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Short communication

Separation optimization in reversed-phase liquid chromatography by using alkanol additives in the mobile phase: Application to amino acids

A. Pappa-Louisi*, P. Agrafiotou, I. Georgiadis

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

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ABSTRACT

In an effort to enhance complex mixture separations by using small amounts of a homologous series of alkanols as additives in the mobile phases, it was proposed an optimization algorithm based on a sixth-parameter retention model. This model considers simultaneously the contents of the main organic modifier and of the alkanol additive in the mobile phase as well as of the number of alkyl chain of the additive. This model is in fact a modification of a previously one derived in a recently published paper for the retention description of a mixture of purely hydrophobic alkylbenzenes under isocratic conditions with mobile phases containing alkanol additives. The effectiveness of the new retention model as well as the optimization algorithm was successfully applied to the separation of ten o-phthalaldehyde (OPA) derivatives of amino acids. Indeed, the new retention model exhibited an excellent prediction performance since the obtained overall predictive error between calculated and experimental times was only 2.8% for all isocratic runs by using a variety of mobile phase compositions containing any alkanol homologue even different than those used in the starting/fitting experiments. Moreover, a perfect resolution of the above amino acid mixture was achieved within only 7.4 min in the chromatogram recorded using the optimal mobile phase determined by means of the simple optimization algorithm proposed in this study.

1. Introduction

In a recent paper [1], it was explored the possibility of controlling retention in reversed-phase liquid chromatography (RP-LC) through the introduction of short and medium straight-chained-length alkanol additives at low concentrations in mobile phases containing methanol as the main organic solvent. Moreover, a sixth-parameter retention model considering simultaneously the contents of the main organic modifier and of the alcohol additive as well as of the number of alkyl chain of additive was derived in that paper and the effectiveness of that model was evaluated in the retention prediction of a mixture of six alkylbenzenes under isocratic conditions with such mobile phases. The prediction was excellent in all cases even when the alkanol additives used in experiments for the fitting procedure were different than those used in chromatographic runs done for testing the prediction ability of the proposed model.

The aim of the present study was to explore the applicability of that retention model to solutes covered a wide range of hydrophobicity instead of the purely hydrophobic alkylbenzenes previously tested. Thus a mixture of ten o-phthalaldehyde (OPA) derivatives of amino acids was selected as model solutes for this study since the analysis of amino acids by derivatization [2–5] or underivatization methods [6,7] is of continued interest due to the inherent difficulties still exist in the chromatographic analysis of these compounds and the important role the amino acids play in several biological processes.

In this paper, it was systematically studied the retention behavior of the above mixture of OPA-derivatives of amino acids under isocratic conditions with mobile phases containing as an additive any member of the homologues series of alkanols (with 2–6 carbon atoms, i.e. ethanol, EtOH, 1-propanol, PrOH, 1-butanol, BuOH, 1-pentanol, PeOH and 1-hexanol, HexOH) at different low concentrations. As a main organic component in the mobile phases was used a mixture of methanol (MeOH) and acetonitrile (MeCN) with a constant volume fraction ratio ($\varphi_{MeOH}/\varphi_{MeCN} = 1/3$) in a range of concentrations ensured the proper miscibility of upper alkanols with the aqueous component of the mobile phases.

Moreover, it is the aim of this study to highlight the important practical separation advantages arising from the diversification of mobile phases with various upper alcohol additives [8–15] and to try to optimize such isocratic separations by means of a computer optimization program.

* Corresponding author. Tel.: +30 2310 997765; fax: +30 2310 997709.
E-mail address: apappa@chem.auth.gr (A. Pappa-Louisi).

