., the shape of the gradient program, may involve a) linear gradients, b) non-linear gradients with curved profile, c) stepwise gradients, and d) segmented gradients which usually include linear with different slopes segments and isocratic periods often at the beginning and/or at the end of the elution. Up to now, single-mode linear solvent gradients which may include an isocratic portion at the beginning and the end in RP-HPLC are, by far, most frequent.[1,3–6] However, the gradient elution becomes more effective if it is combined with temperature,[7–12] and flow-rate programming.[12–14] The theory of multi-mode gradient elution involving combined gradients of the mobile phase composition with flow rate and/or column temperature or combined gradients of flow rate and column temperature is currently under development.[15–17]

The use of linear gradients is imposed, for reasons of simplicity, both at a theoretical and experimental level. However, when gradient elution is used in RP-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 gradients with a linear dependence of $\ln k$ upon φ is called linear solvent strength gradient.[18–27] This approach constitutes the base of DryLab, the most widely used simulation package to date.[25–27] Several other software packages have been marketed and can assist untrained users to set up separations. The most important of them are PREOPT-W,[28] OSIRIS,[29] MICHROM,[30,31] and CHROMSWORD.[32]

This review intends to show the developments in linear gradient elution used for both prediction and optimization in reversed-phase liquid chromatography. The different approaches proposed for both single-mode and multi-mode gradient elution are critically presented and their advantages and disadvantages are indicated. Special attention is devoted to the factors that affect the quality of the prediction and their impact to the optimisation. The most effective algorithms used for fitting and optimisation are also presented and discussed.

THE FUNDAMENTAL EQUATION OF GRADIENT ELUTION

In recent papers,[15–17] we have developed the theory of two-mode gradient elution. From this treatment, the single-mode gradient elution arises as a limited case. Here, we extend this treatment to any multi-mode gradients.

Consider the general case where a multi-mode gradient profile, (x_1, $x_2, \ldots$) vs. t, is formed in the mixer of the chromatographic system. Here, the coordinates x_1, $x_2, \ldots$ may be volume fractions φ_1, $\varphi_2, \ldots$ of the constituents of the mobile phase, the pH of this phase, the flow rate F, and/or the column temperature T. Each change in the values of these variables in the mixer is transformed to the chromatographic column with a velocity that depends on the separation variable. Thus, changes in F and T reach the analyte in the column almost instantaneously, whereas there is a certain delay for any change in φ_i and pH to reach the analyte also inside the column. Moreover, due to the fact that the analyte and the mobile phase are moved with different velocities, if we approximate a φ_i vs. t (or pH vs. t) profile in the mixer by a stepwise curve composed of a large number of infinitesimally small time steps Δt, the analyte feels each Δt step for a time period equal to δt_c different from Δt. Since δt_c is also an infinitesimally small time step, during δt_c all x_1, $x_2, \ldots$ variables may be considered constant. Therefore, the analyte moves at each time step δt_c with a constant velocity $v_a = L/t_{R,i}$ and covers a distance equal to

$$\frac{\delta L_i}{L} = \frac{\delta t_c}{t_{R,i}} = \frac{\delta t_c}{t_{o,i}(1 + k_i)} \tag{1}$$

where $t_{R,i}$, $t_{o,i}$, and k_i are the isocratic retention time of the solute, the column hold-up time, and the solute retention factor during the i-th time step, L is the column length, and δL_i the distance covered by the analyte during time δt_c. Equation (1), upon integration, results in the fundamental equation of gradient elution valid under any single- or multi-mode gradient profile:

$$\int_0^{t_R} \frac{dt_c}{t_o(1 + k)} = 1 \tag{2}$$

Two limiting expressions of this equation are particularly useful:

a) When the mobile phase composition varies at constant flow rate and column temperature, Eq. (2) yields[16,17,33]

$$\int_0^{t_R - t_0} \frac{dt}{t_0 k} = 1 \tag{3}$$

This is the fundamental equation for single-gradient elution and it has been first proposed by Snyder et al.[3,34–38]

b) If only F or T varies, Eq. (2) is transformed to[3,4,16,17,39]

$$\int_0^{t_R} \frac{dt}{t_o(1+k)} = 1 \tag{4}$$

In all other cases, we have to proceed through recursive relationships to estimate the elution time t_R.[16,17] This issue is discussed in the section "Real Multi-Mode Gradients" below.

RETENTION MODELS

The first step for an optimization process is the prediction of the elution times, t_R, of the solutes in the mixture under examination. That is, we should know, explicitly or implicitly, the expression $t_R = f(x_1, x_2, \ldots, x_m)$, where $x_1, x_2, \ldots, x_m$ are gradient parameters. Usually, the elution time t_R is determined by means of the retention factor, k, provided that k is known as a function of the isocratic properties of the system, $k = g(T, pH, \varphi_1, \varphi_2, \ldots)$.

When $k = g(T, pH, \varphi_1, \varphi_2, \ldots)$ is known, t_R is calculated from

$$t_R = t_0(1+k) \tag{5}$$

under isocratic conditions since $k = (t_R - t_0)/t_0$. Under gradient conditions, the expression $k = g(T, pH, \varphi_1, \varphi_2, \ldots)$ is used for the calculation of t_R through the solution of the fundamental equation of gradient elution, Eq. (2), or its various variations discussed in the previous section "The Fundamental Equation of Gradient Elution" and in the section "Real Multi-Mode Gradients" below. Note that k may be also expressed as $k = h(g_1, g_2, \ldots, g_m)$, where $g_1, g_2, \ldots, g_m$ are variables representing the gradient elution conditions, that is, the gradient steepness in linear gradients, the initial and the final composition of the mobile phase, etc. In this case, t_R is again calculated from Eq. (5). The equation that expresses k as a function of either T, pH, $\varphi_1, \varphi_2, \ldots, \varphi_m$ or $g_1, g_2, \ldots, g_m$ is called *retention model* since it is, directly or not, the mathematical expression of the model that expresses the retention mechanism.

For single-mode gradients in φ the most commonly used retention model is the linear one:[2–5,40,41]

$$\ln k(\varphi) = c_0 + c_1\varphi \tag{6}$$

which is, though, valid when the φ range is very narrow. For wider ranges of φ, popular models are the quadratic model:[35,42–46]

$$\ln k(\varphi) = c_0 + c_1\varphi + c_2\varphi^2 \tag{7}$$

and the rational function model:[45–47]

$$\ln k(\varphi) = c_0 - \frac{c_1\varphi}{1 + c_2\varphi} \tag{8}$$

They both exhibit similar applicability. The quadratic Eq. (7) has a simple form, well known properties, and it is computationally easy to use. Its major limitation is that it easily creates over-fitting problems. The rational function model, Eq. (8), exhibits very good fitting performance without over-fitting problems, but it may present convergence problems.

For gradients in T we have[11,48–54]

$$\ln k(T) = c_0 + \frac{c_1}{T} + c_2 g(T) \tag{9}$$

where the function g(T) is given by[48]

$$g(T) = \begin{cases} 0 & \text{when } \Delta H^o = \Delta H_0 \\ \ln T & \text{when } \Delta H^o = \Delta H_0 + \Delta H_1 T \\ T & \text{when } \Delta H^o = \Delta H_0 + \Delta H_1 T^2 \\ 1/T^2 & \text{when } \Delta H^o = \Delta H_0 + \Delta H_1/T \end{cases} \tag{10}$$

Here, ΔH° is the standard enthalpy of the retention process and ΔH_0, ΔH_1 are constants, coefficients of ΔH^o.

The mobile phase pH affects the retention of ionizable analytes.[55–61] Single-mode gradients in pH for a monoprotic acid or base can be described by the familiar expression

$$k = \frac{k_0 + k_1 10^{pH-pK}}{1 + 10^{pH-pK}} \tag{11}$$

which, in combination with Eq. (8), extends to the following retention model to include pH and φ effects[47,61]

$$k = \frac{k_0^0 e^{-c_0\varphi/(1+c_2\varphi)} + k_1^0 e^{-c_1\varphi/(1+c_2\varphi)} 10^{pH-pK^o-r_1\varphi-r_2\varphi^2}}{1 + 10^{pH-pK^o-r_1\varphi-r_2\varphi^2}} \tag{12}$$

where k_0^0, k_1^0, pK°, c_0, c_1, c_2, r_1, and r_2 are adjustable parameters. The same expression is obtained if we include activity coefficients.[61]

For retention in ternary systems, we may use[62–64]

$$\ln k(\varphi_B, \varphi_C) = c_0 + c_1\varphi_B + c_2\varphi_C + c_3\varphi_B^2 + c_4\varphi_C^2 + c_5\varphi_B\varphi_C \tag{13}$$

where φ_B and φ_C are the volume fractions of the two components of the mobile phase. Finally, for retention in systems of variable T and mobile

phase composition φ, we have[47,53,65]

$$\ln k(\varphi, T) = c_0 + c_1\varphi + c_2\varphi^2 + \frac{c_3 + c_4\varphi + c_5\varphi^2}{T} + (c_6 + c_7\varphi + c_8\varphi^2)g(T) \tag{14}$$

or alternatively[47]

$$\ln k = c_0 + \frac{c_1}{T} + c_2 g(T) - \frac{[c_3 + \frac{c_4}{T} + c_5 g(T)][\exp(c_6 + \frac{c_7}{T} + c_8 g(T))]\varphi}{1 + [\exp(c_6 + \frac{c_7}{T} + c_8 g(T)) - 1]\varphi} \tag{15}$$

In general, a simple relationship that can be used to obtain multi-variable expressions of ln$k$ is the following:[47,66]

$$\ln k(\varphi_1, \varphi_2, \cdots T) = \ln k(\varphi_1) \cdot \ln k(\varphi_2) \cdots \ln k(T) \tag{16}$$

Many other models have been proposed and a comprehensive discussion on retention models is presented in Ref. [65].

PREDICTION IN SINGLE-MODE GRADIENT ELUTION

In single-mode gradient elution, only one separation variable, the mobile phase composition φ, the column temperature T, the flow rate F, or the pH varies with time. Figure 1 shows, schematically, the various approaches used to predict the retention time of a sample solute under single-mode linear gradient elution. In particular, we have the following:

Variations in Mobile Phase Composition

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

$$\varphi = \begin{cases} \varphi_{in} & \text{when} \quad t \le t_{in} \\ \varphi_{in} + b(t - t_{in})/t_0 & \text{when} \quad t_{in} < t < t_{fin} \\ \varphi_{fin} & \text{when} \quad t \ge t_{fin} \end{cases} \tag{17}$$

Multilinear gradients involve linear segments with different slopes and isocratic parts often at the end or at the beginning of the elution. The prediction can be attained either by direct fitting to retention data or by solving the fundamental Eq. (3). The first method is limited

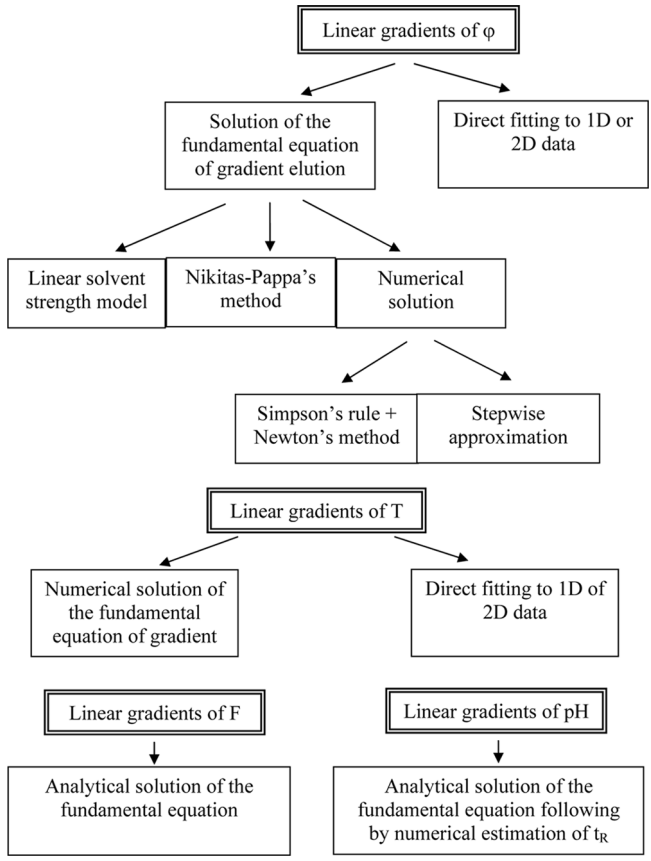

Figure 1. Various approaches used to predict the retention time of a sample solute under single-mode linear gradient elution.

to single gradients, whereas the second one may involve linear and multi-linear gradients.

