Talanta 93 (2012) 279–284

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A simple approach for retention prediction in the pH-gradient reversed-phase liquid chromatography

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ARTICLE INFO

Article history:
Received 24 October 2011
Received in revised form 10 February 2012
Accepted 16 February 2012
Available online 22 February 2012

Keywords:
pH-gradients
Retention prediction
Amino acid derivatives
Reversed-phase liquid chromatography

ABSTRACT

A simple approach for retention modeling of solutes under pH-gradient conditions at various organic contents in the mobile phase is proposed. This approach is based on a retention model arising from the evaluation of the retention data of a set of 17 OPA derivatives of amino acids obtained in two series of 22 pH-gradient runs performed between a given initial and final pH value (between 2.8 and 10.7 or 3.2 and 9.0) with different gradient duration and with different organic modifier content in the eluent. The derived model is a fifth-parameter equation easily manageable through a linear least-squares fitting. It requires only 6 initial pH-gradient experiments, allows a very satisfactory prediction for various pH-changes of the same kind with those used in the fitting procedure and seems to be very promising in separation optimization under pH-gradient conditions. The pH-gradient method appears to be especially suitable and effective for separation of amino acid derivatives whereas the application of pH-gradients from 3.2 to 9.0 proved to be beneficial.

1. Introduction

Retention of ionogenic analytes in reversed-phase liquid chromatography (RP-LC) is known to strongly depend on pH of the eluent. The mobile phase pH modifies separation of ionizable compounds by affecting the degree of analyte dissociation. Different models describing the isocratic retention of ionizable solutes as a function of pH and mobile phase composition were proposed [1,2], and moreover both the pH and organic modifier content for isocratic conditions were optimized [3,4]. However, the performance of a pH-gradient or a combined pH/organic modifier gradient during RP-LC separations extends much more the analytical versatility of this chromatographic technique. A narrow pH-gradient, from pH 3.5 to 6.0, was first reported to improve separation of acidic analytes in 1991 [5]. Since then the increasing availability of modern reversed columns, which can be operated at a wide pH range makes the pH-gradient RP-LC even more attractive. However, to benefit from the pH-gradient separation mode, a simple model enabling retention prediction of solutes under pH-gradient conditions is requested, which would allow a computer-aided optimization of the separation. Recent reports demonstrated a comprehensive theory allowing the prediction of solute retention in the pH-gradient mode as well as the pK_a determination of monoprotic acids and bases [6–11]. Also, a modeling approach allowing the description of retention time and peak width in the combined pH/organic modifier gradient was recently proposed [12].

All the above attempts to describe theoretically the retention of solutes in the pH-gradient mode were based on the solution of the fundamental equation of gradient elution [13,14]

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

where t_R is the solute gradient elution time, t_0 is the column hold-up time and k is the analyte retention factor. The analytical solution of Eq. (1) is feasible in a linear mobile phase pH-gradient for monoprotic acids/bases provided that the retention time of the ionized and non-ionized analyte forms along with the pK_a of the analyte at a given or at any organic modifier content of the mobile phase are known by isocratic experiments [6–12]. Consequently, the above approach is also a means to transfer an isocratic method to a pH-gradient method.

The aim of this study is to propose an alternative, but not so general, simple approach for retention modeling of solutes under pH-gradient conditions at various organic contents in the mobile phase. This approach is based on a model arising from direct fitting of two dimensional (2D) pH-gradient retention data obtained at different but constant values of organic modifier in the eluent. As a result, such a model is applicable exclusively for pH-gradient elution, since it expresses the solute retention times in terms of the variables representing gradient elution conditions, like programmed gradient duration and mobile phase composition. The accuracy of the proposed model is tested by the experimental data

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doi:10.1016/j.talanta.2012.02.034

obtained for 17 o-phthalaldehyde (OPA) derivatives of amino acids at various pH-gradients, since the analysis of amino acids is very important in biological and biomedical research, and as a result, any new analytical approach is worth being applied first to the analysis of these compounds. On the other hand among HPLC methods currently in use to determine amino acids, the most popular is that employing precolumn derivatization of amino acids with OPA, since OPA amino acid derivatives are well resolved on various types of reversed columns and give high fluorescence signals.

2. Experimental

2.1. 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) thermostatted 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 the following 17 OPA/2-mercaptoethanol derivatives of 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), beta-(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-isoleucine (Ile), L-phenylanine (Phe) and L-leucine (Leu). The working concentration of underivatized amino acids used in the derivatization procedure by OPA/2-mercaptoethanol reagent was 1 μg/mL.

