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Expressions for Multilinear Combined pH/Organic Solvent Elution of Ionizable Analytes in Reversed-Phase HPLC

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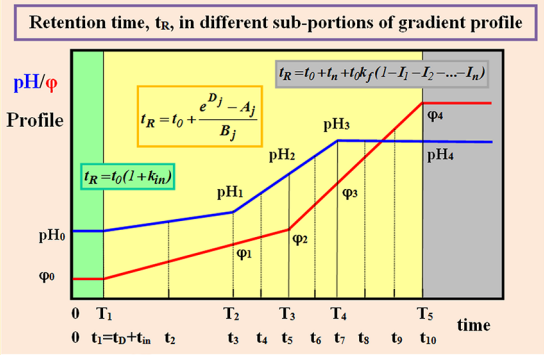
Supporting Information

ABSTRACT: Expressions for the retention time of ionogenic analytes eluted under multilinear double pH/solvent-gradients in reversed-phase liquid chromatography are developed by dividing each gradient profile into a finite number of subportions, where the solute retention factors or their logarithms vary linearly with time. To test the theory, two series of experimental gradient retention data of amino acid OPA derivatives were analyzed: The first one was a monolinear or bilinear pH-gradient data set obtained in eluents with different but constant organic modifier contents, whereas the second data set comprised retention data of combined pH/organic solvent-gradients, where the organic content was changed linearly with time but the variation of pH exhibited a curved form approximated by five linear subportions. It was found that the derived expressions describe these experimental retention data with high accuracy, since under double pH/solvent-gradients the overall errors in the fitted and predicted retention times were 1.9% and 1.7%, respectively, whereas under simple pH-gradients these errors were 0.9% and 2%, respectively.



High-performance liquid chromatography (HPLC) is a fundamental technique used in quantitative and qualitative applications, and numerous attempts have been made in order to model the retention at either isocratic or gradient conditions.[1−11] The most common gradient technique is the mobile phase gradient elution. However, especially for ionogenic analytes, the most crucial chromatographic parameter is the mobile phase pH, since it changes their dissociation degree. Nevertheless, experimental difficulties, such as columns stable over a wide range of pH values and proper control of pH-gradient, delayed the development of this technique. For the same reason, the pH-gradient elution theory has only recently been developed by Kaliszan's group.[12−15] Moreover, the same group has used the combined pH/organic modifier content linear gradient elution as a new approach for the determination of ionogenic analytes in biological liquids.[16] Finally, Kaliszan and his colleagues have developed comprehensive theories, which can describe the simultaneous pH/organic modifier content gradient elution and can be used for the prediction of ionogenic analytes retention.[17−19] As a result of the above work, as well as of the corresponding work carried out in our laboratory,[20,21] mathematical expressions for the solute retention time have been proposed for the case of single linear pH-gradients[20] and organic solvent-gradients at different eluent pH values.[21]

However, the most general and challenging case is that of the simultaneous changes in pH and organic modifier content during the gradient process. Under these conditions, the solute retention time, t_R, could easily be estimated by means of the solution of the fundamental equation:

$$\int_0^{t_R-t_0} \frac{dt}{t_0 k} = 1 \Rightarrow \frac{t_D + t_{in}}{t_0 k_{in}} + \int_{t_D+t_{in}}^{t_R-t_0} \frac{dt}{t_0 k[X(t - t_D - t_{in})]} = 1 \quad (1)$$

where t_0 is the column dead time, t_D is the dwell time, that is, the time needed for the analyte to reach the column inlet, t_{in} is the time the gradient starts in the pump program, k is the analyte retention factor, k_{in} is the value of k at the start of the gradient, and $X(t)$ denotes the gradient profile programmed in the system. The numerical solution of this equation is straightforward provided that k is a known function of time, t.[22] For retention prediction purposes, this approach is very effective. However, for optimization purposes, the numerical solution of eq 1 is inadequate. The optimization algorithms usually demand a great number of iterations to find the best solution, and the incorporation of a subroutine that solves numerically eq 1 increases the computational time considerably. Therefore, the development of mathematical expressions for t_R, apart from its academic value, is particularly useful in optimization schemes.

Recently, we have proposed an approach to predict retention under double pH/organic solvent-gradients, which is based on a ninth parameter empirical model.[23] This model, easily manageable through a linear least-squares fitting, allows a very satisfactory prediction of gradient data obtained in pH/

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organic modifier content double gradients carried out between fixed initial pH/organic modifier values but different final ones and for different gradient durations. However, this empirical model lacks generality, since it is valid only under the restriction of fixed initial pH/organic modifier content values, and this limitation reduces considerably the application of an effective optimization procedure. In addition, this retention modeling cannot be extended to multilinear gradients.

In the present paper we attempt to derive mathematical expressions for t_R for the case of simultaneous linear and multilinear pH/organic solvent double gradients based on eq 1. For this purpose, the theory presented in refs 24−27 is properly extended to include both variables pH and organic modifier content. The expressions for t_R are further used in algorithms for fitting, prediction, and optimization, and their accuracy is tested by the use of the experimental data of *o*-phthalaldehyde (OPA) derivatives of amino acids.