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2. Experimental

2.1. Reagent and solutions

Organic solvents, i.e. MeOH and MeCN, were HPLC grade and obtained from Panreac Quimica Sau (Barcelona, Spain). Buffer solutions were prepared using analytical grade KH_2PO_4 and 85% H_3PO_4 (Sigma–Aldrich, packed in Switzerland). The free amino acids: L-Arginine (Arg), Taurine (Tau), beta-(3,4-dihydroxyphenyl)-L-Alanine (Dopa), L-Alanine (Ala), L-Methionine (Met), L-tryptophan (Trp), L-phenylanine (Phe), L-Valine (Val), L-Isoleucine (Ile) and L-Leucine (Leu) were purchased from Sigma–Aldrich (Steinheim, Germany) as well as the reagents used for the derivatization of the amino acids, i.e. OPA and 2-mercaptoethanol (2-ME).

2.2. Amino acid derivatization

As model compounds in this paper was used a mixture of OPA derivatives of the above aminoacids The derivatives formed by the reaction of OPA with amino acids in the presence of 2-ME according to the previously published non-automated, manual pre-column derivatization procedure [16] with minor modifications. Appropriate working concentrations of underivatized amino acids were used in the derivatization procedure by OPA/2-ME reagent (Arg = 2 μg/mL; Tau = 0.75 μg/mL; Ala = 3 μg/mL; Dopa, Met, Trp, Phe, Val = 4 μg/mL; Ile, Leu = 6 μg/mL) so that the peak heights of the OPA-derivatives recorded by fluorometric detector do not differ significantly.

2.3. Instrumentation and chromatographic conditions

The liquid chromatography system consisted of a Shimadzu LC-20AD pump, a model 7125 syringe loading sample injector fitted with a 20 μL loop, an Agilent Zorbax Eclipse-AAA column (3.5 μm, 150 × 4.6 mm) thermostatted at 30 °C by a CTO-10AS Shimadzu column oven and a Shimadzu spectrofluorometric detector (Model RF-10AXL) working at 455 nm after excitation at 340 nm. The mobile phases were aqueous phosphate buffers (with a total ionic strength of 0.01 M and a pH 2.5) modified with the main organic mixture consisted of a constant ratio of MeOH and MeCN ($\varphi_{MeOH}/\varphi_{MeCN} = 1/3$) and with different low concentrations of alkanol additives varying between $\varphi_A = 0.03$ and 0.07. Two composition of the main organic modifier (i.e. of the mixture of MeOH and MeCN) in the eluent were used, $\varphi_M = 0.36$ and 0.4. The experimental retention data obtained under the above chromatographic conditions are shown in Table 1 (see, the data of the first 20 experiments). The flow rate was 1.0 mL/min and the hold-up time was estimated to be $t_0 = 1.37$ min by injection of water [17].

2.4. Fitting and optimization 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, whereas the optimization algorithm was written in VBA and implemented on Excel spreadsheets. The details concerning the optimization algorithm are given in the Section 3.4. The exe files of all algorithms will be available for free upon request from the authors or alternatively they will be found with instructions in the website of the corresponding author of this paper [18].

3. Results and discussion

3.1. Comparison of different retention models

The model derived in Ref. [1] for the description of the simultaneous effect of the contents of the main organic component (φ_M) and of the alcohol additive (φ_A) as well as of the number of alkyl chain of additive (n) on the solute retention factor, k is the following six-parameter equation:

$$\ln k(n, \varphi_M, \varphi_A) = c_0 + c_1 n + c_3 \varphi_M + c_4 \varphi_A + c_5 n \varphi_M + c_6 n \varphi_A \quad (1)$$