The pH of the mobile phases used in different pH-gradients was measured after mixing the aqueous buffers and the organic modifier, whereas the electrode system was calibrated with the usual aqueous standards [15]. The measurements were done with a Mettler Toledo Seven Easy pH-meter.

2.2. Chromatographic experiments

In order to investigate the effects brought on retention of test solutes by pH-gradients and organic modifier content in the eluent, we performed a series of 22 chromatographic runs. During pH-gradient measurements the organic modifier content was kept constant and pH changed continuously (between a given initial and final pH value) by an applied linear pump program with the same starting time (0 min) but with different gradient duration, t_G. In more details, two series of pH-gradients were conducted in this study. In the first one, pH gradients from 2.8 to 10.7 were carried out by using in the two flow lines of the HPLC pump system two mobile phases with different pH values (2.8 and 10.7), which were consisted of aqueous phosphate buffers with a total ionic strength of 0.02 M and a fixed concentration of organic modifier. Acetonitrile (MeCN) with volume fractions (φ = 0.25, 0.27, 0.3 or 0.35) was used as organic modifier in the eluents during the pH-gradient runs. In the second series of pH-gradients the only difference was that the initial pH value was 3.2 and the final one 9.0. The experimental retention data obtained under the above described pH-gradient runs are shown in Tables 1 and 2, respectively.

2.3. Fitting and prediction algorithms

The algorithms used for fitting and testing the prediction ability of the model derived in this study were written in C++ and based on the theory of linear least-squares. However, the determination of the fitting parameters of the proposed simple retention model additionally with the prediction derived by this retention

Table 1
Experimental retention data (in min) of test solutes obtained in different (from 2.8 to 10.7) pH gradient runs and absolute percentage errors between them and calculated retention data by the approach explained in the text.

No. of grad.	1		2		3		4		5		6		7		8		9		10	
φ	0.35		0.35		0.35		0.35		0.25		0.27		0.3		0.25		0.27		0.3	
t_G, min	10		15		20		30		15		15		15		30		30		30	
Solutes	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error
Arg	3.12	0.4	3.11	0.4	3.13	0.3	3.12	0.4	6.53	0.2	5.89	5.4	4.41	0.6	6.73	0.2	5.97	4.4	4.43	0.6
Asn	4.09	0.3	4.08	0.3	4.07	0.3	4.08	0.3	8.36	0.2	7.61	4.9	5.81	0.4	8.60	0.2	7.76	4.7	5.86	0.4
Gln	4.25	0.3	4.23	0.4	4.24	0.1	4.23	0.4	9.61	0.2	8.57	5.1	6.33	0.6	9.92	0.2	8.72	4.1	6.38	0.6
Ser	5.03	0.3	5.01	0.4	4.99	0.4	5.00	0.4	10.40	0.2	9.45	4.6	7.26	0.5	10.81	0.2	9.64	3.5	7.34	0.5
Asp	5.78	2.6	5.74	2.1	5.61	3.2	5.76	2.0	13.76	0.9	12.13	1.8	9.55	2.5	15.53	0.8	13.10	0.0	9.74	2.4
Glu	6.30	6.3	6.23	4.6	6.26	1.4	6.27	4.6	15.04	1.9	13.73	2.8	11.15	5.1	18.83	1.5	16.21	2.5	11.35	5.1
Thr	6.79	6.2	6.76	4.1	6.76	1.5	6.78	4.1	15.04	1.9	14.21	6.6	11.12	5.0	18.83	1.5	16.21	2.9	11.35	4.9
Dopa	7.90	5.1	7.84	3.4	7.84	0.9	7.89	4.8	15.99	1.7	15.35	4.7	12.71	4.2	25.32	1.5	21.39	0.3	15.48	4.8
Ala	10.98	0.1	11.52	1.5	11.78	0.5	12.31	1.4	16.89	1.0	16.09	4.0	15.39	2.2	26.98	0.6	25.90	2.3	21.84	1.5
Tyr	11.39	1.2	11.67	1.0	12.13	2.4	12.31	1.0	17.99	0.7	16.85	3.1	15.66	1.5	28.25	0.4	26.71	2.9	21.84	1.1
Gaba	11.46	1.6	12.08	3.7	12.13	1.0	12.31	3.6	17.99	2.5	17.42	4.5	16.58	5.3	28.25	1.6	26.71	2.4	24.48	3.6
Met	12.20	3.3	15.72	0.1	19.18	1.6	25.15	0.1	24.85	0.1	21.80	0.3	18.50	0.2	36.25	0.0	32.59	0.8	28.83	0.1
Val	12.34	1.9	15.97	0.2	19.49	0.3	26.33	0.1	24.85	0.1	21.80	0.6	18.67	0.4	36.25	0.1	32.85	0.6	29.35	0.2
Trp	12.97	1.4	16.53	0.0	19.97	0.2	26.69	0.0	31.65	0.0	26.50	0.3	20.91	0.1	43.55	0.0	37.91	0.6	31.90	0.0
Ile	13.41	0.3	16.93	0.3	20.38	0.3	27.12	0.2	33.88	0.1	28.02	1.2	22.00	0.4	45.85	0.1	39.64	1.0	33.35	0.3
Phe	13.41	1.2	17.12	0.2	20.77	0.7	27.64	0.1	34.79	0.1	28.61	0.5	22.00	0.2	46.66	0.1	40.05	0.9	33.35	0.2
Leu	13.41	2.8	17.31	0.3	20.90	0.8	27.64	0.2	37.00	0.1	30.03	1.1	22.84	0.5	49.05	0.1	41.72	1.1	34.34	0.3
Average		2.1		1.4		0.9		1.4		0.7		3.0		1.7		0.5		2.1		1.6