THEORY

The solute retention time, t_R, under gradient elution may be estimated by solving the fundamental eq 1 provided that the dependence of solute retention factor, k, upon time is known. In the case of monoprotic acids, the retention factor is given by[28−30]

$$k = \frac{k_0 + k_1 10^{\mathrm{pH-pK}}}{1 + 10^{\mathrm{pH-pK}}} \tag{2}$$

where k_0 and k_1 are the retention factors of the nonionized and ionized forms of the ionogenic analyte, respectively. These factors, k_0 and k_1, may depend on the organic modifier concentration, and the following simple expressions for this dependence were adopted in the present study[17,20]

$$\ln k_0 = k_{00} + k_{01}\varphi \tag{3}$$

$$\ln k_1 = k_{10} + k_{11}\varphi \tag{4}$$

where k_{00}, k_{01}, k_{10}, and k_{11} are adjustable parameters estimated by fitting to experimental retention times. Note that eqs 3 and 4 express the linear solvent strength model, which is the most commonly used retention model in gradient elution.[4−6,31−33]

Under multilinear double pH/φ-gradient conditions, pH and φ are linear functions of time, t. In this case each gradient profile consisting of the multilinear curves pH = $g_1(t)$ and φ = $g_2(t)$ may be expressed as vectors with common time coordinates as follows. Consider the pH/φ-gradient profiles of Figure 1. They may be written as (t_1, t_{pH_A}, t_{pH_B}, pH_0, pH_A, pH_B) and (t_1, t_{φ_A}, t_{φ_B}, φ_0, φ_A, φ_B). This notation determines precisely the pH vs t and φ vs t-gradients, since they are both multilinear gradients. Thus, for example, at $t = t_1$ the pH in the mobile phase is pH_0 and varies linearly up to the value pH_A, which is attained at $t = t_{\mathrm{pH}_A}$. In order to use common time coordinates, we put the time values t_1, t_{pH_A}, t_{pH_B}, t_{φ_A}, and t_{φ_B} in an ascending order, t_1, t_{pH_A}, t_{φ_A}, t_{pH_B}, t_{φ_B}, and we denote these times by T_1, T_2, T_3, T_4, and T_5. Now, at time T_j a certain pH_{j-1} value as well as a certain φ_{j-1} value correspond. This process is visualized in Figure 2. Thus from the initial vectors (t_1, t_{pH_A}, t_{pH_B}, pH_0, pH_A, pH_B) and (t_1, t_{φ_A}, t_{φ_B}, φ_0, φ_A, φ_B) we obtain the vectors (T_1, T_2, T_3, T_4, T_5, pH_0, pH_1, pH_2, pH_3, pH_4) and (T_1, T_2, T_3, T_4, T_5, φ_0, φ_1, φ_2, φ_3, φ_4), which exhibit common time coordinates. Therefore, in general, we may express each pH vs t and φ vs t multilinear double gradient as (T_1, T_2, ..., T_p, pH_0, pH_1, ..., pH_p) and (T_1, T_2, ..., T_p, φ_0, φ_1, ..., φ_p). Note that when the injection time corresponds to $t = 0$, the dwell time t_D should be included in the time axis.

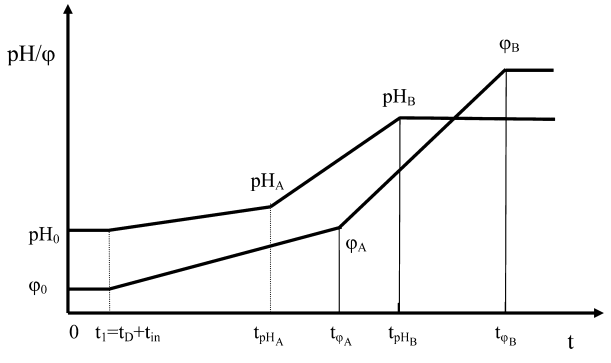

Figure 1. Schematic multilinear gradient profiles of two separation variables pH and φ vs t.

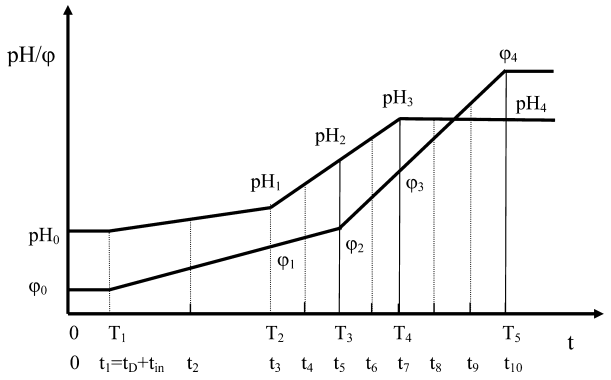

Figure 2. Graphical steps for creating multilinear pH/φ-gradient profiles with common time coordinates T_1, T_2, ..., T_5 and the division of each interval $[T_{i-1}, T_i]$ into subportions $[t_{j-1}, t_j]$ by means of the coordinates t_1, t_2, ..., t_{10}.