This model is in fact a direct combination of equations expressing separately a linear dependence of the retention upon each of these factors. In order to evaluate both the fitting and the prediction performance of this model on the retention of amino acids, the retention data depicted in Table 1 were divided into two groups. In particular, the experimental data nos. 1, 3, 4, 6, 8, 17, 19 and 20 of Table 1 were selected for fitting and the rest for prediction. The experiments selected for the fitting procedure correspond to chromatographic runs obtained using three different members of the homologous series of alkanols (EtOH, BuOH and HexOH) as additives (with $n = 2$, 4 and 6, respectively) in mobile phases containing each additive at a constant $\varphi_A = 0.04$ with two different concentrations of the main organic component ($\varphi_M = 0.4$ and 0.36) as well as using two different members of the homologous series of alkanols (EtOH and BuOH) as additives (with $n = 2$ and $n = 4$, respectively) in mobile phases at two different concentrations ($\varphi_A = 0.04$ and 0.07) with $\varphi_M = 0.4$. The results of this fitting procedure revealed that Eq. (1) exhibits a poor fitting performance since the obtained overall absolute average % error, 4.0, as well as the maximum % error, 14.6, between calculated and experimental retention data, was rather high. A careful evaluation of the experimental data set used for this fitting shows that the dependence of the ln k upon n when the other two parameters, i.e. φ_M and φ_A, are kept constant is rather quadratic instead of linear. This means that the following seven-parameter equation

$$\ln k(n, \varphi_M, \varphi_A) = c_0 + c_1 n + c_2 n^2 + c_3 \varphi_M + c_4 \varphi_A + c_5 n \varphi_M + c_6 n \varphi_A \quad (2)$$

containing a new second order term for the carbon number of straight-chain alkanol additives may present a better fitting performance. Indeed, Eq. (2) describes quite satisfactory the above experimental data set used in the fitting procedure, since the overall average and the maximum percentage error between calculated and experimental retention data were only 0.8 and 2.9%, respectively. However, if the statistical significance of the adjustable parameters of Eq. (2) from their t-ratio values, i.e. the absolute value of the ratio of each parameter to its standard deviation, was examined, it was found that the fitting parameter c_4 was statistically insignificant for all solutes, since its calculated t-ratio parameters were less than 2 [19]. Consequently this regression parameter from the above model, Eq. (2), was dropped, and the same retention data were fitted to the following reduced six-parameter equation:

$$\ln k(n, \varphi_M, \varphi_A) = c_0 + c_1 n + c_2 n^2 + c_3 \varphi_M + c_4 n \varphi_M + c_5 n \varphi_A \quad (3)$$

Indeed, in the majority of cases, all coefficients of Eq. (3) are statistical significant (see Table 2) and additionally the fitting performance of Eq. (3) is comparable to that of Eq. (2) since the overall average and the maximum percentage error between calculated and experimental retention data were 1.0 and 3.7%, respectively.

3.2. Prediction of retention times

Then in order to test the accuracy of retention predictions obtained by both Eqs. (2) and (3) with their corresponding

Table 1
Experimental retention times (in min) of amino acid derivatives obtained at different mobile phase compositions.