Direct Fitting to 1D or 2D Data

This is a simple and quite effective approach when we use single gradients. For each solute, we form a 1D data set of retention times as a function of the slope b of the φ vs. t gradient provided that all gradients have the same initial value of φ, φ_{in} or a 2D table of retention times as a function of φ_{in} and the slope b of the φ vs. t gradient. The data are fitted to appropriate retention models, which arise from the properties of the t_R dependence upon b or upon φ_{in} and b. The following models

may be used:

$$y = c_0 + c_1 b + c_2 b^2 \tag{18}$$

for 1D data, and

$$y = c_0 + c_1 b + c_2 b^2 + c_3 \varphi_{in} + c_4 (\varphi_{in})^2 + c_5 b \varphi_{in} \tag{19}$$

for 2D data, where $y = t_R$ or ln k. From these equations, the prediction of the retention time of a sample solute under a single linear gradient is straightforward provided that b and φ_{in} of this gradient lie within those used in the fitting procedure.

Solution of the Fundamental Equation of Gradient Elution

When there is an initial isocratic portion in the gradient profile, Eq. (3) is written as[33,67]

$$\int_0^{t_R - t_D - t_0 - t_{in}} \frac{dt}{t_o k} = 1 - \frac{t_D + t_{in}}{t_0 k(\varphi_{in})} \tag{20}$$

where t_D is the dwell time, that is, the time needed for a certain change in the mixer to reach the column inlet. Note that, in this relationship, the zero of the time variable t is the point t_{in}. Thus the φ vs. t profile of Eq. (17) is transformed to

$$\varphi = \begin{cases} \varphi_{in} + bt/t_0 & \text{when} \quad t < t_{fin} - t_{in} \\ \varphi_{fin} & \text{when} \quad t \geq t_{fin} - t_{in} \end{cases} \tag{21}$$

For simplicity we denote by I_S the quantity

$$I_S = \frac{t_D + t_{in}}{t_0 k(\varphi_{in})} = \frac{t_D + t_{in}}{t_0 k_{in}} \tag{22}$$

If $I_S > 1$, then the analyte is eluted during the first isocratic portion of the gradient profile of Eq. (17) and, therefore, we have

$$t_R = t_0 (1 + k_{in}) \tag{23}$$

Let us denote by I_g the integral

$$I_g = \int_0^{t_{fin} - t_{in}} \frac{dt}{t_o k} \tag{24}$$

This integral can be calculated either analytically, if it is possible, or numerically using, for example, Simpson's rule. If $I_g < 1 - I_S$, then the analyte is eluted during the last isocratic step, after time t_{fin} and, from Eq. (20), we readily obtain that the retention time may be calculated from

$$t_R = t_0 + t_D + t_{fin} + t_0 k_{fin}(1 - I_S - I_g) \tag{25}$$

We observe that t_R may be easily predicted if the solute is eluted during the isocratic portions of the φ vs. t profile of Eq. (17). If it is eluted during the φ variation, t_R may be estimated by means of the following approaches:

a) Linear Solvent Strength Model. Equation (20) has an analytical solution if ln*k* is given by the linear expression of Eq. (6). The combination of linear gradients with Equation (6) is called linear solvent strength gradient[18–27] and constitutes the basis of DryLab.[25–27] If Eq. (21) is introduced into Eq. (6) when $t < t_{fin} - t_{in}$, we obtain

$$\ln k = A + Bt \tag{26}$$

where

$$A = c_0 + c_1\varphi_{in} \text{ and } B = c_1 b/t_0 \tag{27}$$

Substitution of Eq. (26) into Eq. (20) readily yields

$$t_R = t_0 + t_D + t_{in} - \frac{1}{B}\ln\{1 - t_0 B e^A(1 - I_S)\} \tag{28}$$

Therefore, the retention time of a sample solute is predicted by means of Eq. (23) when $I_S > 1$, Eq. (25) when $I_g < 1 - I_S$, or Eq. (28) in all other cases. The extension to multi-linear gradients is straightforward.

The linear solvent strength model is very simple and leads to an explicit expression for t_R. However, it is based on a rather poor approximation, Eq. (6), which is valid usually in aqueous-methanol mobile phases and in narrow φ ranges. Thus, depending on the experimental system, Eq. (28) may yield notably different retention times from the experimental ones.

b) Nikitas–Pappa's approach. In order to obtain an analytical expression for t_R from Eq. (20) without using the linear retention model of Eq. (6), we may proceed as follows.[67–69] Consider that the retention behavior of a solute has been studied in the φ region from φ_{in} to φ_{fin} 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 $[\varphi_{in}, \varphi_{fin}]$ is divided into m portions, $[\varphi_i, \varphi_{i+1}]$, $i = 0, 1, 2, \ldots,$ m-1, where $\varphi_0 = \varphi_{in}$ and $\varphi_m = \varphi_{fin}$. At each value of φ_i corresponds a certain t_i value through the expression

$$\varphi_{i+1} = \varphi_{in} + bt_{i+1}/t_0 \tag{29}$$

In addition, in each of these portions we have

$$\ln k_i = \ln k_i^0 - b_i\varphi \tag{30}$$

where

$$b_i = \frac{-[f(\varphi_{i+1}) - f(\varphi_i)]}{\varphi_{i+1} - \varphi_i} \tag{31}$$

and

$$\ln k_i^0 = f(\varphi_i) + b_i\varphi_i \tag{32}$$

Now, it can be shown that if $b > 0$, we have

$$t_R = t_0 + t_D + t_{in} + \frac{t_0 C}{bb_{i+n}} \tag{33}$$

where

$$C = \ln\left[\frac{1 - s_{i+n-1} - I_S + A_{i+n}e^{bb_{i+n}t_{i+n}/t_0}}{A_{i+n}}\right] \tag{34}$$

$$s_{i+n-1} = a_i + a_{i+1} + \cdots + a_{i+n-1} \tag{35}$$

$$a_{i+j} = A_{i+j}(e^{bb_{i+j}t_{i+j+1}/t_0} - e^{bb_{i+j}t_{i+j}/t_0}) \tag{36}$$

and

$$A_j = \frac{e^{b_j\varphi_{in}}}{bb_jk_j^0} \tag{37}$$

Here, n is the smaller integer for which the following inequality is fulfilled

$$a_i + a_{i+1} + \cdots + a_{i+n} + I_S > 1 \tag{38}$$

The method practically converges to t_R when $m \geq 10$. Thus, it is very fast, computationally not complicated, and, therefore, suitable for optimization algorithms. Moreover, it is easily extendable to multi-linear gradients.[68,69]

Figure 2 shows an application of the method to a mixture of 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

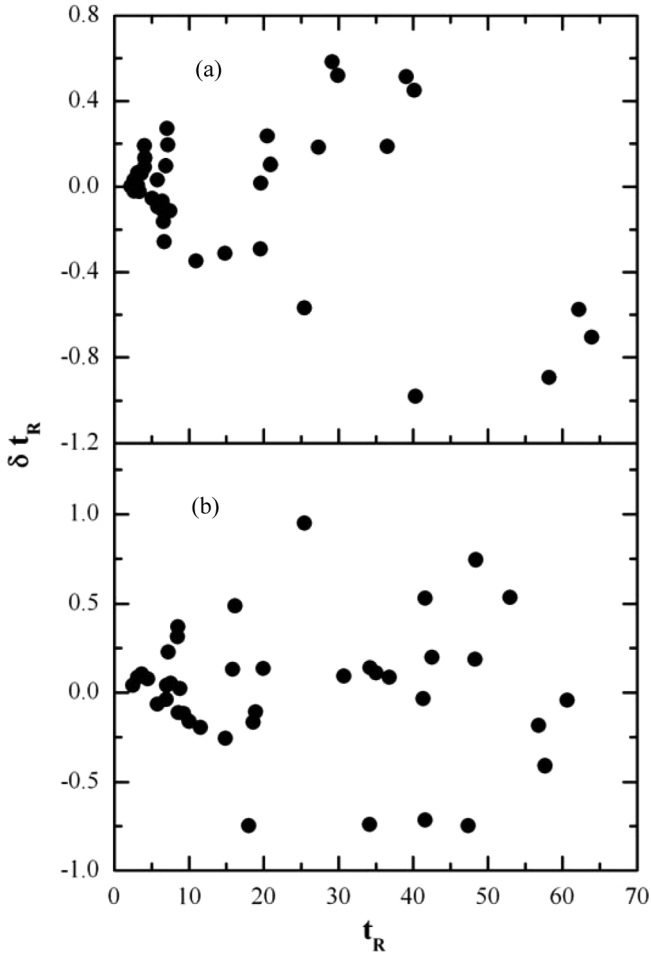

Figure 2. Differences between experimental and predicted retention times under (a) isocratic elution using various φ values, and (b) various linear gradient elution schemes in aqueous mobile phases modified with methanol. (Reprinted from Ref. [67] with permission from Elsevier).

(HTOH), 3,4-dihydroxyphenyl glycol (HPG), and homovanillic acid (HVA) taken from Ref. [67]. This figure depicts differences, δt_R, between experimental and predicted retention times under isocratic elution (a) using various φ values in aqueous mobile phases modified with methanol and the corresponding differences under various linear gradient elution schemes (b). The retention model used in the calculations was

$$\ln k = c_0 - \frac{c_1\varphi}{1 + c_2\varphi} + c_3\varphi \tag{39}$$

It is seen that the maximum deviation of the predicted retention times from the experimental ones is always less than 1 min and that the deviations obtained under gradient conditions is of the same order as those obtained isocratically. This shows that Eq. (33), in combination with Eq. (39), describes absolutely satisfactorily the gradient elution and, consequently, they can be used in optimisation procedures. A chromatogram recorded under optimum conditions is shown in Figure 3. For comparison, the corresponding chromatogram recorded under isocratic conditions is also included in this figure. We observe that that the separation of the constituents of the mixture of catecholamines can effectively take place at a maximum elution time of about 12 min under gradient elution, whereas this is impossible under isocratic elution.

Finally, we should add that two 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 ($b > 0$), and (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.

The capabilities of the method are enhanced especially for separation optimization of complex mixtures if it is extended to multi-linear gradients.[68,69] Such an application is shown in Figure 4, which shows a chromatogram of a mixture of 13 o-phthalaldehyde (OPA) derivatives of amino acids separated by a multi-linear φ vs. t gradient of acetonitrile. The complete study has been carried out in Ref. [69]. The derivatives of amino acids are: l-arginine (Arg), l-glutamine (Gln), l-serine (Ser), l-glutamic acid (Glu), l-threonine (Thr), beta-(3,4-dihydroxyphe nyl)-l-alanine (Dopa), l-alanine (Ala), l-methionine (Met), l-valine (Val), ltryptophan (Trp), l-phenylanine (Phe), l-isoleucine (Ile) and l-leucine (Leu). These derivatives were formed by the reaction of OPA with amino acids in the presence of 2-mercaptoethanol (2-ME).