Thus, the general mathematical expression of a multilinear gradient in X = pH or φ may be expressed as

$$X = \begin{cases} X_1 = X_0 & t < t_D + t_{in} = T_1 \\ X_1 + \lambda_{X2}(t - T_1) & T_1 < t < T_2 \\ \cdots & \cdots \\ X_{p-1} + \lambda_{Xp}(t - T_{p-1}) & T_{p-1} < t < T_p \\ X_p = X_f & t > T_p \end{cases} \tag{5}$$

where λ_{Xi} is the slope of the X vs t profile at the i-th segment, X_0 is the initial value of X, and X_f is the final value of X. For simplicity we may assume that all slopes are different from zero, since a zero slope can be approximated in practice by an infinitesimally small slope.

If eqs 3−5 are introduced into eq 2, the retention factor k becomes a function of time t and in combination with eq 1 it can be used for the calculation of t_R. However, due to the complexity of k, this calculation can be carried out only numerically. Mathematical expressions of t_R may be obtained if we follow the approach developed in refs 24−27.

According to this approach, every nonlinear ln k or k versus t curve can be always subdivided into m linear portions, allowing for the development of mathematical expressions for t_R. In

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Table 1. Expressions of the Retention Time under Multilinear pH/φ-Gradients When the Approximation That $k = A_j + B_j t$ Holds in Each Subportion $[t_{j-1}, t_j]$ Is Adopted

section	condition	t_R
1	$I_1 = \frac{t_{in} + t_D}{t_0 k_{in}} \geq 1$	$t_R = t_0(1 + k_{in})$
j	$I_1 + I_2 + ... + I_{j-1} < 1$	$t_R = t_0 + \frac{e^{D_j} - A_j}{B_j}$
	$I_1 + I_2 + ... + I_{j-1} + I_j \geq 1$	where $B_j = \frac{k(\mathrm{pH}_j, \varphi_j) - k(\mathrm{pH}_{j-1}, \varphi_{j-1})}{t_j - t_{j-1}}$
	where for $i > 1$: $I_i = \frac{1}{t_0 B_i} \ln \frac{A_i + B_i t_i}{A_i + B_i t_{i-1}}$	$A_j = k(\mathrm{pH}_j, \varphi_j) - B_j t_j$
		$D_j = \ln(A_j + B_j t_{j-1}) + B_j(1 - I_1 - ... - I_{j-1})t_0$
$>n$	$I_1 + I_2 + ... + I_{n-1} + I_n < 1$	$t_R = t_0 + t_n + t_0 k_j(1 - I_1 - I_2 - ... - I_n)$

more detail, each interval $[T_{i-1}, T_i]$ in eq 5 is divided into a certain number of subportions, as described in ref 26. This division results in a further division of the time axis, $t_1, t_2, ..., t_n$ (see example in Figure 2). If each of these subportions $[t_{j-1}, t_j]$ is narrow enough, we may reasonably assume that k or/and $\ln k$ varies linearly with t. Let us assume that

$$k = A_j + B_j t \tag{6}$$

holds when t ranges from t_{j-1} to t_j. In this expression, coefficients A_j, B_j can be calculated from the boundary conditions:

$$A_j + B_j t_{j-1} = \frac{k_0 + k_1 10^{\mathrm{pH}_{j-1} - \mathrm{p}K}}{1 + 10^{\mathrm{pH}_{j-1} - \mathrm{p}K}} = k(\mathrm{pH}_{j-1}, \varphi_{j-1}) \tag{7}$$

and

$$A_j + B_j t_j = \frac{k_0 + k_1 10^{\mathrm{pH}_j - \mathrm{p}K}}{1 + 10^{\mathrm{pH}_j - \mathrm{p}K}} = k(\mathrm{pH}_j, \varphi_j) \tag{8}$$

which yield

$$B_j = \frac{k(\mathrm{pH}_j, \varphi_j) - k(\mathrm{pH}_{j-1}, \varphi_{j-1})}{t_j - t_{j-1}} \quad \text{and}$$
$$A_j = k(\mathrm{pH}_j, \varphi_j) - B_j t_j \tag{9}$$

The analyte can be eluted in the first isocratic portion, that is before $t_{in} + t_D$, or in the last isocratic portion when $t_R > T_p$ or at an intermediate linear portion. The condition for an analyte to be eluted at each of these portions and the corresponding expression for the retention time t_R are the following:

(A) When the analyte is eluted just at the end of the first isocratic portion, that is, at $t_{in} + t_D$, then from eq 1 we obtain that $I_1 = (t_{in} + t_D)/t_0 k_{in} = 1$, whereas if the elution occurs just after $t_{in} + t_D$, I_1 becomes less than 1 because the integral in eq 1 from $t_D + t_{in}$ to $t_R - t_0$ is always a positive number. Therefore, the condition for the analyte to be eluted in the first isocratic portion is given by the inequality

$$I_1 = \frac{t_{in} + t_D}{t_0 k_{in}} \geq 1 \tag{10}$$

In this case the retention time is given from the well-known expression

$$\int_0^{t_R - t_0} \frac{dt}{t_0 k_{in}} = 1 \Rightarrow \frac{t_R - t_0}{t_0 k_{in}} = 1 \Rightarrow t_R = t_0(1 + k_{in}) \tag{11}$$

where k_{in} is the retention factor when pH and φ are equal to their initial values; that is, k_{in} is calculated from eqs 2−4 by using $\mathrm{pH} = \mathrm{pH}_0$ and $\varphi = \varphi_0$.