No. of exp.	Alcohol additive	No. of C atoms	φ_M	φ_A	Arg	Tau	Dopa	Ala	Met	Trp	Phe	Val	Ile	Leu
1	EtOH	2	0.4	0.07	1.72	1.93	2.61	3.90	7.09	7.83	9.75	9.21	14.96	15.70
2	PrOH	3	0.4	0.07	1.67	1.84	2.32	3.35	5.53	5.98	7.32	7.37	11.38	11.84
3	BuOH	4	0.4	0.07	1.60	1.71	1.97	2.74	4.08	4.23	5.12	5.40	7.92	7.89
4	EtOH	2	0.4	0.04	1.78	2.03	3.04	4.67	9.16	10.93	13.28	12.04	20.11	21.62
5	PrOH	3	0.4	0.04	1.77	1.99	2.86	4.33	8.14	9.39	11.55	10.86	17.84	18.79
6	BuOH	4	0.4	0.04	1.71	1.90	2.65	3.81	6.52	7.28	8.87	8.87	14.08	14.42
7	PeOH	5	0.4	0.04	1.65	1.76	2.34	3.10	4.88	5.34	6.33	6.58	10.05	9.89
8	HexOH	6	0.4	0.04	1.55	1.62	2.12	2.58	3.51	3.96	4.31	4.72	6.60	6.34
9	EtOH	2	0.4	0.03	1.78	2.03	3.12	4.77	9.56	11.52	14.07	12.46	21.17	22.58
10	PrOH	3	0.4	0.03	1.77	2.00	2.99	4.51	8.69	10.26	12.56	11.52	19.17	20.26
11	BuOH	4	0.4	0.03	1.73	1.93	2.84	4.14	7.41	8.52	10.36	10.01	16.27	16.82
12	PeOH	5	0.4	0.03	1.67	1.80	2.51	3.38	5.58	6.30	7.49	7.61	12.00	11.97
13	HexOH	6	0.4	0.03	1.59	1.68	2.29	2.90	4.20	4.81	5.36	5.78	8.50	8.28
14	EtOH	2	0.36	0.07	1.84	2.15	3.21	5.01	10.22	12.14	15.11	13.77	23.82	25.31
15	PrOH	3	0.36	0.07	1.76	1.96	2.74	4.06	7.30	8.28	10.24	10.24	16.51	17.11
16	BuOH	4	0.36	0.07	1.65	1.79	2.22	3.03	4.84	5.29	6.37	6.75	10.30	10.34
17	EtOH	2	0.36	0.04	1.92	2.26	3.71	5.88	13.25	16.96	20.80	17.82	31.92	34.22
18	PrOH	3	0.36	0.04	1.87	2.14	3.40	5.23	10.70	13.23	16.21	14.77	25.52	26.94
19	BuOH	4	0.36	0.04	1.78	1.97	2.84	4.24	7.96	9.42	11.50	11.25	18.81	19.19
20	HexOH	6	0.36	0.04	1.59	1.61	2.33	2.79	4.06	4.97	5.25	5.65	8.15	7.71
21	HexOH	6	0.39	0.035	1.57	1.65	2.23	2.76	3.88	4.45	4.88	5.32	7.65	7.39

adjustable parameters, all the results of Table 1 except those used for the fitting procedure were tested. Both equations enable equivalent predictive ability, which is very satisfactory indeed since the overall predictive % error between calculated and experimental retention data was only 2.8, (see Table 3 for the predictive % errors obtained by Eq. (3)). Consequently, it seems that starting from eight initial isocratic runs, both Eqs. (2) and (3) enable an accurate prediction for other isocratic runs obtained by using a variety of mobile phase compositions containing any alkanol homologue even different than those used in the starting/fitting experiments. However, at least in our experimental system, Eq. (3) with one adjustable parameter less than Eq. (2) can be successfully used for an accurate optimization procedure.

Table 2
Values of adjustable parameters of Eq. (3), their standard deviations and *t*-statistic values.