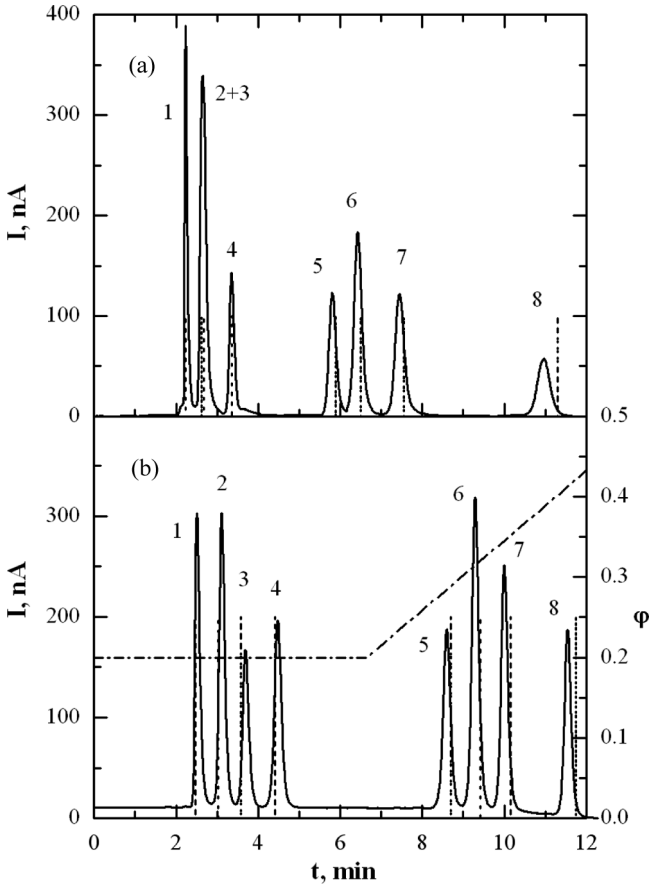

Figure 3. ED chromatograms of an eight-component mixture composed of (1) DA, (2) HPG, (3) 5HT, (4) VMA, (5) DOPAC, (6) HTOH, (7) HIAA, and (8) HVA. They are recorded under (a) isocratic conditions in an aqueous mobile phase modified with methanol using $\varphi = 0.29$, and (b) gradient conditions using $\varphi_{in} = 0.2$, $\varphi_{fin} = 0.5$, $t_{in} = 0$, $t_0 = 1.844$ min, $b/t_0 = 0.043$, $t_D = 4.6$ min. The dotted vertical lines indicate the predicted retention times by means of Eq. (39) (A) and Eqs. (33) and (39) (B), whereas the dash-dotted line shows the variation pattern of φ when it reaches the electrochemical detector. (Reprinted from Ref. [67] with permission from Elsevier).

c) Numerical Solution of the Fundamental Equation. The fundamental equation of gradient elution, Eq. (20), is in fact a non-linear equation with one unknown, i.e., the retention time t_R. Therefore, it can be solved by means of an appropriate numerical method for computing the real roots of an equation, like Newton's method. In this approach, the

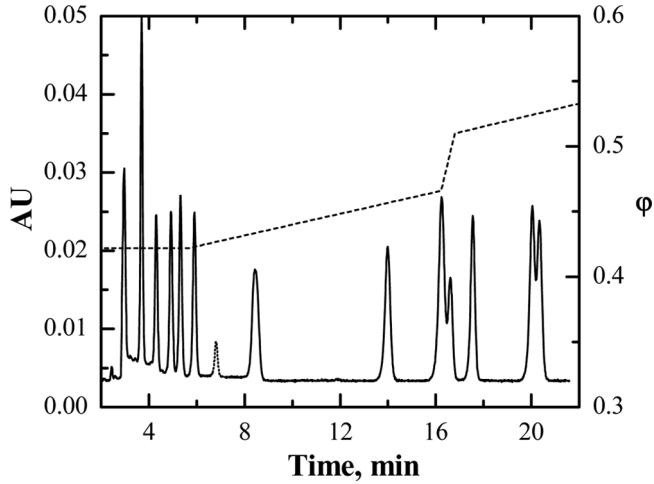

Figure 4. UV detected chromatogram of Arg, Gln, Ser, Glu, Thr, Dopa, Ala, Met, Val, Trp, Phe, Ile, Leu (from left to right) under the optimal gradient. The small peak shown by dots corresponds to OPA and the crooked line shows the variation pattern of φ vs. t when it reaches the UV detector. (Reprinted from Ref. [69] with permission from Elsevier).

integral may be estimated using Simpson's rule. The method is computationally very simple, but it needs a great number of iterations. The computational time is small for prediction, but it may become extremely great when this method is used in an optimization algorithm.

Alternatively, linear and non-linear gradients can be approximated by stepwise gradients consisting of p equidistant steps, where p is a relatively great number. Cela et al.[7,28,70,71] propose p = 11; however, in a recent paper, we found that good results are obtained when p is of the order of 100.[72] Under this approximation, the retention time is calculated from[73]

$$t_R = t_0(1 + k_{\varphi_n}) + (t_D + \delta t)\frac{k_{\varphi_1} - k_{\varphi_n}}{k_{\varphi_1}} + \delta t\frac{k_{\varphi_2} - k_{\varphi_n}}{k_{\varphi_2}} + \ldots + \delta t\frac{k_{\varphi_{n-1}} - k_{\varphi_n}}{k_{\varphi_{n-1}}} \tag{40}$$

where n is the least number of terms of the sum that makes the following inequality valid:

$$\frac{\delta t + t_D}{t_0 k_{\varphi_1}} + \frac{\delta t}{t_0 k_{\varphi_2}} + \ldots + \frac{\delta t}{t_0 k_{\varphi_n}} \geq 1 \tag{41}$$

In the above equations, δt is the time step of the stepwise gradient, k_{φ_j} is the retention factor when the composition of the mobile phase is φ_j at the j-th step. The method is also computationally simple and very fast. Therefore, it can be used in optimization algorithms.

Variations in Column Temperature

Although the preferred factor in single-mode reversed-phase liquid chromatography separations is generally the organic solvent volume fraction, φ, temperature, T, is now recognized as an important variable in controlling separations.[74] However, temperature has not yet been adequately explored as a parameter to tune separation and shorten analysis time in RPLC. As in the case of φ variations, there are two approaches for predicting the elution time of a sample solute under temperature programming conditions: a) by direct fitting to 1D or 2D data, and b) by the numerical solution of the fundamental equation, i.e., Equation (4).

Direct Fitting to 1D or 2D Data

We form a series of 1D data of the retention times of an analyte at various b values, b being the slope of T-gradients with the same initial, T_{in}, and final, T_{fin}, temperature. That is, the linear T-gradients are of the form

$$T = \begin{cases} T_{in} + bt & \text{when} \quad t < t_{fin} \\ T_{fin} & \text{when} \quad t \geq t_{fin} \end{cases} \tag{42}$$

These data may be fitted to

$$\ln k = c_0 + c_1 b + c_2 b^2 \tag{43}$$

or to

$$\ln k = c_0 + c_1 b \tag{44}$$

allowing the direct estimation of t_R.

2D data of the retention times of an analyte as a function of the initial value T_{in} and the slope b may be also used and fitted to retention models, like the model of Eq. (19) with T_{in} in place of φ_{in}.

Numerical Solution of the Fundamental Equation

The fundamental equation for T-gradients is Eq. (4). The numerical solution of this equation presents the following difficulty. Consider that a

linear T-gradient

$$T_{ov} = T_{in} + bt \tag{45}$$

is formed in the oven. Then, the effective gradient that experiences the analyte is different from that of Eq. (45), due to hysterisis phenomena. If we assume that the hysteresis is described by Newton's law

$$\frac{dT}{dt} = -h(T - T_{ov}) \tag{46}$$

where h is a constant characteristic of the system, the effective gradient is obtained by solving the above linear differential equation. Thus, we find

$$T_{ef} = T_{in} + bt + ce^{-ht} - \frac{b}{h} \tag{47}$$

where c is an integration constant calculated from the initial condition

$$T_{ef}(t = 0) = T_{in} \Rightarrow c = b/h.$$

When the T-gradient reaches the upper limit of T_{fin} at $t = t_{fin}$, where the temperature becomes constant, then T_{ef} is given by

$$T_{ef} = T_{fin} + c^* e^{-ht} \tag{48}$$

where the integration constant c^* is estimated by equating Eqs. (47) and (48) at $t = t_{fin}$.

At this point, we should stress the following. Apart from the above hysteresis between the actual temperature of the oven and the effective temperature inside the column, there may be a lag between the programmed and the actual temperature formed in the oven if the oven does not respond fast to the programmed changes of the temperature.

It is evident that, due to these delay phenomena, the effective temperature given by Eq. (47) should be used in the solution of Eq. (4). Additionally, when running T-programs, the elution time can be calculated simpler if the effective T-gradient experienced by the analyte is approximated by a stepwise gradient.[12] To derive an analytical expression for t_R, we may proceed as follows. The distance L_n inside the column that the analyte travels under the influence of the n-th step is given by

$$L_n = v_{T_n}\delta t = \frac{L\delta t}{t_{T_n}} = \frac{L\delta t}{t_0(1 + k_{T_n})} \tag{49}$$

where L is the length of the chromatographic column, $v_{T_n} = L/t_{T_n}$ is the velocity of the analyte under isocratic conditions at constant temperature equal to that of the n-th step, t_{Tn} is the retention time of the analyte if the temperature was constant and equal to that of the n-th step, and $k_{Tn} = (t_{T_n} - t_0)/t_0$ is the corresponding retention time. If the solute is eluted during the n-th step, the distance traveled by the solute is smaller and equal to

$$L_{n,final} = \frac{L[t_R - (n-1)\delta t]}{t_0(1 + k_{T_n})} \tag{50}$$

Now, from the relationship

$$L_1 + L_2 + \cdots + L_{n,final} = L \tag{51}$$

we obtain that t_R is estimated from

$$t_R = t_0(1 + k_{T_n}) + \delta t \frac{k_{T_1} - k_{T_n}}{1 + k_{T_1}} + \delta t \frac{k_{T_2} - k_{T_n}}{1 + k_{T_2}} + \cdots + \delta t \frac{k_{T_{n-1}} - k_{T_n}}{1 + k_{T_{n-1}}} \tag{52}$$

Where, again, n is the least number of terms of the sum that makes the following inequality valid

$$\frac{\delta t}{t_0(1 + k_{T_1})} + \frac{\delta t}{t_0(1 + k_{T_2})} + \ldots + \frac{\delta t}{t_0(1 + k_{T_n})} \geq 1 \tag{53}$$

The application of this theory to predict retention times under T-gradients requires, first, the evaluation of the hysteresis constant h. This can be done experimentally by recording one or two T-gradients. For these gradients, t_R is calculated by means of Eqs. (47), (48), (52), and (53) using the proper k(T) expression selected from Eq. (9), $\delta t = 0.1$ min, and a grid search for h in order to determine the value of h that yields the minimum deviations between experimental and calculated retention times.[12]