Table 2
Experimental retention data (in min) of test solutes obtained in different (from 3.2 to 9.0) pH gradient runs and absolute percentage errors between them and calculated retention data by the approach explained in the text.

No. of grad.	1		2		3		4		5		6		7		8		9		10		11		12	
φ	0.25		0.25		0.25		0.25		0.27		0.3		0.35		0.3		0.35		0.27		0.3		0.35	
t_G, min	10		15		20		30		15		15		15		20		20		30		30		30	
Solutes	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error	t_R	% error
Arg	6.03	2.8	5.91	0.7	5.88	0.2	5.82	0.7	4.99	1.1	3.78	2.8	3.02	0.2	3.99	2.8	3.02	0.5	5.03	1.8	3.99	2.7	3.00	0.2
Asn	6.94	4.4	7.13	1.3	7.03	2.9	6.32	1.5	6.15	1.7	5.00	3.8	3.75	2.6	4.91	4.3	3.74	0.5	5.95	2.6	5.17	3.7	3.75	2.6
Gln	7.81	5.5	8.29	0.6	8.19	0.7	8.20	0.6	7.02	1.2	5.28	2.0	3.99	1.3	5.44	0.6	3.97	0.2	7.14	2.6	5.55	1.9	3.99	1.3
Ser	8.08	6.4	8.80	0.1	8.80	2.5	9.44	0.1	7.66	2.1	6.00	0.3	4.58	0.2	6.16	1.2	4.58	0.0	7.85	2.0	6.27	0.3	4.58	0.2
Asp	9.06	2.7	9.85	1.2	10.72	0.8	12.09	1.0	8.76	3.3	6.96	3.5	4.98	2.4	7.17	2.1	5.01	0.3	9.72	3.2	7.37	3.3	5.01	2.4
Glu	9.57	1.9	11.03	0.6	12.73	2.4	15.15	0.4	10.22	4.9	8.08	1.6	5.67	1.1	8.61	0.8	5.69	2.1	12.45	3.0	9.59	1.3	5.30	1.2
Thr	10.19	1.9	11.49	0.7	12.73	0.0	15.15	0.5	10.07	1.8	8.66	1.7	6.10	1.2	9.02	0.1	6.10	1.1	12.45	4.2	9.90	1.5	5.82	1.3
Dopa	10.27	3.6	12.47	0.8	14.25	1.7	18.44	0.5	11.88	2.5	10.21	2.0	7.02	1.4	11.14	1.7	7.12	0.5	16.14	0.2	12.64	1.6	7.12	1.4
Ala	11.10	0.4	13.43	1.1	15.29	1.5	19.83	0.8	12.65	4.5	12.23	2.5	9.98	1.5	13.86	0.1	10.59	3.6	19.04	1.2	16.76	1.8	10.84	1.4
Tyr	12.11	1.4	14.46	1.5	16.20	2.1	20.92	1.0	13.24	5.3	12.51	3.4	9.98	2.2	14.15	1.1	10.59	3.8	19.61	0.6	17.47	2.5	10.84	2.0
Gaba	12.67	1.4	15.48	0.6	17.65	1.6	22.93	0.4	14.36	3.8	13.77	1.3	11.80	0.8	15.40	0.3	12.32	1.4	21.51	1.1	18.60	1.0	12.92	0.7
Met	18.40	2.7	21.46	0.1	23.49	2.5	29.27	0.1	18.54	2.1	14.60	0.3	12.25	0.2	16.84	0.2	14.40	0.6	25.24	1.4	21.44	0.2	18.41	0.1
Val	18.40	2.3	21.46	0.2	23.49	2.9	29.56	0.1	18.54	1.3	14.94	0.6	12.58	0.3	17.29	0.2	14.77	0.7	25.90	0.7	22.15	0.4	19.43	0.2
Trp	24.86	0.4	27.84	0.3	29.57	4.5	36.94	0.2	22.60	0.3	17.15	1.1	13.18	0.7	19.41	0.7	15.33	0.7	30.78	1.1	24.50	0.7	19.87	0.5
Ile	26.81	1.5	30.16	0.2	31.95	4.1	39.35	0.2	24.48	0.0	18.31	0.8	13.61	0.5	20.58	0.9	15.66	1.2	33.16	0.4	25.79	0.6	20.25	0.4
Phe	27.78	0.3	30.87	0.0	32.43	4.5	39.91	0.0	24.48	1.5	18.31	0.1	13.96	0.0	20.75	1.1	16.07	1.4	33.16	1.0	26.31	0.0	20.98	0.0
Leu	29.80	1.0	33.18	0.0	34.78	4.3	42.44	0.0	26.25	1.0	19.18	0.1	14.24	0.1	21.67	1.2	16.35	1.5	35.20	0.4	27.38	0.1	21.29	0.0
Average		2.4		0.6		2.3		0.5		2.3		1.6		1.0		1.1		1.2		1.6		1.4		0.9