Solutes	Values of adjustable parameters					
	c_0	c_1	c_2	c_3	c_4	c_5
Arg	1.756	0.091	−0.048	−7.737	0.508	−3.033
Tau	3.240	−0.354	−0.063	−10.507	1.884	−3.562
Dopa	3.652	−0.067	−0.013	−7.922	0.497	−5.775
Ala	4.587	−0.135	−0.041	−9.212	0.982	−4.587
Met	6.804	−0.224	−0.049	−12.511	1.253	−5.165
Trp	7.857	−0.281	−0.034	−14.229	1.177	−5.932
Phe	8.099	−0.282	−0.051	−14.632	1.420	−5.630
Val	7.177	−0.174	−0.050	−12.858	1.219	−5.053
Ile	8.478	−0.239	−0.056	−14.722	1.457	−5.386
Leu	8.624	−0.259	−0.060	−14.881	1.546	−5.673
Standard deviations						
Arg	0.518	0.133	0.005	1.310	0.307	0.263
Tau	0.550	0.141	0.006	1.390	0.325	0.279
Dopa	1.242	0.319	0.012	3.142	0.735	0.630
Ala	0.578	0.148	0.006	1.461	0.342	0.293
Met	0.652	0.167	0.007	1.648	0.386	0.330
Trp	0.997	0.256	0.010	2.522	0.590	0.506
Phe	0.736	0.189	0.007	1.862	0.436	0.373
Val	0.545	0.140	0.005	1.378	0.322	0.276
Ile	0.475	0.122	0.005	1.201	0.281	0.241
Leu	0.452	0.116	0.005	1.142	0.267	0.229
Values of *t*-statistics						
Arg	3.4	0.7	9.2	5.9	1.7	11.5
Tau	5.9	2.5	11.5	7.6	5.8	12.8
Dopa	2.9	0.2	1.1	2.5	0.7	9.2
Ala	7.9	0.9	7.0	6.3	2.9	15.7
Met	10.4	1.3	7.5	7.6	3.2	15.6
Trp	7.9	1.1	3.4	5.6	2.0	11.7
Phe	11.0	1.5	6.9	7.9	3.3	15.1
Val	13.2	1.2	9.1	9.3	3.8	18.3
Ile	17.9	2.0	11.7	12.3	5.2	22.4
Leu	19.1	2.2	13.3	13.0	5.8	24.8

3.3. Benefits of using alkanol additives in mobile phases

Chromatograms depicted in Fig. 1, as well as the retention data depicted in Table 1 illustrate the usefulness of such mobile phases in the separation of a mixture of ten amino acid derivatives. In more details, the retention order of Phe, Val, Ile and Leu seems to depend on the type and in some case on the concentration of the alkanol additive. For example there is an alteration of the elution order of the above four amino acids when the same content ($\varphi_A = 0.04$) of EtOH or PrOH instead of PeOH or HexOH is added in mobile phases with $\varphi_M = 0.4$ (see, experimental data nos. 4, 5, 7 and 8 of Table 1), whereas the retention order of the same amino acids in case BuOH is used as an additive depends on its concentration (compare for example in Fig. 1 the chromatograms recorded in mobile phases with $\varphi_M = 0.4$ containing BuOH at $\varphi_A = 0.03$ or 0.07, respectively). Consequently, it is clear that the introduction of alkanol additives in commonly used mobile phases provides more pertinent options to reversed-phase isocratic separations of OPA amino acid derivatives, which could be compared to those previously revealed in gradient mode by using ternary gradients [3] or multimode gradients involving simultaneously variations of mobile phase composition, flow

Table 3
Absolute percentage error between experimental and calculated retention times obtained by Eq. (3).

No. of exp.	Arg	Tau	Dopa	Ala	Met	Try	Phe	Val	Ileu	Leu
2	0.2	0.4	3.1	1.2	0.3	2.2	0.4	1.1	0.2	1.5
5	0.6	1.1	0.9	0.7	2.1	4.1	3.1	1.4	1.4	1.5
7	1.1	0.1	0.5	1.5	0.9	1.3	0.6	0.5	1.0	0.0
9	1.9	3.2	6.1	5.3	5.4	4.6	4.6	6.1	5.7	6.7
10	1.6	3.9	4.2	5.0	4.3	2.3	3.5	6.1	6.6	7.1
11	0.9	2.5	2.1	2.2	1.9	1.6	1.7	3.6	3.5	3.9
12	0.2	1.8	6.7	6.8	5.0	7.9	5.3	6.8	5.0	6.0
13	0.2	0.1	7.4	3.0	3.2	7.0	3.8	3.1	1.9	1.8
14	0.8	3.1	6.9	5.2	2.5	0.7	0.7	2.9	2.3	3.2
15	0.5	1.5	8.9	4.8	0.6	0.4	0.9	3.1	0.2	1.4
16	0.6	1.8	3.4	1.2	3.8	4.0	5.1	2.1	3.9	2.2
18	0.0	0.6	4.0	1.7	0.3	1.1	0.2	0.5	1.5	1.0
21	0.4	0.3	4.6	2.4	4.2	7.2	5.2	4.0	4.0	3.8
Overall average % error for prediction: 2.8										

