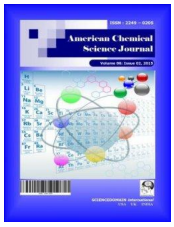

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Efficiency of Solvatic Sorption Model for Predicting the Retention in Multi-step Gradient RP-LC with Different Stationary Phases

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Gradient elution is widely applied in analytical chromatography to reduce separation time and improve selectivity. However, the development and optimization of high-performance liquid chromatography (HPLC) gradient methods is arduous and time-consuming. In this paper, we demonstrate a solvatic sorption model to predict the retention time for phenylisothiocyanate derivatives of amino acids in a multi-step gradient reversed-phase HPLC. This model uses zero approximation level predictions. Rather, we use structural formulae and column and mobile phase properties as a "first guess" to develop the HPLC method before further optimization and prediction of the best multi-step gradient profile. The gradient elution mode with mobile phases modified with methanol and acetonitrile was used and verified the efficiency of different stationary phases. This approach provides good predictions of retention time values achieved after the first approximation step—this uses the data from only one experimental run.

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1. INTRODUCTION

Gradient elution is a powerful method that markedly enhances the separation and peak detection capabilities of many branches of chromatography [1,2]. Reversed-phase high-performance liquid chromatography (RP-HPLC) gradient mode enables the analysis of a mixture with a broad range of polarity and retention characteristics in a single run. It has many advantages over isocratic methods. In conventional gradient liquid chromatography, the eluent strength increases with constant flow rate during the run [1-3]. Until recently, linear gradient has been the most widely used; it is based on simple theoretical relationships [3].

Providing the gradient performance in time units is crucial in obtaining the best trade-off between separation power (peak capacity or maximum number of peaks that can possibly be solved) and analysis time [4]. The design of suitable gradient shapes is a difficult task and optimization is frequently performed empirically. Unfortunately, this makes the optimization process expensive and time consuming [3-5].

Mathematically modeling of the chromatographic processes under overloaded conditions and optimizing the operating parameters have improved considerably in recent years. Many models and numerical methods are now available to optimize the isocratic preparative chromatography [6,7]. In parallel, considerable efforts have been undertaken to use the available mathematical models and tools to analyze and design gradient processes for preparative purposes [8-14].

In recent papers, we have presented a solvatic sorption model for the prediction of retention parameters of phenylisothiocyanate derivatives of natural amino acids in gradient mode for linear gradient profiles [15-16]. Prediction of retention times for multi-segment gradients is more complicated. We next predicted the retention of multi-segment gradients as an example of one C18 stationary phase. Good predictions of retention time can be achieved with complete information including the chemical structure of the analyte, physicochemical properties of the mobile phase, physicochemical properties of the stationary phase, and preliminary data from one run [17]. The purpose of this paper is to present the solvatic retention model for the optimization of multi-segment gradients in RP-HPLC for use with different stationary phases. Acetonitrile-aqueous and methanol-aqueous mobile phases were used for these experiments.

2. EXPERIMENTAL DETAILS

2.1 Preparation of Solutions

HPLC-grade methanol (MeOH) and acetonitrile (MeCN) were obtained from LabScan (Gliwice, Poland). Ultrapure water (18.2 $M\Omega \cdot cm^{-1}$ at 25ºC) was obtained with a Milli-Q water system (Millipore Merck, Darmstadt, Germany).

Buffers solutions were prepared using sodium acetate trihydrate (analytical grade), 6 $mol \cdot L^{-1}$ hydrochloric acid and glacial acetic acid obtained from Penta (Chrudim, Czech Republic); HPLC–grade triethylamine (TEA) was obtained from Fluka (Buchs, Switzerland). Solutions A and B were prepared for gradient elution. Solvent A was pH 6.4 and was prepared by dissolving 19.0 g sodium acetate trihydrate in 1 L water with 0.5 mL TEA and adjusting the pH with glacial acetic acid. The solution was filtered through 0.45 µm filter (Millex-LH, Millipore, Germany). Finally 940 mL of the resulting solution was mixed with 60 mL MeCN or MeOH. Solution B was prepared from MeCN or MeOH and water at a ratio of 3:2.

The following 25 natural amino acids were of analytical grade or better and were purchased from Sigma Aldrich (St. Louis, MO, USA): L-α-Amino-n-butyric acid (Aab), L-α-Aminoadipic acid (Aad), DL- Alanine (Ala), DL-Arginine (Arg), DL-Asparagine (Asn), DL-Aspartic acid (Asp), DL-β-Aminoisobutyric acid (βAib), DL-Citrulline (Cit), γ-Amino butyric acid (γAba), L-Glutamine (Gln), DL-Glutamic acid (Glu), Glycine (Gly), DL-Histidine (His), DL-Isoleucine (Ile), DL-Leucine (Leu), DL-Methionine (Met), DL-Ornithine (Orn), DL-Phenylalanine (Phe), DL-Proline (Pro), DL-Serine (Ser), Taurine (Tau), DL-Threonine (Thr), DL-Tryptophan (Trp), DL-Tyrosine (Tyr), and DL-Valine (Val). The other reagents used for the derivatization of amino acids and HPLC-grade phenyl isothiocyanate (PITC) was also from Sigma. These reagents were dissolved in 0.1 $mol \cdot L^{-1}$ HCl before use.