The above theory has been tested using six alkylbenzes, benzene (B), toluene (T), ethylbenzene (EB), isopropylbenzene (*i*PB), propylbenzene (PB) and *tert*-butylbenzene (*t*BB) in eluting systems modified by acetonitrile.[12] The chromatographic column was a conventional Zorbax SB-C_{18} column (3.5 μm, 150 × 4.6 mm), stable at temperatures ≤90°C. The initial temperature in all T-gradients was 15°C and the final 75°C. Due to an oven limitation, the programmed T-gradient profiles were stepwise. However, the actual oven temperatures can be approximated by the linear gradients depicted in Figure 5. These gradients are denoted by gT_1, gT_2, gT_3, gT_{3b}, and gT_4 from left to right. Based on these gradients,

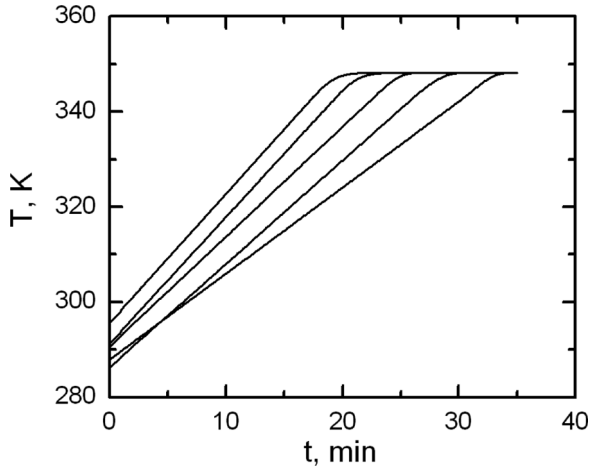

Figure 5. Five linear gradients of the actual temperature inside the oven indicated by gT_1, gT_2, gT_3, gT_{3b} and gT_4 (from left to right). They have been used to treat data of Table 1.

we have recalculated the data in Ref. [12] to estimate the percentage error between experimental and calculated retention times. In these calculations, we used Eqs. (52), (53), the effective temperature from Eqs. (47), (48) using $h = 0.12\,min^{-1}$, and the retention model of Eq. (15) with $g(T) = 0$. The results obtained are listed in Table 1, which depicts the percentage error between experimental and calculated retention data of the solutes under T-gradient conditions at constant φ values. It is seen, and the agreement is excellent, in all cases provided that hysteresis phenomena are properly embodied into the treatment. In this case, the average percentage error between experimental and predicted retention times is less than 4%. Note that, if we adopt a more accurate representation of the effective temperature, the error falls to less than 2.4%.[12] In contrast, it has been shown that, if we ignore the hysteresis between the oven and the effective temperature, the overall prediction error may reach 11%.[12]

Variations in Flow Rate

Flow programming is rather rarely used,[75–79] whereas this gradient mode is sometimes combined with solvent gradient elution[80–85] or with temperature programming.[86–88] When only the flow rate F varies, the retention factor k is constant and Eq. (4) yields[3,4,16,17,39]

$$\frac{1}{t_{o1}(1+k)}\int_0^{t_R} F(t)dt = 1 \tag{54}$$

Table 1. Absolute percentage error between experimental and calculated from Eqs. (52), (53) retention data of the solutes under the T-gradients of Figure 5 and at constant φ values shown in the Table

T-gradient	gT_1	gT_2	gT_4	gT_1	gT_2	gT_3	gT_{3b}	gT_4	gT_1	gT_2	gT_3	gT_{3b}	gT_4	gT_1	gT_2	gT_{3b}	gT_4
φ	0.4	0.4	0.4	0.45	0.45	0.45	0.45	0.45	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.6	0.6
Solute																	
B	0.7	2.3	4.9	0.6	2.7	0.3	2.5	5.3	0.5	2.5	0.0	2.4	5.3	0.3	1.8	0.4	4.6
T	0.6	0.9	2.6	0.7	2.1	0.7	1.7	3.8	0.6	2.4	0.5	2.2	4.6	0.4	2.1	0.2	4.9
EB	0.1	0.1	1.5	0.4	0.8	0.4	0.4	1.9	0.6	1.7	0.7	1.3	3.0	0.5	2.1	0.2	4.4
iPB	0.3	0.2	1.1	0.2	0.0	0.2	0.1	1.3	0.3	0.7	0.3	0.3	1.6	0.5	1.7	0.5	3.7
PB	0.2	0.2	1.0	0.4	0.1	0.2	0.1	1.2	0.2	0.4	0.2	0.1	1.4	0.5	1.6	0.6	3.5
tbb	0.2	0.1	0.8	0.3	0.2	0.1	0.1	1.0	0.0	0.0	0.2	0.0	1.2	0.5	1.3	0.6	2.8
Average	0.3	0.6	2.0	0.4	1.0	0.3	0.8	2.4	0.4	1.3	0.3	1.0	2.8	0.4	1.8	0.4	4.0

where t_{o1} is the column hold-up time that corresponds to $F = 1$ in arbitrary units. This equation is easily solved with respect to t_R, especially when a linear or multi-linear gradient is used. For example, if $F = F_{in} + bt$, we obtain

$$(b/2)t_R^2 + F_{in}t_R - t_{o1}(1 + k) = 0 \tag{55}$$

which yields

$$t_R = \frac{-F_{in} + \sqrt{F_{in}^2 + 2bt_{o1}(1 + k)}}{b} \tag{56}$$

If the analyte is eluted after F reaches its upper limit, say F_{fin} at $t = t_{fin}$, then the elution time is calculated from

$$\int_0^{t_{fin}} F(t)dt + \int_{t_{fin}}^{t_R} F(t)dt = t_{o1}(1 + k) \tag{57}$$

which yields

$$t_R = t_{fin} + \frac{1}{F_{fin}}\{t_{o1}(1 + k) - F_{fin}t_{fin} - bt_{fin}^2/2\} \tag{58}$$

The above equations have been tested in Ref. [39], where different types of flow rate gradients were implemented in the separation of five 1,4-dihydropyridines: amlodipine (AML), nitrendipine (NIT), nimodipine (NIM), felodipine (FEL), and lacidipine (LAC). Some of the linear flow rate gradients used in that study are shown in Figure 6. The prediction of the retention times of the sample compounds was found to be very satisfactory in all different types of flow-rate gradients tested, as shown in Table 2. The average percentage error between experimental and predicted retention times was less than 3.5% for all gradient flow rate profiles used. Thus, it is clear that a variation in mobile phase flow-rate leads to a predictable, by means of Eq. (54), rearrangement of peaks within the chromatogram and can be used as a complementary technique for peak identification.

A simple linear flow rate gradient can hardly be used in optimization separation. Substantial optimizing effect can be achieved on a separation by multilinear or more complicated flow rate gradients, where the applying variation in mobile phase flow rate can change peak retention both absolutely and relatively, i.e., it can lead to an optimum rearrangement of peaks within the chromatogram. Figure 7b shows such a chromatogram, where the overall separation of nine underivatized amino acids, beta-(3,4-dihydroxyphenyl)-l-alanine (dopa), l-tyrosine (tyr), l-a-methyl-dopa (me-dopa), dl-mtyrosine (m-tyr), dl-alpha-methyltyrosine

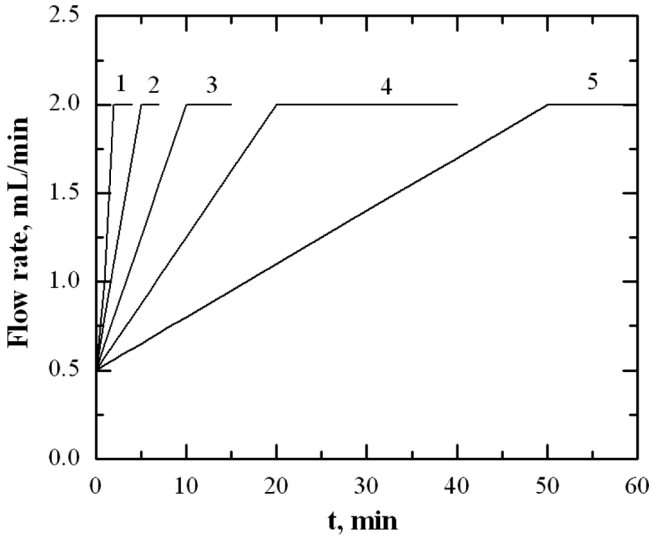

Figure 6. Linear flow-rate gradients used for the prediction of the 1,4-dihydropyridines retention times. (Reprinted from Ref. [39] with permission from Elsevier).

(me-tyr), 5-hydroxytryptophan (5htp), l-phenylanine (phe), 3-nitro-l-tyrosine (ntyr), and l-tryptophan (trp) in aqueous phosphate buffers (pH 2.5) modified with acetonitrile was significantly improved, even compared to the lowest constant flow rate of 0.5 mL/min separation depicted in Figure 7a. The chromatograms were taken from Ref. [39]. It is seen that the separation capability of the above flow rate plot is given by the mild starting decrease of the flow rate, followed by a sharp increase of the flow rate at the correct time point, which after a perfect time length is kept constant to the end of the run.

Variations in Mobile Phase pH

The theory of linear pH gradients has been developed by Kaliszan et al.[89–94] However, up to now, pH gradients in reversed phase chromatography are of limited use only. A linear mobile phase pH gradient is described by the general equation:

$$pH = pH_0 + bt \tag{59}$$

where pH_0 is the initial pH and b denotes gradient steepness. If this equation is introduced into Eq. (11), we obtain

Table 2. Comparison of experimental and predicted by Eq. (54) retention data of 1,4-dihydropyridines at the linear flow-rate gradients depicted in Figure 6. (Reprinted from Ref. [39] with permission from Elsevier)

Gradient	1 ($\varphi = 0.7$)			2 ($\varphi = 0.7$)			3 ($\varphi = 0.7$)			4 ($\varphi = 0.6$)			5 ($\varphi = 0.5$)		
solute	t(exp)	t(th)	error (%)	t(exp)	t(th)	error (%)	t(exp)	t(th)	error (%)	t(exp)	t(th)	error (%)	t(exp)	t(th)	error (%)
AML	2.332	2.28	2.2	3.168	3.16	0.3	3.935	3.88	1.4	4.704	4.76	1.2	6.530	6.84	4.7
NIT	3.686	3.58	2.9	4.765	4.70	1.4	6.013	5.98	0.5	9.999	10.02	0.2	20.75	21.48	3.5
NIM	3.946	3.84	2.7	5.037	4.96	1.5	6.375	6.34	0.5	10.94	10.96	0.2	23.76	24.60	3.5
FEL	4.785	4.66	2.6	5.869	5.78	1.5	7.450	7.40	0.7	13.26	13.26	0.0	29.93	30.93	3.3
LAC	6.902	6.70	2.9	7.971	7.82	1.9	9.791	9.68	1.1	19.53	19.48	0.3	51.24	52.44	2.4
Average			2.7			1.3			0.9			0.4			3.5