(B) When the analyte is eluted in the j-th linear segment ($j > 1$), that is between t_{j-1} and t_j, the conditions are the following

$$\int_0^{t_{j-1}} \frac{dt}{t_0 k} < 1 \quad \text{and} \quad \int_0^{t_j} \frac{dt}{t_0 k} \geq 1 \tag{12}$$

The last integral may be expressed as

$$\int_0^{t_j} \frac{dt}{t_0 k} = \int_0^{t_1} \frac{dt}{t_0 k} + \int_{t_1}^{t_2} \frac{dt}{t_0 k} + ... + \int_{t_{j-1}}^{t_j} \frac{dt}{t_0 k} = I_1 + I_2 + ... + I_j \tag{13}$$

Therefore, the conditions for the analyte to be eluted in the j-th linear segment may be expressed as

$$I_1 + I_2 + ... + I_{j-1} < 1 \quad \text{and}$$
$$I_1 + I_2 + ... + I_{j-1} + I_j \geq 1 \tag{14}$$

where I_1 is calculated from eq 10 and I_i, $i > 1$, from

$$I_i = \frac{1}{t_0} \int_{t_{i-1}}^{t_i} \frac{dt}{A_i + B_i t} = \frac{1}{t_0 B_i} \ln \frac{A_i + B_i t_i}{A_i + B_i t_{i-1}} \tag{15}$$

In what concerns the retention time t_R of the analyte, we have

$$\int_{t_{j-1}}^{t_R - t_0} \frac{dt}{k} = \int_{t_{j-1}}^{t_R - t_0} \frac{dt}{A_j + B_j t} = (1 - I_1 - ... - I_{j-1})t_0 \tag{16}$$

which results in

$$t_R = t_0 + \frac{e^{D_j} - A_j}{B_j} \tag{17}$$

where

$$D_j = \ln(A_j + B_j t_{j-1}) + B_j(1 - I_1 - ... - I_{j-1})t_0 \tag{18}$$

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Table 2. Expressions of the Retention Time under Multilinear pH/φ-Gradients When the Approximation That $\ln k = A_j + B_j t$ Holds in Each Subportion $[t_{j-1}, t_j]$ Is Adopted

section	condition	t_R
1	$I_1 = \frac{t_{in} + t_D}{t_0 k_{in}} \geq 1$	$t_R = t_0(1 + k_{in})$
	$I_1 + I_2 + \cdots + I_{j-1} < 1$	$t_R = t_0 - \frac{\ln D_j + A_j}{B_j}$
	$I_1 + I_2 + \cdots + I_{j-1} + I_j \geq 1$	where
j		$B_j = \frac{\ln[k(pH_j, \varphi_j)/k(pH_{j-1}, \varphi_{j-1})]}{t_j - t_{j-1}}$
	where for $i > 1$:	$A_j = \ln k(pH_j, \varphi_j) - B_j t_j$
	$I_i = \frac{1}{t_0 B_i (e^{A_i + B_i t_{i-1}} - e^{A_i + B_i t_i})}$	$D_j = \frac{1}{\exp(A_j + B_j t_{j-1})} - B_j(1 - I_1 - \ldots - I_{j-1})t_0$
$>n$	$I_1 + I_2 + \cdots + I_{n-1} + I_n < 1$	$t_R = t_0 + t_n + t_0 k_f(1 - I_1 - I_2 - \ldots - I_n)$

(C) When the analyte is eluted in the last isocratic portion, that is, when pH and φ are equal to their final values, $\varphi = \varphi_f$ and pH = pH_f, the condition is

$$\int_0^{t_n} \frac{dt}{t_0 k} < 1 \Rightarrow I_1 + I_2 + \ldots + I_{n-1} + I_n < 1 \quad (19)$$

and the retention time t_R arises from

$$\int_{t_n}^{t_R - t_0} \frac{dt}{t_0 k} = 1 - I_1 - I_2 - \ldots - I_n \quad (20)$$

which yields

$$t_R = t_0 + t_n + t_0 k_f(1 - I_1 - I_2 - \ldots - I_n) \quad (21)$$

since $k = k_f = k(t_n)$ is constant.

Table 1 summarizes the above results. Similar results are obtained when we assume that $\ln k = A_j + B_j t$ holds in each subportion $[t_{j-1}, t_j]$. The results of this approximation are given in Table 2.

EXPERIMENTAL SECTION

Instrumentation and Solutes. The liquid chromatography system consisted of a Shimadzu LC-20AD pump, a Shimadzu DGU-20A_3 degasser, a model 7125 syringe loading sample injector fitted with a 20 μL loop, a 250 mm × 4.6 mm MZ-Analytical column (PerfectSil Target ODS-3 HD 5 μm) thermostated at 25 °C by a CTO-10AS Shimadzu column oven, and a Shimadzu RF-10AXL spectrofluorometric detector (Shimadzu, Model) working at 455 nm after excitation at 340 nm. The solutes were 17 OPA/2-mercaptoethanol derivatives of the following amino acids: L-arginine (Arg), L-asparagine (Asn), L-glutamine (Gln), L-serine (Ser), L-aspartic acid (Asp), L-glutamic acid (Glu), L-threonine (Thr), β-(3,4-dihydroxyphenyl)-L-alanine (Dopa), L-alanine (Ala), L-tyrosine (Tyr), 4-aminobutyric acid (GABA), L-methionine (Met), L-valine (Val), L-tryptophan (Trp), L-phenylanine (Phe), L-isoleucine (Ile), and L-leucine (Leu).