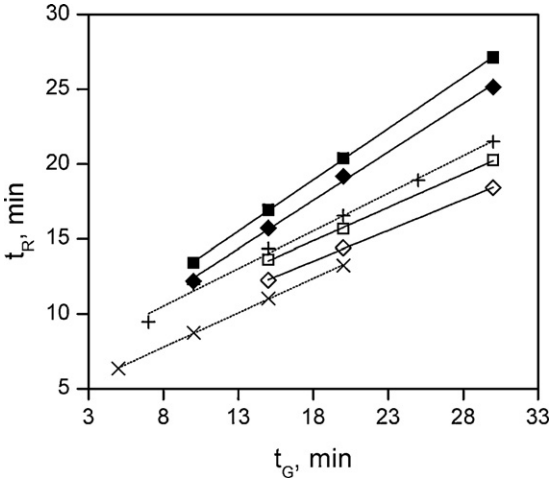

Fig. 1. Variation of t_R as a function of the duration of linear pH-gradients, t_G, for Met (♦, ◊) and Ile (■, □) obtained at φ_{MeCN} = 0.35 and for morphine obtained at φ_{MeOH} = 0.03 (+) and at φ_{MeOH} = 0.07 (×). Filled symbols (♦, ■) refer to pH gradients from 2.8 to 10.7 and open symbols (◊, □) to pH gradients from 3.2 to 9.0. Points are experimental data (received from Tables 1 and 2 of the present paper for Met and Ile and from Table 6 of Ref. [6] for morphine) and lines are linear fittings.

equation could also be easily done on Excel spreadsheets using the Regression tool.

3. Results and discussion

3.1. Modeling pH-gradient elution at various constant organic modifier contents in the eluent

In an effort to develop a simple model describing the retention of solutes in pH-gradient runs carried out between a given initial and final pH value with different programmed gradient time (or duration), t_G, and with different organic modifier content, φ, in the eluent, we followed the approach adopted in Ref. [14,16–18]. In those references, the simultaneous effect of two parameters such as the column temperature and organic modifier content or the ion-pairing reagent concentration and organic modifier content under single-mode gradient conditions was treated by empirical models arising from the experimental properties of the system. In our case, there are two series of 2D pH-gradient retention data obtained at different φ values (see Tables 1 and 2 for pH-gradients from 2.8 to 10.7 and from 3.2 to 9.0, respectively). For each set of gradient runs, the retention is governed by two variables, i.e. t_G and φ, and consequently the combined effect of these factors on the solute retention, t_R (t_G, φ) may be written as a product of two equations [19]. That is,

$$t_R(t_G, \varphi) = t_R(t_G) t_R(\varphi) \quad (2)$$

In order to find out the proper equations that express separately the dependence of the retention upon each of these factors, the retention times of solutes obtained in each series of chromatographic runs were evaluated. Based on these retention data we found that a linear dependence of t_R upon t_G, i.e.