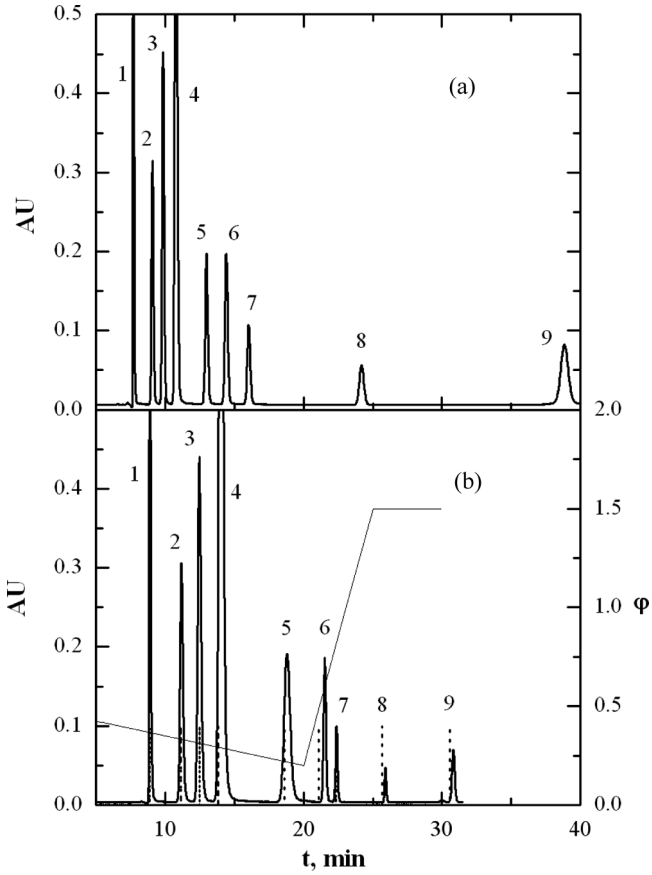

Figure 7. UV chromatograms of a mixture of underivatized amino acids obtained at a constant flow-rate of 0.5 ml/min (a) and by flow rate program indicated by the segmented line (b); the volume fraction of acetonitrile in the mobile phase is $\varphi = 0.06$. Peaks are: (1) dopa; (2) tyr; (3) me-dopa; (4) m-tyr; (5) me-tyr, (6) 5htp; (7) phe; (8) n-tyr; and (9) (trp). The dotted vertical lines indicate the predicted retention times by means of Eq. (54). (Reprinted from Ref. [39] with permission from Elsevier).

$$k = \frac{k_0 + k_1 10^{pH_0 - pK + bt}}{1 + 10^{pH_0 - pK + bt}} \tag{60}$$

which, by substitution in Eq. (20) and integration, yields

$$t_R^* + \frac{k_0 - k_1}{bk_1 \ln(10)} \ln \frac{k_0 + k_1 10^{pH_0 - pK + bt_R^*}}{k_0 + k_1 10^{pH_0 - pK}} = k_0 t_0 (1 - I_S) \tag{61}$$

where

$$t_R^* = t_R - t_0 - t_D - t_{in} \tag{62}$$

Equation (61) can be solved numerically with respect to t_R and, therefore, it can be used for prediction and optimization in linear gradients of pH.

PREDICTION IN MULTI-MODE GRADIENT ELUTION

In multi-mode gradient elution, two or more separation variables are used for prediction and optimization. The multi-mode gradients may be divided into two categories: a) Gradients related exclusively to the mobile phase composition, that is, the gradient parameters are the volume fractions φ_1, $\varphi_2, \ldots$ of the constituents of the mobile phase and/or the pH of this phase, and b) combined gradients of the mobile phase composition with flow rate and/or column temperature. The fundamental equation for the gradients of the first category is still Eq. (3), whereas this equation, as well as Eq. (4), are inapplicable for the gradients of the second category. The theory of the multi-mode gradients of the second category is still under development in our laboratory.[15–17]

Multi-Mode Gradients Associated with Changes in Mobile Phase Composition

This category includes linear gradients in ternary mobile phases and double gradients of pH and organic solvent concentration in the mobile phase. The theory of multilinear gradient elution in reversed-phase liquid chromatography using ternary solvent mixtures and its application in optimisation separation of a mixture of 13 o-phthalaldehyde (OPA) derivatives of amino acids with mobile phases modified by acetonitrile and methanol has been developed in.[64] The theory is an extension of the corresponding theory of linear single-gradients. Indicative results of this study are the following. If we use the linear gradients of Table 3, we observe in Table 4 that the prediction of the retention times is very satisfactory; the average percentage error of the predicted values is very low, lower than 4%, if we exclude the prediction of Arg. The problem with Arg is that its predicted retention times are far away from the corresponding values used in the fitting procedure.[64]

In the same paper the optimum separation of the mixture of 13 o-phthalaldehyde (OPA) derivatives of amino acids was attempted by

Table 3. Linear gradient elution modes used to test the theory of multilinear gradient elution in ternary solvent systems. (Reprinted from Ref. [64] with permission from Elsevier)

Gradient	g1	g2	g3	g4	g5	g6
φ_{MeCN}	0.24 → 0.36	0.30 → 0.42	0.30 → 0.30	0.42 → 0.42	0.30 → 0.36	0.21 → 0.30
φ_{MeOH}	0.21 → 0.28	0.21 → 0.21	0.21→ 0.35	0.0 → 0.21	0.21 → 0.28	0.245 → 0.35
t, min	0 → 10	0 → 10	0 → 20	0 → 10	0 → 5	0 → 10

multilinear gradients. One of the optimum gradients obtained is shown in Table 5 and Figure 8 shows that there is a good separation of the mixture of the thirteen solutes when the maximum gradient time is 20 min. Attempts to reduce this time were not successful because of the limited range of φ_{MeCN}, φ_{MeOH} values which are available due to limited solubility of the phosphate buffer.

The combined effect of a gradient of pH and of the organic modifier in the mobile phase has been theoretically and experimentally studied by the group of Kaliszan.[93] The proposed theoretical model allows determination of both pK and the lipophilicity parameter of the ionized and the nonionized form of the analyte and prediction of the retention times at specific separation conditions.

Table 4. Percentage error between experimental and calculated retention times of o-phthalaldehyde derivatives of amino acids under the linear gradients of Table 3. (Reprinted from Ref. [64] with permission from Elsevier)

Solute	g1	g2	g3	g4	g5	g6
arg	(17.2)	(6.8)	(7.2)	(11.7)	(7.5)	(15.3)
gln	4.2	2.7	2.6	4.6	2.3	6.2
ser	0.9	0.7	0.1	3.2	0.6	0.7
glu	5.2	2.6	2.3	5.1	2.1	6.6
thr	3.1	1.9	2.5	9.1	1.9	4.4
dopa	0.3	1.2	0.0	7.2	0.4	0.9
ala	3.1	3.2	3.5	7.8	2.5	3.8
met	1.9	2.4	3.1	4.3	2.9	2.6
val	2.6	2.3	3.0	0.2	2.4	3.3
trp	1.2	2.2	2.6	4.0	2.9	2.3
phe	1.9	2.4	2.5	1.7	3.1	2.6
ile	2.2	2.1	3.0	0.0	1.8	3.1
leu	1.9	2.0	2.9	0.1	1.8	3.0
Average	2.4	2.1	2.3	3.9	2.1	3.3

Table 5. Optimum gradient obtained using the retention model of Eq. (13). (Reprinted from Ref. [64] with permission from Elsevier)

φ_{MeCN}	0.332	0.332	0.420	0.420	0.420
φ_{MeOH}	0.123	0.123	0.162	0.166	0.210
t, min	0	3.29	6.32	9.73	29.72

Multi-Mode Gradients with Changes in Mobile Phase Composition, Flow Rate, and/or Column Temperature

The gradients of this category may be further subdivided into pseudo and real multi-mode gradients. In the pseudo multi-mode gradients, a variable, say x_1, takes a constant value and a second variable, say x_2, varies with time following a certain profile. Then x_1 takes another constant value, whereas x_2 performs the previous x_2 vs. t profile, and so on. In the real multi-mode gradient elution, all variables x_1, x_2, ... vary simultaneously with time following a pre-set profile.

Pseudo Multi-Mode Gradients

They are usually two-mode gradients and the prediction may be easily achieved by direct fitting to 2D data. Below, we examine gradients of

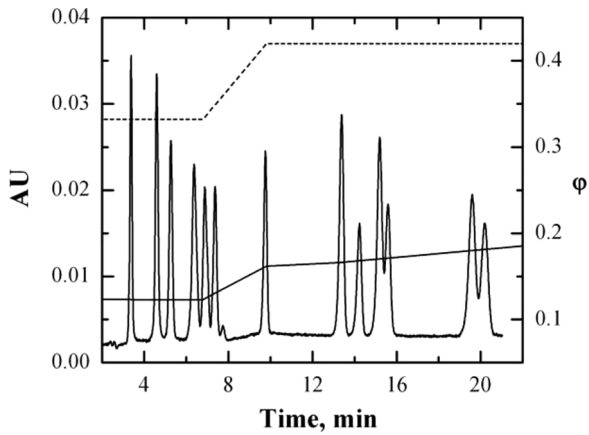

Figure 8. UV detected chromatogram of OPA derivatives of Arg, Gln, Ser, Glu, Thr, Dopa, Ala, Met, Trp, Val, Phe, Ile, Leu (from left to right) under the optimal gradient of Table 5. The crooked lines (- - -) and (—) show the variation pattern of φ_{MeCN} vs. t and φ_{MeOH} vs. t, respectively, when they reach the UV detector. (Reprinted from Ref. [64] with permission from Elsevier).

φ at constant T and gradient of T at constant φ values. The treatment of all other cases is similar.

a) Gradients of φ *at constant T.* Suppose that we have a series of 2D data, which are the elution times of an analyte at various φ-gradients at constant T values. All φ-gradients should exhibit the same initial and final value of φ, but different gradient steepness b. These data are likely to be fitted to the following equations

$$t_R = c_1 + c_2T + (c_3 + c_4T)b + (c_5 + c_6T)b^2 \tag{63}$$

or

$$t_R = c_1 + c_2T + (c_3 + c_4T)b \tag{64}$$

since it is known that, at constant b value, t_R varies linearly with T.[8,9,11,49–51,73] If the fitting is satisfactory, Eq. (63) or (64) can be used to predict the elution time of the analyte under φ-gradient conditions at various constant T values, provided that the φ-gradients are of the same type with those used in the fitting procedure and the values of b and T used lie within those used for fitting.

b) Gradients of T at constant φ. In this case, we should have a series of 2D data of the elution times of an analyte at constant φ values, but at various T-gradients with the same initial and final temperature and different slopes, b. These data may be fitted to

$$\ln k = c_1 + c_2\varphi + c_3\varphi^2 + (c_4 + c_5\varphi + c_6\varphi^2)b + (c_7 + c_8\varphi + c_9\varphi^2)b^2 \tag{65}$$

or to

$$\ln k = c_1 + c_2\varphi + c_3\varphi^2 + (c_4 + c_5\varphi + c_6\varphi^2)b \tag{66}$$

or to

$$\ln k = c_1 + c_2\varphi + (c_3 + c_4\varphi)b \tag{67}$$

Then these equations can be used for prediction under prerequisites similar to those for Eqs. (63), (64).

The above approaches have been tested in Ref. [12] using a conventional column Zorbax SB-C_{18} (3.5 μm, 150 × 4.6 mm). The solutes were a mixture of six non-polar solutes: benzene (B), toluene (T), ethylbenzene (EB), isopropylbenzene (*i*PB), propylbenzene (PB) and *tert*-butylbenzene (*t*BB) in acetonitrile–water mixtures. The gradient conditions were

Table 6. Percentage error between experimental and calculated from Eq. (63) retention data of the solutes under φ-gradient conditions at constant T values. (Reprinted from Ref. [12] with permission from Wiley-VCH Verlag GmbH & Co. KGaA)

φ-gradient	$g\varphi_4$	$g\varphi_1$	$g\varphi_3$	$g\varphi_5$	$g\varphi_2$
T, K	303.15	318.15	318.15	318.15	333.15
Solute					
B	1.1	1.0	2.5	0.3	2.1
T	0.2	0.2	1.1	1.2	1.3
EB	0.0	0.8	0.1	1.4	0.6
*i*PB	0.2	1.2	0.8	1.4	0.1
PB	0.8	1.0	0.7	1.2	0.1
*t*BB	1.0	1.2	1.0	1.0	0.5
Average	0.6	0.9	1.0	1.1	0.8

the following: In all φ-gradients, the initial acetonitrile φ value was 0.4, the final one was 0.7, and the time of φ variation, t_{fin}, was 10, 15, 20, 30, and 40 min. These gradients are denoted by $g\varphi_1$, $g\varphi_2$, $g\varphi_3$, $g\varphi_4$, $g\varphi_5$, respectively. The initial temperature in all T-gradients was 15°C, the final 75°C and the time between the initial and the final temperature was 6, 15, 20, and 30 min. They are denoted by gT_1, gT_2, gT_3, and gT_4. Table 6 depicts the absolute percentage error between experimental and calculated from Eq. (63) retention data of the solutes under φ-gradient conditions at constant T values, and Table 7 shows the corresponding error between experimental and calculated from Eq. (66) retention data of the solutes under T-gradient conditions at constant φ values. It is seen that, in all cases, the prediction of the retention times is excellent.