The mobile phase pH was measured after mixing the aqueous buffers and the organic modifier (acetonitrile), whereas the electrode system was calibrated with the usual aqueous standards.[34] The measurements were done with a Mettler Toledo Seven Easy pH-meter.

Note that the aqueous carboxylic pK values of free or underivatized common amino acids are about 2.3, with the exception of GABA, which exhibits a pK value equal to 4.23. However, in the present study the analytes are OPA derivatives of amino acids. For these compounds, direct pK measurements, either in aqueous or in hydro-organic solutions, are not available in the literature, but from Table 1 of ref 23 we can easily see that their retention time is strongly affected by the mobile phase pH variation. See for example in Table 1 of ref 23 the gradient experiments no. 2 and 20, 3 and 21, 4 and 22, etc. In these gradients the effect of the pH on the solute retention is so strong that in no case could it be justified if the amino acids−OPA derivatives had pK values lower than 3.2, which is the initial pH value in all pH-gradients of the present study in eluents containing acetonitrile (MeCN) at a concentration range of φ = 0.25−0.5. This increase in the pK of carboxylic groups of the OPA derivatives of amino acids may be due to the presence of MeCN in the mobile phase, irrespective to the pH increase in phosphoric or citric buffers in the same organic modifier content range.[35]

Chromatographic Experiments. For the validation of the expressions derived for multilinear pH/φ-gradients, two series of experimental gradient retention data were analyzed: The first one was monolinear and bilinear pH-gradient data obtained in eluents with different but constant φ values, whereas the second data set comprised retention data of combined pH-/φ-gradients, where φ was varied linearly with time but the variation of pH exhibited a curved shape.

The first data set was obtained from 10 chromatographic runs using 11 amino acid derivatives of concentration 5 μg/mL. During these gradients the concentration of acetonitrile, used as organic modifier, was kept constant at a value of φ = 0.25, 0.30, or 0.50, whereas two buffers (0.1 M citric acid and 0.2 M disodiumphoshate) were mixed at various proportions, providing monolinear or bilinear changes of eluent pH. The total ionic strength ranged between 10 and 18 mM. All the chromatographic experiments were carried out with a flow rate equal to 1 mL/min, where the hold-up time and the dwell time were estimated to be t_0 = 1.9 and t_D = 1.2 min, respectively. The obtained experimental retention data are presented in the Supporting Information (SI) in Table S-1.

The second series of double pH-/φ-gradient data set was taken from Table 1 of ref 23. This table contains the retention times of 17 amino acid derivatives obtained from 27 chromatographic runs generated by automatically mixing two mobile phases with different pH and organic (acetonitrile)

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content, according to a linear pump program with the same starting time (t_{in} = 0 min) but with different gradient duration, t_G = 10, 20, or 30 min, which was the same for both φ and pH changes. However, although in these gradient runs φ was changed in a linear manner, the actual pH-changes were curved, as they were found by measuring the pH values in separate solutions prepared according to programmed gradient profiles. We found that the shapes of the pH-gradients depend mainly on the value of pH_f and not on the value of φ_f, at least in the range of φ_f tested. See for example in Figure 3 the real pH

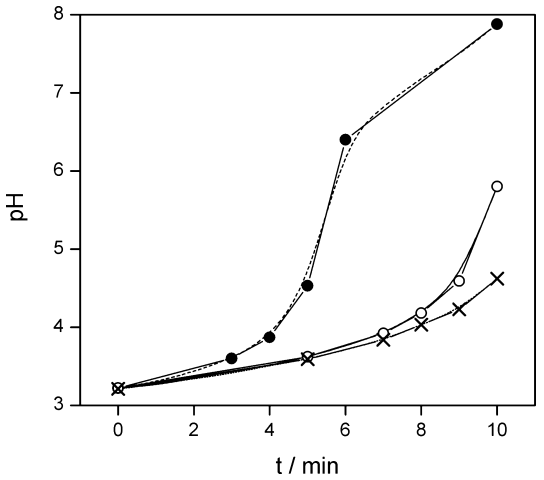

Figure 3. Actual shapes of pH vs t curves and their approximation by five linear portions, when pH is changed between 3.2 and 7.9 (●), 3.2−5.9 (○), and 3.2−4.7 (×) in double pH/φ-gradient experiments in Table 1 of ref 23 within t_G = 10 min.

changes that correspond to double pH/φ-gradient experiments in Table 1 of ref 23 within t_G = 10 min. The shapes of these pH-gradients may be explained by the variation of the buffer capacity of phosphate, as discussed by Subirats, Bosch, and Roses.[36] These curved pH-profiles were approximated by five linear portions, as shown in Figure 3, allowing for the application of the multilinear theory developed in the present paper.