$$t_R(t_G) = c_{0t} + c_{1t} t_G \quad (3)$$

describes quite satisfactory the experimental data obtained by pH-gradient runs with different gradient duration but with constant MeCN content in the eluent. Examples for the linear variation of t_R against t_G when φ is kept constant, are depicted in Fig. 1. Moreover, the same linearization has been observed in several other cases, like in certain experimental pH-gradient retention data obtained by Kaliszan et al. in chromatographic conditions totally different

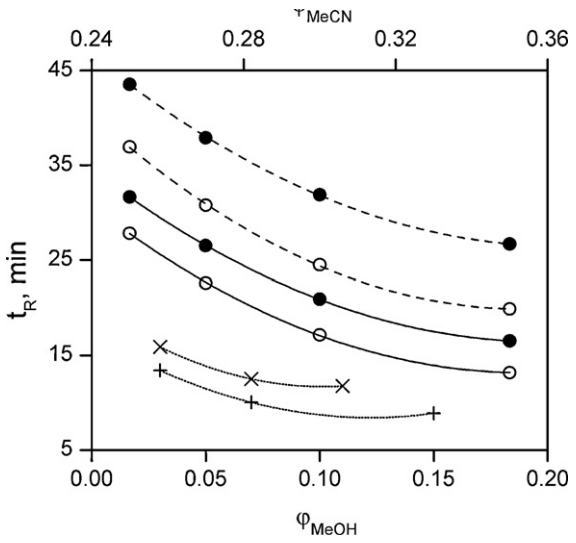

Fig. 2. Variation of t_R as a function of MeCN content in the eluent, φ_{MeCN}, for Trp obtained under pH gradients from 2.8 to 10.7 (●) and from 3.2 to 9.0 (○) at a gradient time t_G = 15 min (solid line) and at 30 min (dashed line), respectively, as well as variation of t_R as a function of MeOH content in the eluent, φ_{MeOH}, for 2-methylobenzimidazol obtained under pH gradients from 10.5 to 3.0 at a gradient time t_G = 15 min (×) and at 10 min (+), respectively. Points are experimental data (received from Tables 1 and 2 of the present paper for Trp and from Table 6 of Ref. [6] for 2-methylobenzimidazol) and lines are second order polynomial fittings.

than those used in the present study [6,8]. An example of such linear dependence of t_R on t_G is shown in Fig. 1 for morphine (a basic solute). These experimental data, obtained on XTerra MS C-18 column for pH-gradient runs from 10.5 to 3.0 with various gradient times, t_G, in two concentrations of methanol (MeOH) in mobile, are given in Ref. [6] (Table 6) and also suggest that the linear dependence of t_R on t_G holds true.

As far as it is concerned the dependence of t_R upon the organic modifier content, φ, in the eluent during pH-gradient runs with a fixed gradient duration, t_G, the evaluation of the experimental data in Tables 1 and 2 reveals a quadratic expression for this dependence:

$$t_R(\varphi) = c_{0\varphi} + c_{1\varphi}\varphi + c_{2\varphi}\varphi^2 \quad (4)$$

Examples for the quadratic variation of t_R against φ when t_G is kept constant, are depicted in Fig. 2. A similar quadratic dependence seems to be valid for the retention of data previously obtained under pH-gradient experiments between 10.5 and 3.0 with different MeOH contents in the eluent but with a certain gradient duration, t_G. Examples of this retention behavior are constructed for 2-methylobenzimidazol by results given in Table 6 of Ref. [6] and they are shown in Fig. 2.