Table 7. Percentage error between experimental and calculated from Eq. (66) retention data of the solutes under T-gradient conditions at constant φ values. (Reprinted from Ref. [12] with permission from Wiley-VCH Verlag GmbH & Co. KGaA)

T-gradient	gT_1	gT_2	gT_3	gT_4	gT_1	gT_2	gT_3	gT_4
φ	0.45	0.45	0.45	0.45	0.5	0.5	0.5	0.5
Solute								
B	0.6	1.4	2.1	0.7	0.2	1.0	1.4	0.0
T	1.2	1.8	2.8	1.4	0.1	1.4	2.1	0.1
EB	1.5	1.5	2.8	2.0	0.1	1.8	2.8	0.1
*i*PB	0.7	0.0	1.9	1.8	0.2	1.3	2.6	0.1
PB	1.3	1.7	0.5	1.3	2.2	0.5	1.3	0.1
*t*BB	1.9	2.5	0.3	0.4	2.3	0.9	1.0	0.3
Average	1.2	1.5	1.7	1.3	0.9	1.1	1.8	0.1

Real Multi-Mode Gradients

The theory of this type of multi-mode gradient elution is currently under development. Up to now, detailed studies have been performed on dual-mode gradient elution involving simultaneous changes in flow rate and mobile phase composition,[15,16] and temperature and mobile phase composition.[17] The study of the simultaneous variations in φ, T, and F has also been completed.[95] According to the theory presented in Refs. [16,17], all φ_i vs. t and pH vs. t profiles at the inlet of the chromatographic column are approximated by stepwise plots with time steps Δt and the T vs. t or the F vs. t profile is approximated by a stepwise curve with time steps δt, where $\Delta t/\delta t = m >> 1$. Then, it can be shown that the distance from the inlet of the chromatographic column travelled by the mobile phase for p steps of Δt duration to meet the analyte is given by

$$\ell_p = \sum_{i=pm+1}^{n_p} \frac{L\delta t}{t_{o,i}} \tag{68}$$

where L is the column length, $t_{o,i}$ is the hold up time during the i-th step, and n_p is an integer estimated from the recursive relationship

$$\sum_{i=pm+1}^{n_p} \frac{1}{t_{o,x}} = \sum_{i=n_{p-1}+1}^{n_p} \frac{1}{t_{R,x}} + \sum_{i=(p-1)m+1}^{n_{p-1}} \frac{1}{t_{o,x}} \tag{69}$$

where $t_{R,i}$ is the retention time of the sample solute during also the i-th step. The analyte is eluted when

$$\ell_{p-1}/L < 1 \text{ and } \ell_p/L \geq 1 \tag{70}$$

and, therefore, the retention time may be calculated from

$$t_R = n_p \delta t \tag{71}$$

Note that the temperature that should be used in the T-gradients is the effective temperature given by Eqs. (47), (48), or the relevant Equations presented in Refs. [12,17]. In addition, we should point out that, if the flow rate varies, then an arbitrary φ versus t gradient profile formed in the mixer of the chromatographic system is transformed to a new one, φ versus t^*, at the inlet of the chromatographic column, since an event that takes place at the time t_p in the mixer and it is associated with the composition of the mobile phase is transferred to the inlet of the column at t_p^*, where t_p and t_p^* are interrelated through the following

equation[15,16]

$$t_D(F=1) = \int_{t_p}^{t_p^*} F(t)dt \tag{72}$$

Here, F(t) is the function of flow rate upon t and $t_D(F=1)$ is the product of the dwell time when the flow rate F is equal to 1, in arbitrary units, by the unit flow rate ($F=1$). Therefore, the φ_i vs. t (as well as the pH vs. t) plot at the inlet of the chromatographic column is the transformed gradient profile through Eq. (72) and not that formed in the mixer.

Up to now, the above theory has been applied only for prediction under gradient conditions involving simultaneous changes a) in flow rate and mobile phase composition,[16] and b) temperature and mobile phase composition.[17] For changes in flow rate and mobile phase composition, the theory was tested using 18 o-phthalaldehyde derivatives of amino acids in eluting systems modified by acetonitrile or methanol.[16] Figure 9 shows the effect of flow rate variation on a linear gradient profile (- - -) programmed and formed in the mixer. The flow rate

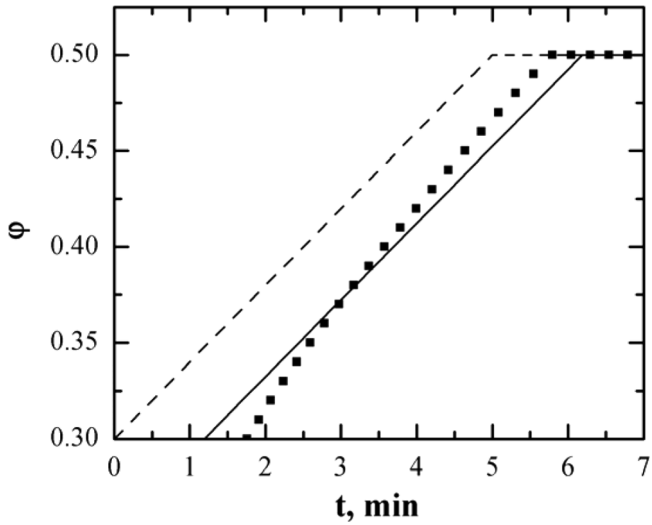

Figure 9. Effect of flow rate variation on a gradient profile (- - -) programmed and formed in the mixer. The flow rate (in mL/min) changes linearly from 0.5 at $t=0$ to 1.5 at $t=5$ min. Line (—) represents the gradient profile transformed at the inlet of the chromatographic column when F is constant and equal to 1 mL/min. Points represent the gradient profile at the inlet of the chromatographic column when it is calculated from Eq. (72). (Reprinted from Ref. [16] with permission from ACS).

Table 8. Dual-mode gradients used in mobile phases modified with acetonitrile (time in min, F in mL/min).[17]

Gradient	I	II	III	IV	V	VI
φ_1	0.3	0.3	0.3	0.3	0.3	0.3
φ_2	0.5	0.5	0.5	0.4	0.4	0.4
t_{φ_1}	0	0	10	0	0	0
t_{φ_2}	10	25	16	20	20	20
F_1	0.5	0.5	0.5	0.5	1	1.0
F_2	1.5	1.5	1.5	1.5	1.5	1.5
t_{F_1}	0	0	2	0	0	10
t_{F_2}	10	10	9	10	10	10.01
Average % error	2.5	2.2	1.3	1.5	2.4	2.4

(in mL/min) changes linearly from 0.5 at $t = 0$ to 1.5 at $t = 5$ min. Line (---) represents the gradient profile transformed at the inlet of the chromatographic column when F is equal to 1 mL/min and $t_D = 1.19$ min for $F = 1$ mL/min. Points represent the gradient profile at the inlet of the chromatographic column when it is calculated from Eq. (72).

The dual-mode gradients used in mobile phases modified with acetonitrile are shown in Table 8, where the average percentage error in

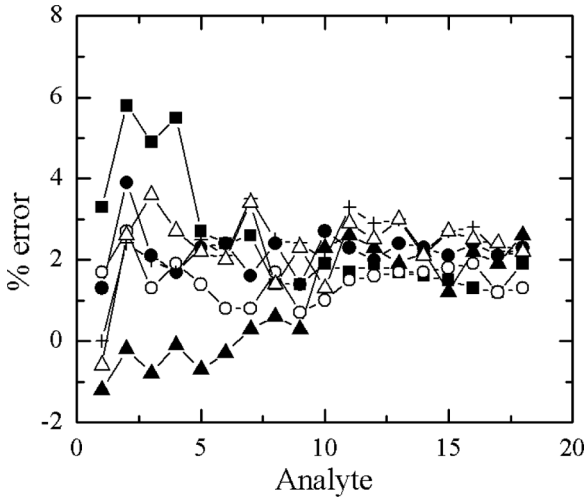

Figure 10. Percentage error between experimental and calculated retention times of the Amino Acids Derivatives Arg, Asn, Gln, Ser, Tau, Asp, Glu, Thr, Gly, Dopa, Ala, GABA, Met, Trp, Phe, Val, Ile, Leu (from 1 to 18) under the dual-mode gradients of Table 8: (■) I, (●) II, (▲) III, (o) IV, (+) V, and (Δ) VI.

the retention prediction is also shown for each type of gradient program. The agreement is very good in all-different types of dual-mode gradient programs tested, with average errors below 2.5%. Figure 10 depicts the percentage error between calculated and experimental retention times for the individual sample compounds. This error is lower than 6%, depending on the gradient profile. Although the differences between experimental and calculated retention times are small, there is a kind of bias and the calculated retention times are systematically lower than the experimental ones. This bias depends on the organic modifier used, since it is small for mobile phases modified by acetonitrile and practically negligible in methanol solutions and, according to a recent study,[96] it should be attributed to two conflicting processes, i.e., a solvent "demixing" process and a slow change in stationary-phase conformation as a result of change in mobile-phase composition during gradient elution.

The theory of the dual-mode gradient elution involving changes in column temperature and mobile-phase composition was tested for retention prediction of six alkylbenzenes in aqueous eluting systems modified by acetonitrile.[17] Table 9 summarizes the comparison between calculated and experimental retention times obtained when we take into account the temperature hysteresis (see Eq. (47)). The initial temperature in all T-gradients was 15°C, the final was 75°C and the gradient time, i.e., the time between the initial and the final temperature, was 6 (gT_1), 15 (gT_2), 20 (gT_3), and 30 (gT_4) min. It is seen that the agreement is excellent in all types of dual-mode gradient programs tested. The average

Table 9. Predicted retention data obtained under dual-mode gradient conditions and percentage absolute error between experimental and predicted data when the hysteresis between actual and effective temperature is taken into account. (Reprinted from Ref. [17] with permission from ACS)

T-gradient	gT_1		gT_2		gT_3		gT_4	
φ_{min}	0.4		0.4		0.4		0.4	
φ_{max}	0.7		0.7		0.7		0.7	
t, min	20		30		40		50	
Solute	t_R (calc)	% error	t_R (calc)	% error	t_R (calc)	% error	t_R (calc)	% error
B	8.594	1.0	9.071	3.4	9.591	0.7	9.890	1.7
T	11.473	0.0	12.886	2.2	13.772	1.5	14.678	2.1
EB	14.586	0.8	16.883	1.8	18.571	0.8	20.380	1.2
*i*PB	17.183	0.7	20.376	1.6	22.977	0.1	25.570	1.0
PB	17.985	0.9	21.483	1.2	24.277	0.1	27.171	0.8
*t*BB	19.288	0.9	23.283	1.1	26.582	0.4	29.978	0.3
Average % error		0.7		1.9		0.6		1.2

percentage error between experimental and predicted retention times is less than 2%.