Data Analysis. The application/evaluation of the theory presented above requires the knowledge of the pK of each analyte, as well as the parameters k_{00}, k_{01}, k_{10}, and k_{11} that determine the dependence of the retention factors of the nonionized, k_0, and ionized, k_1, forms of the analyte upon φ via eqs 3 and 4. Thus, the first step is the determination of these parameters through a nonlinear fitting procedure of the expressions presented in Tables 1 and 2 to experimental t_R data.

For fitting, an algorithm based on the R_LM algorithm proposed in ref 26 was written in C++. The objective (cost) function used for fitting was

$$CF = \sum_{j=1}^{N} (t_{R_j,exp} - t_{R_j,calc})^2 \quad (22)$$

where $t_{R_j,exp}$ is the experimental retention time of a certain solute under the j-th gradient and $t_{R_j,calc}$ is the corresponding retention time calculated from the equations of Table 1 or 2. The fitting performance is evaluated from the overall percentage error between experimental and calculated t_R values. The fitting procedure was applied to the whole data set as well as to a part of it. In the latter case, the rest of the data set was used for prediction.

Finally, a very simple optimization scheme was adopted to determine the optimum separation conditions under double pH-/φ-gradient conditions. The algorithm searched for the best φ_0, φ_f, and t_G values in the ranges $\varphi_0 \geq 0.25$, $\varphi_f \leq 0.50$, and 10 min $\leq t_G \leq$ 30 min. In what concerns the optimum pH-gradient, the algorithm used pH_0 = 3.2 and searched for the best pH_f only among the values 4.7, 5.9, and 7.9. The search of φ_0, φ_f, and t_G values was random over the domain defined by the above inequalities. The objective function (OF) used was

$$\text{maximize OF} = \delta t_1 \text{ subject to } t_{R,max} < t_{max} \quad (23)$$

where δt_1 is the minimum value of $\delta t_{R,ij} = |t_{R,i} - t_{R,j}|$ between all possible pairs of adjacent solutes, i and j, $t_{R,max}$ is the elution time of the most distant solute, and t_{max} is a preset maximum gradient elution time. In the present study t_{max} was set equal to 25 min.

RESULTS AND DISCUSSION

As mentioned above, for the evaluation of the models developed in the present study, two types of data were adopted: (1) pH-gradient data at different but constant φ values, and (2) double pH/φ-gradient data. However, we should first clarify (a) which of the approximations $k = A_j + B_j t$ or $\ln k = A_j + B_j t$ performs better, and (b) what is the optimum division of the time axis into subportions $[t_{j-1}, t_j]$. In the current study, the number of subportions $[t_{j-1}, t_j]$ is defined by the pH step, ΔpH, which is used to divide each linear portion of the pH curve into m equidistant portions, where a minimum value of m equal to 10 was adopted under all circumstances.

Figure 4 shows the variation of the average percentage error between experimental and calculated retention times upon

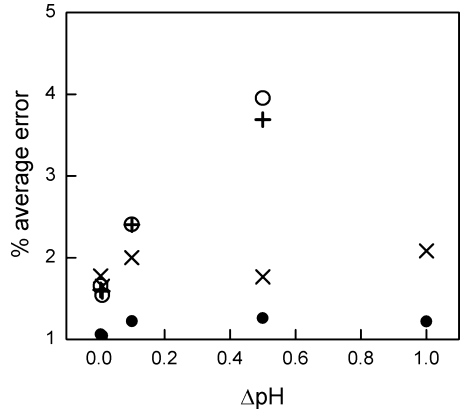

Figure 4. Dependence of the average percentage error between experimental and calculated retention times upon ΔpH when the approximation $k = A_j + B_j t$ (○, ●) and the approximation $\ln k = A_j + B_j t$ (+, ×) are adopted under single pH-gradients (×, ●) and double pH/φ-gradients (+, ○).

ΔpH, when the approximations, $k = A_j + B_j t$ and $\ln k = A_j + B_j t$, are used. It is seen that both approximations perform equally well, although $k = A_j + B_j t$ seems to give better results when we use single pH-gradients under any constant eluent organic content. It is interesting to note that in this case the approximation $k = A_j + B_j t$ gives an average error in t_R lower than 1.3%. We also observe that, under double pH/φ-gradients, a step ΔpH < 0.1 suppresses the error in t_R below to 2.5%.

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Nevertheless, in order to achieve the maximum accuracy, in all comparisons carried out below we used the approximation $k = A_j + B_j t$ with ΔpH = 0.01.

pH-Gradient Data Obtained at Different φ Values. Two tests were performed. In the first one, all data sets of Table S-1 of the Supporting Information (SI) were used for fitting to the model of Table 1. The five adjustable parameters, k_{00}, k_{01}, k_{10}, k_{11}, and pK, of eqs 2−4 were determined through the fitting algorithm and they are given in Table S-2 of the SI. The percent absolute error between calculated and experimental retention times is presented in Table S-3 of the SI, whereas Figure 5A shows the plot of the calculated retention times as a function of the experimental ones. In this plot the expected straight line with a slope of 1 and an intercept of 0 is also presented. The proximity of the individual points to this line indicates the quality of the model, which is further confirmed by the fact that the overall average and maximum percentage errors are 1.0% and 5.1%, respectively.