Consequently, having experimentally verified that Eqs. (3) and (4) describe separately the dependence of the retention upon each of the two factors involved in the present experiments, i.e. t_G and φ, then Eq. (2) results in:

$$t_R(t_G, \varphi) = c_0 + c_1\varphi + c_2\varphi^2 + c_3 t_G + c_4\varphi t_G \quad (5)$$

in case we want the final expression of t_R (t_G, φ) not to have terms of order higher than 2. In Eq. (5), coefficients $c_0 \ldots c_4$ are adjustable parameters determined by fitting a series of 2D pH-gradient retention data obtained in programmed linear changes of the mobile phase pH between two fixed pH values for different programmed gradient time t_G, and at different (but constant during the separation) MeCN contents, φ, in the eluent. In order to evaluate both the fitting and the prediction performance of Eq. (5) on the retention of test solutes, each series of retention data depicted in Tables 1 and 2 was divided into two groups. In particular, the experimental data nos. 2, 4, 5, 7, 8 and 10 in Table 1 and nos. 2, 4, 6, 7, 11 and 12 in Table 2 were selected for fitting and the rest for prediction. The retention data selected for the fitting procedure correspond to pH-gradient experiments carried out at two different t_G values, 15 and 30 min, and at three φ values, 0.25, 0.3 and 0.35, since Eq. (5) presumes a linearity between t_R and t_G but a quadratic dependence of t_R upon φ. In other words, for fitting Eq. (5), at least a 2 × 3 table of t_R data is needed. Thus, a direct fitting of the above selected 2-D pH-gradient retention data to Eq. (5) gave the values of fitting parameters, $c_0 \ldots c_4$, listed in Table 3 for each series of pH-gradient experiments. Most of these coefficients are statistically significant, as it arises from their *t*-ratio values, i.e. the absolute values of the ratio of each parameter to its standard deviation, which were found to be greater than 2 [20]. In some cases, however, such as the retention data obtained for Dopa in pH-gradient runs from 2.8 to 10.7 as well as those obtained for Arg in pH-gradient runs from 3.2 to 9.0, in order to obtain statistically significant parameters, simpler dependences of t_R upon t_G or upon φ should be considered, see Table 3. Nevertheless, as it can be seen from Tables 1 and 2, the fitting performance of Eq. (5) is quite satisfactory since the overall average percentage error between calculated and experimental retention data used in the fitting procedure was only 1.2 and 1.0% for pH-gradients runs from 2.8 to 10.7 and from 3.2 to 9.0, respectively.

3.2. Retention prediction

In order to test the accuracy of retention predictions obtained by Eq. (5) with adjustable parameters in Table 3 for each series of pH-gradient experiments, all the experimental retention data in Tables 1 and 2, except those used for the fitting procedure, were tested. It is seen that Eq. (5) enables equivalent predictive ability for both sets of retention data, which is very satisfactory indeed, since the overall predictive % error between calculated and experimental retention data was only 2.0 for the pH-gradient retention data from 2.8 to 10.7 (see Table 1 for each solute retention prediction) and 1.8% for the pH-gradient retention data from 3.2 to 9.0 (see Table 2 for details of prediction). Consequently, it seems that starting from six pH-gradient runs conducted with two different gradient duration, t_G, between a given initial and final pH value of mobile phases containing three different MeCN contents, φ, Eq. (5) enables an accurate prediction for any other pH-gradients with different t_G and φ values but with the same initial and final pH value with those used in the fitting procedure. This accurate description of all retention data obtained in this study by Eq. (5), verifies also the linear and the quadratic dependence of t_R upon t_G and φ, respectively, adopted in this experimental system.

In conclusion, although the proposed equation is very simple, empirical in nature and it is easily manageable through a linear least-squares fitting, it allows predicting analyte retention in pH-gradient mode very well. Other advantages of the proposed approach over currently employed procedures [6–12] are the following: no solution of Eq. (1), i.e. of the fundamental equation of gradient elution is required; there is no need of the knowledge or the determination of the pK_a of analytes along with the retention of the ionized and non-ionized forms of analytes at any organic content of the mobile phase; deviations of actual pH-gradients from the linear programmed gradients of pH have no effect on the accuracy of prediction ability of Eq. (5), since the measurement of the true pH changes occurring after mixing together two phosphate buffers (used in pH gradients) in linearly changing proportions showed slight deviations from the straight lines; the use of a model exclusively for gradient elution, such as Eq. (5), reduces errors related to non-equilibria phenomena due to the use of similar experimental gradient data throughout the procedure [18]. However, the practical potential of Eq. (5) is not universal but it is restricted in an accurate prediction of retention behavior of solutes under

Table 3
Values of adjustable parameters of Eq. (5).