Table 4
Spreadsheet containing the solutes $c_0, c_1, \ldots, c_5$ coefficients, the optimization parameters and optimum solutions.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1		Arg	Tau	Dopa	Ala	Met	Trp	Phe	Val	Ile	Leu							
2	*c0*	1.756	3.240	3.652	4.587	6.804	7.857	8.099	7.177	8.478	8.624							
3	*c1*	0.091	-0.354	-0.067	-0.135	-0.224	-0.281	-0.282	-0.174	-0.239	-0.259							
4	*c2*	-0.048	-0.063	-0.013	-0.041	-0.049	-0.034	-0.051	-0.050	-0.056	-0.060							
5	*c3*	-7.737	-10.507	-7.922	-9.212	-12.511	-14.229	-14.632	-12.858	-14.722	-14.881							
6	*c4*	0.508	1.884	0.497	0.982	1.253	1.177	1.420	1.219	1.457	1.546							
7	*c5*	-3.033	-3.562	-5.775	-4.587	-5.165	-5.932	-5.630	-5.053	-5.386	-5.673							
8																		
9	Equation:	$\ln k(n, \varphi_M, \varphi_A) = c_0 + c_1 n + c_2 n^2 + c_3 \varphi_M + c_4 n\varphi_M + c_5 n\varphi_A$																
10																		
11	Optimization parameters			Results														
12	n(min)=	2		n	φ_A	φ_M	dt_R	$t_{R,max}$	Arg	Tau	Dopa	Ala	Met	Trp	Phe	Val	Ile	Leu
13	n(max)=	6		6	0.035	0.39	0.067	7.96	1.58	1.65	2.33	2.83	4.04	4.77	5.13	5.54	7.96	7.66
14	φ_A(min)=	0.03		5	0.064	0.39	0.067	6.21	1.56	1.63	1.89	2.44	3.36	3.52	4.07	4.43	6.21	6.01
15	φ_A(max)=	0.07		5	0.060	0.38	0.066	7.18	1.58	1.65	1.98	2.60	3.72	4.00	4.63	4.99	7.18	6.95
16	$d\varphi_A$=	0.001		6	0.036	0.39	0.065	7.75	1.57	1.64	2.29	2.79	3.96	4.65	5.00	5.41	7.75	7.45
17	φ_M(min)=	0.36		5	0.065	0.39	0.065	6.08	1.56	1.62	1.87	2.42	3.31	3.46	3.99	4.35	6.08	5.88
18	φ_M(max)=	0.4		5	0.061	0.38	0.064	7.03	1.58	1.64	1.96	2.57	3.66	3.93	4.54	4.90	7.03	6.79
19	$d\varphi_M$=	0.01		5	0.066	0.39	0.063	5.96	1.55	1.62	1.86	2.39	3.26	3.40	3.92	4.28	5.96	5.75
20	$t_{R(max)}$=	8		6	0.037	0.39	0.063	7.54	1.57	1.63	2.26	2.75	3.88	4.54	4.88	5.29	7.54	7.25
21	N_solutions=	100		5	0.062	0.38	0.063	6.88	1.58	1.64	1.95	2.54	3.60	3.85	4.45	4.81	6.88	6.64
22	t_0=	1.37		5	0.067	0.39	0.062	5.84	1.55	1.61	1.84	2.37	3.21	3.34	3.85	4.21	5.84	5.63

rate and temperature [5]. However, in order to get full advantage from the retention benefits of using such mobile phases, a proper simple optimization algorithm is necessary based on the retention model proposed in this study, i.e. Eq. (3).