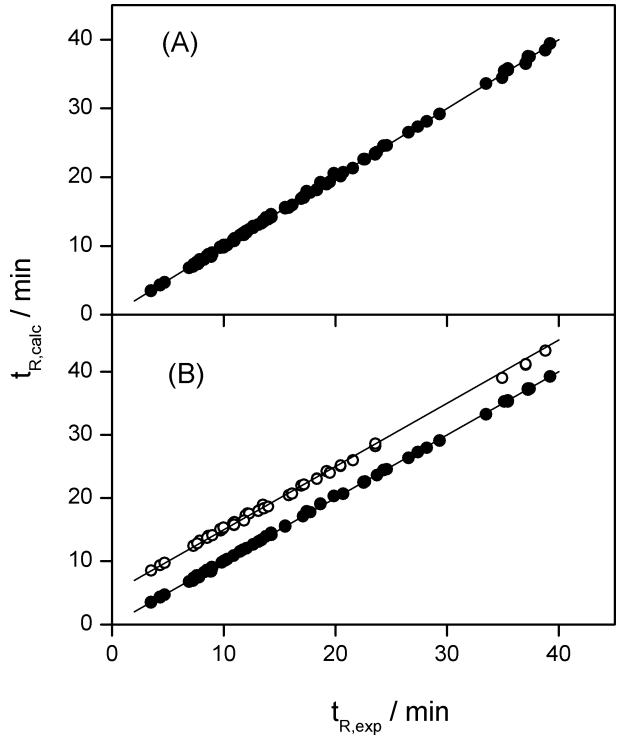

Figure 5. Plots of calculated vs experimental retention times obtained under the following conditions: (A) single pH-gradients; (B) single pH-gradients where part of the data set of Table S-1 of the SI was used for fitting (●) and the rest for prediction (○). Points (○) are shifted along the y axis by 5 min. Lines correspond to the ideal change when the slope is 1 and the intercept 0.

In the second test, part of the data set of Table S-1 was used for fitting and the rest of them for prediction. In more detail, six gradient data points from Table S-1 (No. 1, 2, 6, 7, 9, and 10) were fitted to the model of Table 1. Note that the data which can be used in this procedure should be chosen properly, and in particular, they should have been obtained under extreme gradient conditions. This is because, for example, it is improbable to obtain satisfactory prediction of gradients No. 8, 9, and 10, where $\varphi = 0.5$, if the fitting parameters have been calculated by the use of gradient data No. 1−6, where the organic modifier fraction is 0.25 or 0.3. The adjustable parameters obtained from the fitting procedure are given in Table S-4 of the SI and were used for the prediction of the retention time of the other four gradients. The obtained results concerning the percentage error in the fitted and predicted retention times are presented in Tables S-5 and S-6, whereas Figure 5B shows the dependence of the calculated retention times from the fitting procedure and the predicted retention times versus the experimental ones. We observe, again, that the model describes very accurately the experimental data. The overall absolute average percentage error between experimental and fitted t_R values is 0.9%, while the maximum percentage error is 5.3%. The prediction is also very satisfactory, since the overall absolute average and maximum percentage errors between experimental and predicted solute retention times are 2.0% and 5.1%, respectively.

Note that the values of the same adjustable parameters of Tables S-2 and S-4 of the SI are not identical. This is expected because they are calculated by using different numbers of experimental data points. However, if the 95% confidence intervals of the adjustable parameters are calculated, then the observed differences become statistically insignificant. Nevertheless, taking into account the assumptions adopted for the derivation of the various expressions of t_R, it arises that these parameters do not have a clear physical meaning and their values are to make the fitting better.

Double pH-/φ-Gradient Data. The test of the theory was completed by using experimental data of 17 OPA derivatives of amino acids, which were obtained under simultaneous pH and organic modifier content gradients.[23] For this purpose, the nonlinear least-squares fitting procedure was first applied to the whole data of Table 1 of ref 23. The values of the adjustable parameters as well as the percent errors in the fitted t_R values are presented in Tables S-7 and S-8 of the SI, respectively. The overall average and maximum percentage errors between calculated and experimental retention data are 1.6% and 9.8%, correspondingly.

Furthermore, 18 gradient data from Table 1 of ref 23 (No. 1, 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18, 19, 21, 22, 24, 25, 27) were fitted to the model of Table 1. In fact, for reasons of comparison, we have adopted the same data set as that used in ref 23 to test the ninth parameter empirical model. Table S-9 shows the adjustable parameters, k_{00}, k_{01}, k_{10} k_{11}, and pK, determined through the fitting procedure, whereas the percent error in the fitted t_R values is presented in Table S-10. It is seen that the fitting procedure gives very satisfactory results, since the overall average percentage error between calculated and experimental retention data is 1.9%, whereas the maximum absolute percentage error in t_R values is 10.2%. When the adjustable parameters of Table S-9 are used for prediction, the percent error in the predicted t_R values is given in Table S-11. Now, the overall average and the maximum errors are 1.7% and 5.5%, correspondingly. The very satisfactory description of the experimental retention times by the proposed theory is also depicted in Figure 6, which shows the relationship between experimental and calculated retention time in both cases of fitting and prediction.