Solutes	pH-gradients from 2.8 to 10.7					pH-gradients from 3.2 to 9.0				
	c_0	c_1	c_2	c_3	c_4	c_0	c_1	c_2	c_3	c_4
Arg	30.3	−141	181	0.04	−0.13	32.4	−162	222	–	–
Asn	33.9	−148	179	0.05	−0.16	23.5	−79.1	62.0	−0.18	0.54
Gln	44.5	−205	257	0.07	−0.21	43.8	−212	281	−0.01	0.06
Ser	40.2	−171	202	0.09	−0.28	42.1	−207	285	0.15	−0.43
Asp	47.3	−195	221	0.40	−1.17	43.1	−222	325	0.50	−1.48
Glu	36.7	−133	137	0.84	−2.50	25.1	−117	181	1.01	−2.99
Thr	45.0	−193	247	0.84	−2.51	24.2	−100	143	0.89	−2.63
Dopa	4.78	11.2	–	2.12	−6.18	0.12	42.9	−65	1.36	−3.91
Ala	−62.7	445	−676	2.24	−6.20	−32.8	255	−391	1.37	−3.69
Tyr	−42.8	320	−479	2.30	−6.42	−28.3	237	−376	1.39	−3.73
Gaba	−105	732	−1150	2.42	−6.70	−13.0	124	−162	1.56	−4.22
Met	95.3	−510	730	1.08	−1.31	114	−634	931	0.79	−1.11
Val	97.5	−519	735	0.93	−0.69	110	−606	883	0.74	−0.83
Trp	165	−902	1280	1.08	−1.16	177	−993	1452	1.00	−1.61
Ile	174	−937	1308	1.10	−1.18	189	−1051	1518	1.03	−1.71
Phe	199	−1094	1552	1.02	−0.90	203	−1138	1648	0.94	−1.35
Leu	215	−1178	1664	1.10	−1.15	223	−1249	1803	0.99	−1.47

pH-gradient conditions between a given initial and final pH value of mobile phases but for different gradient duration and at various organic contents in the eluent.

3.3. The usefulness of the pH-gradient mode in the separation of OPA derivatives of amino acids

In organic modifier mobile phase gradients, φ-gradients, the retention of solutes decrease during the gradient run by increasing the elution power of the mobile phase, i.e. by increasing the concentration of organic solvent in the eluent. A similar effect can be obtained for ionizable analytes in RP-LC by using a pH-gradient of mobile phase that gives a continuous increase in the concentrations of the ionized forms of the analytes. In general, in the pH-gradient mode the eluting strength of the mobile phase increases due to its changing pH: increasing in case of acids and decreasing in case of bases. The increase of pH for acids and the decrease of pH for bases affect the degree of analyte dissociation and lead to an increase of charged species of analyte and consequently to a decrease of analyte retention [21]. In case of amino acids and/or OPA-derivatives of amino acids, such as the test analytes in this study, increasing the pH converts the singly positive charged solute (cation form) to the zwitterion form and finally to the anion form. Thus the retention of amino acids in RP-LC passes through a minimum at intermediate pH due to the greater polarity of the zwitterionic form [22]. Taking into account the big difference between the pK_a values of the carboxyl group (≈2.2 in water) and of the ammonium group (≈9.5 in water) of common amino acids, the ranges of pH-gradients tested in this study, from 2.8 to 10.7 and from 3.2 to 9.0, lead to similar changes of solute retention affected mainly by the carboxyl group pK_a. In other words, the programmed pH-gradient runs performed at the ranges between 2.8 and 10.7 or 3.2 and 9.0 provide in general a suppression of ionization of carboxyl group of amino acids at the beginning of the gradient and its total ionization and formation of zwitterion at gradient end. The only difference is that the application of pH-gradients from 2.8 to 10.7 leads to an increase of analyte retention in comparison to that obtained with the use of pH-gradients from 3.2 to 9.0 (compare the retention data in Tables 1 and 2 for the same MeCN content and gradient duration). This increase may be due to the fact that the degree of ionization of the carboxyl group of OPA derivatives of amino acids should be different at the beginning of these two types of pH-gradient runs since their initial values of pH may be around of the pK_a value of OPA derivatives of amino. This seems to be true if it is taken into account that the formation of the OPA derivatives and the presence of organic modifier in the eluent slightly increase the pK_a value of the carboxyl group of free amino acids, which is similar and about 2.2 in a neat water eluent [23]. Consequently, at the start of pH gradient runs from 2.8 to 10.7 the ionization fraction of carboxyl group of OPA derivatives of amino acids should be less than in those gradients starting at 3.2 pH and as a result the solutes should be less polar at pH 2.8 showing greater retention.