The above method for estimating the retention time practically converges to the elution time if $\Delta t \leq 0.1$ min and $\delta t \leq 0.0001$ min ($m = \Delta t/\delta t \geq 1000$). That is, the computational time is rather long and this is the main disadvantage of this approach. An alternative approach, much easier and less time consuming for the estimation of t_R, arises from the numerical solution of Eq. (2).

Consider that, in the mixer, the following profiles are formed: $\varphi_i = \varphi_i(t)$, $pH = pH(t)$, $F = F(t)$, and $T = T(t)$. From the latter, the effective profile $T_{ef} = T_{ef}(t)$ is easily calculated. Then, we divide the time axis into small segments, δt, and consider the i-th time step. During this time step, the analyte covers a distance equal to δL_i and its position from the inlet of the chromatographic column can be estimated from

$$\ell_i = \ell_{i-1} + \delta L_i \tag{73}$$

where $\ell_0 = 0$. In order to calculate δL_i, we may use Eq. (1) with δt in place of δt_c. In addition, for the calculation of δL_i, we need to know the values of the separation variables that affect the analyte during the i-th step, that is, the values of $\varphi_{1i}, \varphi_{2i}, \ldots, pH_i, F_i$ and T_{efi}. Since any change in F and T is transferred almost immediately from the mixer to the analyte, we have: $F_i = F(t)$ and $T_{efi} = T_{ef}(t)$, where $t = i\delta t$. For the values of $\varphi_{1i}, \varphi_{2i}, \ldots$, and pH_i, we have to take into account the delay in any change in φ_i and/or pH created in the mixer to reach the analyte. This delay can be calculated by means of Eq. (72) which, in the present case, is extended to

$$t_D + t_o \ell_i / L = \int_{t^*}^{t} F(t)dt \tag{74}$$

where t^* is the time that a change in φ_i and/or pH takes place in the mixer and t is the time needed for this change to be transferred to the analyte inside the column. Therefore, in order to calculate the values of φ_{1i}, $\varphi_{2i}, \ldots$, and pH_i we put in the upper limit of Equation. (74) $t = i\delta t$ and determine from this equation the value of t^*. If $t^* < 0$, we put $t^* = 0$. Using this value of time, the effective separation variables are calculated from $\varphi_{1i} = \varphi_1(t^*)$, $\varphi_{1i} = \varphi_1(t^*), \ldots$, and $pH_i = pH(t^*)$. Now, δL_i is determined by means of Eq. (1) using $\delta t_c = \delta t$, $t_{o,i} = t_o/F_i$ and $k_i = k(\varphi_{1i}, \varphi_{2i}, \ldots, pH_i, F_i, T_{efi})$ and the position of the analyte inside the column is estimated from the recursive Eq. (73). The analyte is eluted when Eqs. (70) are fulfilled and, therefore, the retention time is calculated from

$$t_R = p\delta t \tag{75}$$

The theory is successfully applied to the separation of 12 o-phthalal dehyde derivatives of amino acids in eluting systems modified by acetonitrile.[95] Average errors below 1.4% have been found in the retention prediction for all types of gradient programs involving simultaneous changes of the modifier content, flow rate, and temperature (see Table 10). Additionally, the use of three-mode gradients has been shown to have a big potential for producing well balanced optimized chromatograms in the minimum analysis time. Figure 11c shows the chromatogram selected as optimum three-mode gradient profile. A regular peak distribution is shown in the optimized gradient chromatogram, leaving a relatively large void in the initial part of the chromatogram, which is very useful for the analysis of real samples. For comparison, Figure 11a shows the chromatogram obtained under isocratic conditions and Figure 11b the chromatogram recorded under one-mode gradient conditions.

Table 10. Experimental retention data (in min) of amino-acid derivatives obtained in different three-mode gradient runs and absolute percentage errors between them and calculated retention data by the predictive approach explained in the text. (Reprinted from Ref. [95] with permission from ACS)

Gradient	$g(\varphi,F,T)_1$		$g(\varphi,F,T)_2$		$g(\varphi,F,T)_3$	
φ	$0.34 \rightarrow 0.4$		$0.3 \rightarrow 0.4$		$0.3 \rightarrow 0.4$	
t, min	$3 \rightarrow 4$		$0 \rightarrow 30$		$0 \rightarrow 30$	
F, mL/min	$0.5 \rightarrow 1.5$		$0.5 \rightarrow 1.5$		$0.5 \rightarrow 1.5$	
t, min	$6 \rightarrow 6.1$		$0 \rightarrow 30$		$0 \rightarrow 30$	
Temp. °C	$20 \rightarrow 80$		$20 \rightarrow 80$		$15 \rightarrow 75$	
t, min	$0 \rightarrow 2$		$0 \rightarrow 2$		$0 \rightarrow 30$	
Solutes	t_R(exp)	error (%)	t_R(exp)	error (%)	t_R(exp)	error (%)
Arg	3.604	4.6	3.987	0.8	4.057	0.2
Tau	4.007	2.8	4.450	2.2	4.510	2.2
Asn	4.420	0.2	4.842	0.8	4.937	1.3
Gln	4.692	0.2	5.246	0.5	5.397	0.0
Ser	5.387	2.4	5.850	0.1	6.031	0.1
Thr	6.161	2.3	7.773	0.9	8.075	0.8
Dopa	6.877	0.1	9.112	1.0	9.726	0.9
Met	9.978	0.3	18.27	0.1	20.16	1.1
Val	11.02	0.6	20.41	0.1	22.31	1.1
Phe	12.03	1.0	22.31	0.3	24.68	0.9
Ile	13.92	1.0	24.93	0.1	27.33	1.2
Leu	14.24	1.6	25.30	0.8	27.80	0.8
Average % error		1.4		0.6		0.9

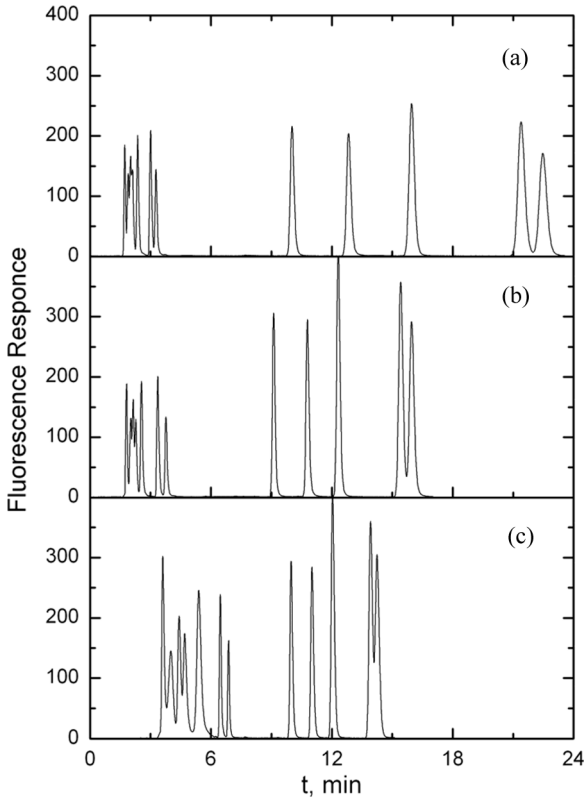

Figure 11. Fluorescence detection chromatograms of a 12-component mixture of derivatized amino acids obtained (a) under isocratic/isorheic/isothermal conditions at $\varphi = 0.36$, $F = 1$ mL/min and 40°C, (b) by using at 40°C a single-mode φ-gradient from $\varphi = 0.34$ to $\varphi = 0.4$ between 3 and 4 min, and (c) by using the selected as optimum three-mode gradient $g(\varphi,F,T)_1$ shown in Table 10. The elution order of the amino acid derivatives is the following: Arg, Tau, Asn, Gln Ser, Thr, Dopa, Met, Val, Phe, Ile, Leu. (Reprinted from Ref. [95] with permission from ACS).

FITTING APPROACHES FOR RETENTION PREDICTION

Objective Functions

The prediction of the solute elution time requires, always, a fitting procedure for the determination of the adjustable parameters of a retention model. The fitting may be performed to either isocratic or gradient data. In both cases, the objective (cost) function for fitting may be either

$$CF = \sum_{j=1}^{N} (t_{R_j,\mathrm{exp}} - t_{R_j,calc})^2 \tag{76}$$

or

$$CF = \sum_{j=1}^{N} (\ln k_{j,\mathrm{exp}} - \ln k_{j,calc})^2 \tag{77}$$

where $t_{R_{j,exp}}$, $\ln k_{j,exp}$ are the experimental retention time and the logarithm of the retention factor of a certain solute under the j-th measurement, respectively, and $t_{R_{j,calc}}$, $\ln k_{j,calc}$ are the corresponding calculated from the retention model values. The suggestion by Torres-Lapasio et al.[97] to use weighted least squares when we fit ln k data with weights calculated from

$$w = \frac{1}{\left(\frac{\partial \ln k}{\partial k}\right)^2} = k^2 \tag{78}$$

is equivalent to the use of the objective function of Equation (76).

The two above expressions, i.e., Eqs. (76) and (77) of the objective function, do not give always practically converged results. If the φ range used is not narrow, Eq. (76) reduces the error in the high t_R values and increases it in the small values of t_R. Quite the opposite is the behaviour of Eq. (77). An example is shown in Figure 12, which shows the percentage absolute error between experimental and calculated retention times of benzene in iPrOH (a) and MeCN (b) as a function of the mobile phase composition in φ. We observe that the use of weighting factors is, indeed, equivalent to using Eq. (76) and that the use of this equation has, as a result, small errors at low φ values, i.e., at great retention times, and great errors at high φ values, i.e., at low retention times. At any rate, the use of Eq. (77) seems to have a more balanced behaviour.

Finally, statistical tests for the significance of the various adjustable parameters of a retention model or for the choice of the proper retention model may be used,[81] although the ultimate criteria for a retention model is the percentage error between calculated and experimental retention times and the lack of over-fitting problems.

Fitting Algorithms

The fitting to isocratic data is usually easy and can be performed using commercial software, like the Regression and Solver programs of Excel. The fitting to gradient data may require home-made software. Previous study[68] has shown that the fitting problem can be solved using

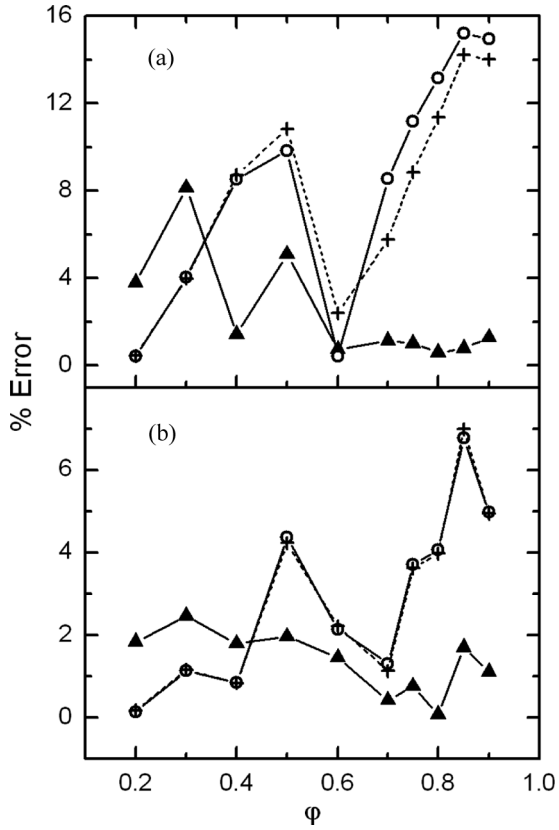

Figure 12. Percentage absolute error between experimental and calculated retention times of Benzene in iPrOH (a) and MeCN (b) as a function of φ. Retention times were calculated from Eq. (7) using the objective function of Eq. (76) (o), and Eq. (77) with (+) and without (▲) weighting factors from Eq. (78). (Reprinted from Ref. [65] with permission from Elsevier).

algorithms based on the Levenberg-Marquardt (LM) method.[98–101] However, the direct application of the LM algorithm exhibits several problems, the most important of which is the trapping of the algorithm into local minima other than the global one. For this reason, in recent papers,[69,102] we proposed two variations of this algorithm denoted by RND_LM and R_LM, respectively.