It is interesting to note that the above performance of the present method is much better than that of the empirical approach proposed in ref 23, despite the fact that the empirical model is a ninth parameter expression for t_R whereas the present model is based on only five adjustable parameters. Thus, the errors 1.6% and 9.8%, recorded by the present method for the average and maximum error between calculated

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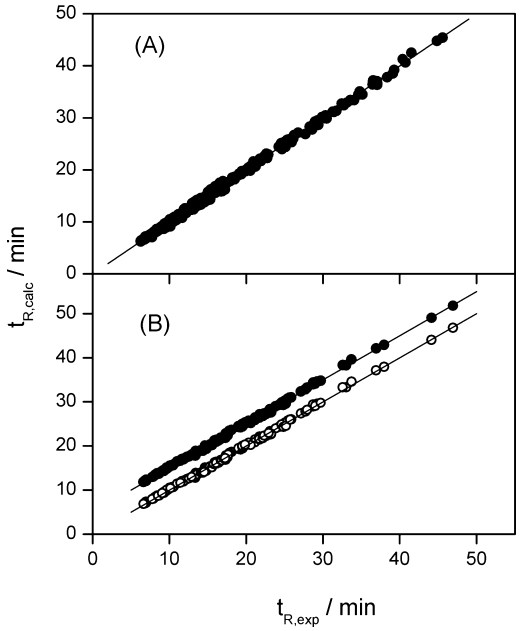

Figure 6. Plots of calculated vs experimental retention times obtained under the following conditions: (A) double pH/φ-gradients where all the data set of Table 1 in ref 37 was used for fitting (●); (B) double pH/φ-gradients where part of the data set of Table 1 in ref 23 was used for fitting (●) and the rest for prediction (○). Points (●) are shifted along the y axis by 5 min. Lines correspond to the ideal change when the slope is 1 and the intercept 0.

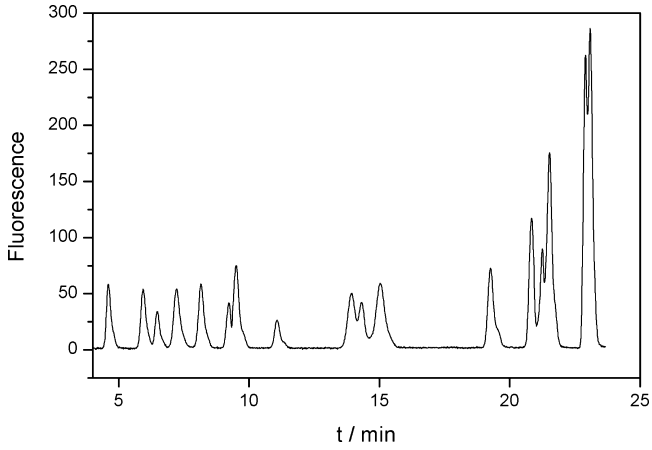

Figure 7. Optimum chromatogram recorded according to a linear pump program from pH_0/φ_0 = 3.2/0.30 to pH_f/φ_f = 5.9/0.50 within t_G = 20 min. The solutes elution order is as follows: arg, asn, gln, ser, asp, glu, thr, dopa, ala, tyr, gaba, met, val, trp, phe, ile, and leu (from left to right).

and experimental retention data, are increased to 2.9% and 18.9%, respectively, in the empirical model of ref 23. In addition, the fitting errors 1.9% and 10.2% obtained above become 2.7% and 19.2% in the empirical model, whereas the average and the maximum prediction errors 1.7% and 5.5% become 3.5% and 11.8%.[23] Therefore, the proposed method for obtaining mathematical expressions for the retention time of ionogenic analytes eluted under multilinear double pH/solvent-gradients in reversed-phase liquid chromatography is much superior to the empirical method presented in ref 23. In particular, the proposed approach (a) is more rigorous, since it is based on the solution of the fundamental equation of gradient elution, eq 1, (b) is more flexible, since it is not restricted to constant pH_0, φ_0, and t_{in} values, (c) can be applied to single and multilinear gradients, and (d) exhibits better fitting and prediction performance. Nevertheless, we should note that the empirical model developed in ref 23, despite its limitations, gives also satisfactory fitting and prediction results, and its main advantage is that it is easily manageable through a linear least-squares fitting which can be done on every commercial statistical package.

Finally, the optimum gradient profile obtained by the optimization algorithm is a monolinear variation of φ from 0.3 to 0.5 at 20 min combined with multilinear pH variation: 3.2 (t = 0), 3.6 (t = 10), 3.9 (t = 14), 4.2 (t = 16), 4.6 (t = 18), and 5.9 (20 min). The chromatogram recorded under the above conditions is shown in Figure 7. It is seen that there is no baseline separation of all analytes. However, all analytes give clear peaks, and therefore, the determination of their area through a deconvolution algorithm is straightforward.[37]

To sum up, the theoretical approach presented in this paper resulted in the development of mathematical expressions for the retention time of ionogenic analytes eluted under multilinear double pH/solvent-gradients in reversed-phase liquid chromatography. These expressions can describe the experimental retention times with high accuracy, especially in the case of single multilinear pH-gradients at any constant φ values. Thus, in the latter case, the overall error in the fitted t_R values is 0.9% and in the predicted ones 2%. These errors become 1.9% and 1.7%, respectively, under double pH/solvent-gradients.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables of experimental retention times, adjustable parameters of eqs 2−4, and absolute percentage error between experimental and calculated t_R. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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