However, except for the retention, the fluorescence sensitivity also depends on the eluent pH. In a recent paper [24], the role of mobile phase pH on fluorescence detection of OPA-derivatives of amino acids recorded under isocratic and/or φ-gradient conditions at different constant pH values (2.5, 5 and 7) was investigated. It was found that, in general, increasing the mobile phase pH resulted in a considerable increase of fluorescence responses. However, the extreme fluorescence signal enhancement was observed between pH 2.5 and 5 due to the different degree of ionization of carboxyl group of amino acids at that pH range. In particular, at pH 2.5 dominates the protonated (nonionized) form of carboxyl group of amino acids, whereas at pH 5 dominates its deprotonated (ionized) form. For this reason the pH-gradients tested in the present paper increase detection sensitivity of more retained amino acids (see Fig. 3). It is clear from this figure that the fluorescence response of poorly retained analytes, which probably elute in the acidic region

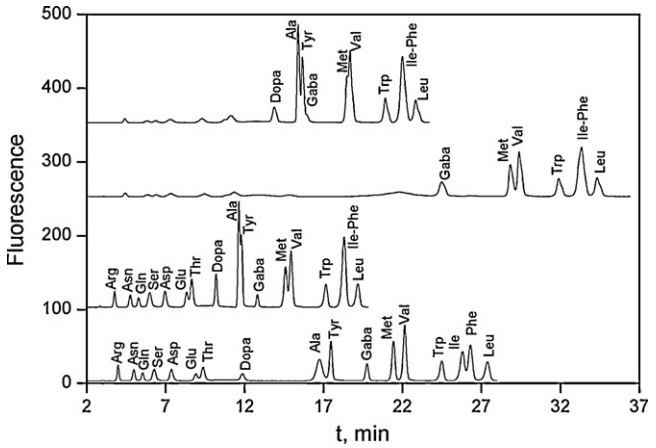

Fig. 3. Chromatograms of a mixture of 17 amino acids derivatives obtained in mobile phases with φ_{MeCN} = 0.30 under the following pH-gradients from the top to bottom: linear programmed variation of pH from 2.8 to 10.7 at 15 and 30 min (nos. of gradients 7 and 10 in Table 1) and variation of pH from 3.2 to 9.0 at 15 and 30 min (nos. of gradients 6 and 11 in Table 2). The elution order of solutes is shown in the figure.

of pH during a pH-gradient run, is smaller than the strongly retained solutes, which elute in the basic region of pH. This difference in the peak sensitivity is more evident in the pH-gradients from 2.8 to 10.7 in comparison to those performed at the pH-range between 3.2 and 9.0, and this seems to be consistent with our assumption that the degree of deprotonation of amino acids is different at the beginning of these two types of pH-gradients tested. Moreover, Fig. 3 shows that the difference in peak sensitivity is also more evident for relative slow pH gradients (big gradient duration) since then the effect of acidic pH affect analytes for a longer period of time.

Generally, the pH-gradient method appears to be especially suitable and effective for separation of amino acid derivatives. Indeed, a good resolution of the mixture of 17 amino acid derivatives was achieved for example in the chromatogram in Fig. 3 obtained under pH-gradient from 3.2 to 9.0 within 30 min at constant concentration of MeCN in the eluent ($\varphi_{MeCN} = 0.30$). Finally, from the point of view of fluorescence signal improvement the application of pH-gradients from 3.2 to 9.0 proved to be more beneficial than that of pH-gradients from 2.8 to 10.7 in the analysis of amino acids.

4. Conclusions

An approach requiring 6 preliminary pH-gradients carried out between a given initial and final pH value with two different gradient times and at three different organic modifier contents in the eluent was proposed to mathematically describe the retention behavior of solutes for any other pH-gradients of the same kind with those used in the fitting procedure. This approach is based on a fifth-parameter retention model, Eq. (5), easily manageable through a linear least-squares fitting. The positive results of this study open the practical possibility of using Eq. (5) for retention prediction and separation optimization under pH-gradient conditions. In general the pH-gradient method offers a convenient means to improve separation of amino acid derivatives whereas the application of pH-gradients from 3.2 to 9.0 proved to be beneficial.

Acknowledgments

This research has been co-financed by the European Union (European Social Fund – ESF) and Greek National Funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) – Research Funding Program: Heracleitus II. Investing in Knowledge Society through the European Social Fund.

The authors would also like to thank MZ-ANALYSENTECHNIK GMBH for the gift of the HPLC column "PerfectSil® Target HD", enabling permanent operation under conditions from pH = 2–11 without any noticeable loss of performance or sign of degradation.

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