The RND_LM modification of the LM algorithm arises if we combine LM with an initial random search of the domain adopted for the adjustable parameters (c_0, c_1, c_2, ...) of the retention model.[69,102] Thus, a number of vectors (c_0, c_1, c_2, ...) are randomly selected from the search domain and the vector that yields the minimum value of the objective

function is selected and its coordinates c_0, c_1, c_2, ... are used as initial estimates in the LM algorithm, which determines the minimum of the objective function. In the R_LM modification, the LM algorithm is repeated several times. In particular, the algorithm starts with an initial vector (c_0, c_1, c_2, ...) randomly selected from the search domain and the LM method using a small number of iterations (100 in our applications) determines the local minimum, which is stored. Then, a new initial vector (c_0, c_1, c_2, ...) is randomly selected and the whole algorithm is repeated for a preset number of iterations. The minimum of the stored local minima is determined and it presumably corresponds to the global minimum of the objective function. Apart from the LM based algorithms, genetic algorithms (GA)[103–105] as well as algorithms based on the descent method (D),[105] have been also used to solve fitting problems.[106,107]

OPTIMIZATION

Objective Functions

The prediction of the elution times of a mixture of solutes is the first step towards the separation optimisation. The final goal is to find the optimum gradient profile that yields the best separation of all solutes to the minimum gradient time. From the retention times of all solutes, we can calculate either the absolute difference

$$\delta t_{\mathrm{R,ij}} = |t_{\mathrm{R,i}} - t_{\mathrm{R,j}}| \tag{79}$$

between all possible pairs of adjacent solutes, i and j, or the resolution

$$R_{\mathrm{S,ij}} = 2\frac{t_{R,j} - t_{R,i}}{w_j + w_i} \tag{80}$$

of adjacent solutes. Here, $t_{\mathrm{R,j}} > t_{\mathrm{R,i}}$ and w_i, w_j are the peak widths. Note that Eq. (79), which considers only peak position, is the simplest one and it is suited for comparing chromatograms where the peaks are relatively narrow with regard to the peak distance.

For the optimization, we may adopt single- or multi-objective optimization criterion. The most general formulation of an optimization procedure may be the following[108]

$$\begin{cases} \text{Maximize/minimize } \mathrm{CF_m} = \mathrm{f_m}(\mathbf{x}), \mathrm{m} = 1,2,\ldots \mathrm{M} \\ \text{or Maximize/minimize } \mathrm{CF} = \sum_{1}^{\mathrm{M}} \mathrm{w_m f_m}(\mathbf{x}), \mathrm{m} = 1,2,\ldots \mathrm{M} \\ \text{subject to } \mathrm{g_j}(\mathbf{x}) \geq 0 \text{ and } \mathrm{h_k}(\mathbf{x}) = 0, \mathrm{j} = 1,2,\ldots,\mathrm{J}, \mathrm{k} = 1,2,\ldots,\mathrm{K} \\ \text{when } x_i^L \leq x_i \leq x_i^U, \mathrm{i} = 1,2,\ldots,n \end{cases} \tag{81}$$

Here, we have M objective functions $f_1(\mathbf{x})$, $f_2(\mathbf{x}), \ldots$. or one, which is the weighted sum of $f_1(\mathbf{x})$, $f_2(\mathbf{x}), \ldots$, subject to J+K constraints. w_1, $w_2, \ldots$ are weighting factors, and $\mathbf{x}$ is the vector of the independent separation variables x_i, i.e., the variables that are chosen in the optimisation problem and which vary from x_i^L to x_i^U.

In the single-objective optimization problem, $M = 1$. Two typically adopted single-objective functions are

$$\text{Minimize } CF = t_{R,\max} \text{ subject to } R_{s,\min} > R_{\min} \tag{82}$$

$$\text{Maximize } CF = \delta t_1 \text{ subject to } t_{R,\max} < t_{g,\max} \tag{83}$$

where δt_1 is the minimum value of $\delta t_{R,ij}$ or $R_{S,ij}$, $t_{R,max}$ is the elution time of the most distant solute, $t_{g,max}$ is the maximum gradient elution time preset by the researcher, $R_{s,min}$ is the smaller $R_{S,ij}$ value, and R_{min} is a preset constant usually equal to 1.5.

For multi-objective optimization, we may use either the popular approach of the weighted sum method or we examine more than one of the objective functions separately. Thus, in the weighted sum method, the objective function is written as

$$\text{Maximize/minimize CF} = \sum_{i=1}^{M} w_i f_i(x) \tag{84}$$

The first objective function of this form was suggested by Berridge[109]

$$\text{Minimize } CF = -\sum_i R_{S,ij} - P^{w_1} + w_2|t_{g,\max} - t_{R,\max}| + w_3|t_{R,0} - t_{R,1}| \tag{85}$$

where P is the number of peaks detected, $t_{R,0}$ is the minimum retention time desired for the first eluted peak, $t_{R,1}$ is the real retention time for that first peak. Factors w_1, w_2, w_3 are operator-selectable weightings that, according to Berridge, are usually set to values between 0 and 3.

Alternatively, we may use:

$$\begin{aligned}\text{Minimize CF} = &-(w_1\delta t_1 + w_2\delta t_2 + w_3\delta t_3) + t_{R,\max} \\ &- t_{g,\max} \text{ when } t_{R,\max} > t_{g,\max}\end{aligned} \tag{86}$$

and

$$\text{Minimize CF} = -(w_1\delta t_1 + w_2\delta t_2 + w_3\delta t_3) \text{ when } t_{R,\max} \leq t_{g,\max} \tag{87}$$

where δt_1, δt_2, δt_3 are the first three minimum values of $\delta t_{R,ij}$ or $R_{S,ij}$. If we want the most distant solute to elute close to $t_{g,max}$, then the objective functions (86) and (87) should be replaced by

$$\text{Minimize CF} = -(w_1\delta t_1 + w_2\delta t_2 + w_3\delta t_3) + |t_{g,max} - t_{R,max}| \qquad (88)$$

An alternative approach for multi-objective optimization is to examine more than one objective functions

$$\text{Maximize/minimize } CF_m = f_m(\mathbf{x}), m = 1, 2, \ldots, M \qquad (89)$$

subject to certain constraints. In this case, methods of multi-criteria decision making should be applied for estimation of the optimum. The most popular of them is Pareto optimality,[108,110–113] in which one compares all the experimental results with each other. A point is called Pareto optimal if there exists no other experiment which has a better result in one objective without having a worse result in another objective. The Pareto optimal points form the Pareto front, which, by definition, contains the optimal solutions to a certain optimization problem. It is seen that there is no one optimum point, but there are several Pareto-Optimal points. Among these Pareto-optimal solutions, one or several are selected for the optimum separation of the sample mixture, either with secondary criteria or personal criteria decided by the chromatographer.

Other criteria for separation and optimisation, like the desirability function, a multi-criterion decision-making method proposed by Derringer, has been proposed and discussed in Refs. [114–117].

Optimization Algorithms

Although the problems of fitting and optimization are interrelated, there is a striking difference. The fitting objective functions, Eqs. (76), (77), are differentiable functions, whereas this property is not valid for the objective functions used for optimization. Thus, the LM-based algorithms cannot be used for optimization. For this reason, genetic algorithms (GA), as well as algorithms based on the descent method (D), are usually used for the determination of the optimum gradient.

In a recent study,[69] we found that the classical GA suggested by Michalewicz[103] with linear scaling,[104] combined crossover and Gaussian mutation[103] performs very well. For its application, we may use population size 100, crossover probability 0.8, and probability of mutation 0.02.

In the same study,[69] we have proposed a variant of the descent method, the RND_D algorithm, which can be applied for both fitting

and optimization with very good results. This algorithm involves the following steps:[64,69]

1. A number of N_{rnd} vectors $\mathbf{x} = (x_1, x_2, \ldots, x_m)$, where $x_1, x_2, \ldots, x_m$ are either the adjustable parameters of a retention model or the coordinates of the gradient profile that leads to the best separation of the chromatographic peaks of a mixture of analytes, are randomly selected from the search domain and the vector that yields the minimum value of the cost function is selected.
2. N_{neigh} ($= N_{neigh1} + N_{neigh2}$) neighbour vectors $\mathbf{x}_N$ are created around $\mathbf{x}$ as follows: N_{neigh1} vectors are randomly selected from the search domain and N_{neigh2} are produced by means of $x_{N,i} = x_i + N(0, \sigma_i)$, where $N(0, \sigma_i)$ is a random number that follows the normal (Gaussian) distribution with 0 mean value and standard deviation equal to σ_i.
3. If $CF(\mathbf{x}_N) < CF(\mathbf{x})$, then $\mathbf{x} = \mathbf{x}_N$ and go to 2. If $CF(\mathbf{x}_N) \geq CF(\mathbf{x})$, then again go to 2 without changing $\mathbf{x}$.
4. Steps 2 to 3 are repeated for N_D times and $\mathbf{x}_N$ is the final solution.

The RND_D algorithm exhibits the following advantage: If the standard deviations σ_i are small enough, it searches thoroughly the area close to the current solution $\mathbf{x}$ by means of the N_{neigh2} vectors but, at the same time, explores far distant areas using the N_{neigh1} vectors to determine the possibility of better solutions which might lead to the global minimum. For the application of the RND_D algorithm, we used the following control parameters: $N_D = 2000$, $\sigma_i = 0.01$, $N_{neigh1} = 100$, and $N_{neigh2} = 10$. Note that the adjustable parameters should be scaled to vary in the range [0, 1].

The RND_D algorithm has been adopted to determine the optimum separation conditions in Refs.[64,69] and some of the obtained results are shown in Figures 4 and 8. In contrast, the optimum conditions in Figure 3 were determined by means of a grid search technique.[67] Such simple techniques are quite effective when the number of the separation variables is small, as in linear gradients. Thus, in the example of Figure 3, the optimum conditions are determined by the values of t_{in}, φ_{in}, b, which may be estimated following the 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 at a certain pair of φ_{in} and b values and, from these values, we calculate the quantities δt_1 and $t_{R,max}$. It is evident that the best pair of φ_{in}, b values is that which corresponds to the maximum δt_1, 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.

If we use the simple Pareto optimal approach with two criteria, δt_1 and $t_{R,max}$, we may adopt the objective function of Eq. (88) with $w_1 = 1$ and $w_2 = w_3 = 0$ to construct the Pareto front. Indeed, using this objective function at each value of $t_{R,max}$ we determine the corresponding δt_1 value and the plot of δt_1 vs. $t_{R,max}$ is, in fact, the Pareto front from which the chromatographer will select the optimum solution(s). If the system is such that no practical solution can be found, one may examine the plots δt_2 vs. $t_{R,max}$ or δt_3 vs. $t_{R,max}$.

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