3.4. Computer-aided separation optimization

The optimization algorithm, written in VBA and implemented on Excel spreadsheets, uses the values of $c_0, c_1, \ldots, c_5$ parameters of all solutes and makes a 3D lattice search during which at each lattice point (n, φ_M, φ_A) it calculates and stores the total elution time and the minimum value of the quantity $\Delta t_R = |t_R(\text{solute i}) - t_R(\text{solute j})|$ when i and j ($i \neq j$) range all the solutes. The optimum point (n, φ_M, φ_A) is the one that corresponds to the maximum value of Δt_R provided that the total elution time at this point is less than a preset value (see Table 4).

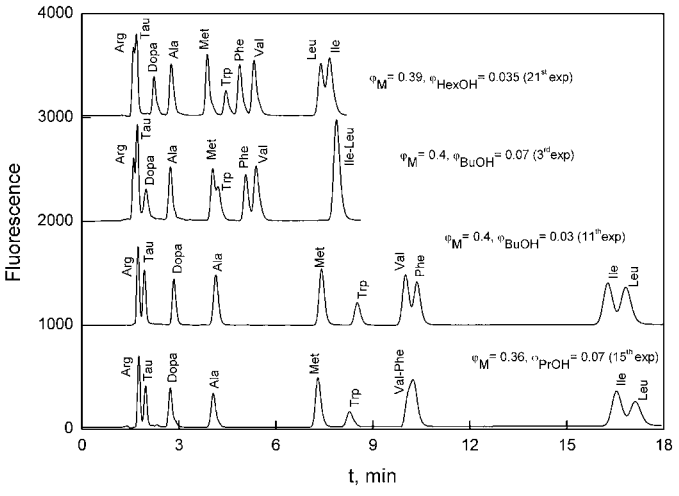

Fig. 1. Chromatograms of a mixture of ten amino acid derivatives recorded (from bottom to top) during the 15th, 11th, 3rd, and 21st experiment, respectively, shown in Table 1. The mobile phase composition used in each experiment is also shown in the figure as well as the elution order of the amino acids obtained.

Thus, the above described optimization algorithm using the adjustable parameters of Table 2 and searching for n between 2 and 6, for φ_M between 0.36 and 0.4 (with steps 0.01) and for φ_A between 0.03 and 0.07 (with steps 0.001) gave as an optimal mobile phase for the amino acid separation at a total elution time $\lesssim$8 min that containing HexOH at $\varphi_A = 0.035$ in mobile phases with $\varphi_M = 0.39$ (see, 21st experiment in Table 1). Indeed, a perfect resolution of the amino acid mixture is achieved within only 7.4 min in the chromatogram recorded under the above conditions and shown in Fig. 1. In the same figure the superiority of the optimal mobile phase is also illustrated, since, for example, it is clear that the mixture of 10 amino acids was not able to be separated neither in 7.9 min by using BuOH additive in the mobile phase at $\varphi_A = 0.07$ with $\varphi_M = 0.4$ since Leu and Ile co elute, nor even in 17.1 min by using PrOH additive at $\varphi_A = 0.07$ with $\varphi_M = 0.36$, where Val and Phe co elute.

4. Conclusion

In this paper, a simple optimization algorithm capable of optimizing isocratic separation by using small amounts of alkanols as additives in the mobile phases was developed. This algorithm was based on a on a sixth-parameter retention model, Eq. (3), considering simultaneously the contents of the main organic modifier and of the alkanol additive as well as of the number of alkyl chain of additive. Starting from eight initial isocratic runs, Eq. (3) enabled an accurate retention prediction, with an average error 2.8%, for other isocratic runs obtained by using a variety of mobile phase compositions containing any alkanol homologue even different than those used in the starting/fitting experiments. Moreover, it was demonstrated in this study that the diversification of mobile phases with alkanol additives enhanced complex mixture separations in RPLC. However, in order to get full advantage from the retention benefits of using such mobile phases, a proper simple optimization algorithm, like the one proposed in this study, is necessary.

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