2.2 Derivatization of Amino Acids

A mixture of the phenylisothiocyanate derivatives of the 25 natural amino acids (PITC) was used as model compounds. The derivatives were formed via the reaction of PITC with amino acid solution according to a widely used non-automated, manual pre-column derivatization procedure [16,18-19].

The PITC derivatives of the amino acids were used in this study and considered that the compounds differ substantially in their hydrophobicity and eluted in a wide range of concentrations in an organic solvent in a mobile phase. This method also has several other advantages including pre-column derivatization possibilities and stability of the derivates for up to 48 hours (at 5ºC – 8ºC). This approach also allows the use of a UV detector [18,19].

The appropriate working concentrations of the naive amino acids was 2 μmol·mL^{-1}. At these conditions, the peak heights of the phenylisothiocyanate derivatives do not differ significantly in the UV detector.

2.3 Instrumentation and Chromatographic Conditions

A pH meter (Oakton Instruments, Vernon Hills, IL USA) equipped with a glass electrode was used to measure pH in buffer solutions. The electrode was calibrated daily with the appropriate standard buffer solutions. Hydrolysis and derivatization of samples were carried out with the Pico•Tag Workstation (Waters, Milford, MA, USA).

All chromatographic experiments were conducted using an Alliance Waters 2695 chromatographic system and controlled by Empower 2 software (Waters, Milford, MA, USA). This instrument was equipped with a Waters 2487 UV detector and a thermo-controlled oven for the column and autosampler (at 6ºC). All chromatographic measurements were performed at 46ºC with an eluent flow rate of 1 mL·min^{-1}. The injected sample volume was 10 μL. Analyte detection was at 254 nm.

We used the ChromSword computer simulation system (version 4.8.3.2010; ChromSword, Germany) to predict the retention parameters; this system also was used for result processing. The experiments used standard RP-HPLC columns but with a wide difference in the stationary phase efficiency, hydrophobicity, silanol activity, ion-exchange capacity, etc. The columns have the same internal diameter and particle size, but different lengths and pore sizes. This also changed the retention time of the compounds. The parameters of 8 RP-HPLC columns are presented in Table 1. All columns contained 5-micron particles in a 4.6 mm internal diameter column.

Table 1. RP-HPLC columns used to study solvatic sorption model

Column	Stationary phase	Internal diameter, mm	Length, mm	Particle size, μm	Pore size, Á	Vendor
SunFire C18	Silica/C18	4.6	250	5	100	Waters, USA
Zorbax CN	Silica/CN	4.6	250	5	60	Agilent Technologies, USA
Zorbax SB-C8	Silica/C8	4.6	150	5	80	Agilent Technologies, USA
Nucleosil 100 C8	Silica/C8	4.6	250	5	100	MACHEREY-NAGEL GmbH & Co, Germany
Alltima C8	Silica/C8	4.6	250	5	100	GRACE, USA
YMC-Pack C4	Silica/C4	4.6	150	5	300	YMC, USA
Mixed Mode RP-C18/Cation	Polymer/C18 /Cation	4.6	250	5	100	GRACE, USA
Mixed Mode RP-C8/Cation	Polymer /C8/Cation	4.6	250	5	100	GRACE, USA

3. RESULTS AND DISCUSSION

The 25 phenylisothiocyanate derivatives of natural amino acids were used to test the retention prediction potency of the solvatic sorption model. The prediction model used methanol and acetonitrile as the mobile phase.

There are three factors determining the distribution of an analyte between the mobile and stationary phases in liquid chromatography. At a constant separation temperature, these factors are: chemical structure of the analyte, physical-chemical properties of the mobile phase and physical-chemical properties of the stationary phase. The solvatic sorption model gives possibility to calculate the retention time from the structural formulae and the properties of the mobile and stationary phases. This model has been described in detail elsewhere [13-14]. We also derived an equation for calculating retention in the reversed-phase chromatography and calibration of columns:

$$\ln k_x = aV_x^{2/3} + b\Delta G_{e.s.x.H_2O} + c \quad (1)$$

Where

$\Delta G_{e.s.x.H_2O}$ is the energy of electrostatic interaction of analyte with water;

V is the partial molar volume of the substance in water that determines an energy value to create a cavity in the mobile and in stationary phases;

a=16.48 ($\gamma_m - \gamma_s$) is a coefficient where γ_m and γ_s are the surface tension of a mobile and stationary phase respectively;

b=0.8234×[f(ε_m) – f(ε_s)] is coefficient, where ε_m and ε_s are dielectric permittivity values of a mobile and stationary phase respectively, where f(ε)=(ε-1)/(2ε+1); and

c is a parameter that includes the ratio of phases and some other characteristics of the stationary and mobile phases.

Accordingly, the model considers both the column and mobile phase properties (a, b, c), as well as characteristics of chemical structure (V and ΔG). This is used to calculate the retention and prediction via initial gradient conditions.

Two experiments were performed to verify the efficiency of the solvatic sorption model. The first type verified the optimization model for retention time. The second evaluated the effectiveness of the optimization of separation. In the first type, we used a “first guess” method. This method usually consists of a linear gradient method with some initial/final concentrations of an organic solvent in a mobile phase and a gradient time. A “first guess” method can be predicted from the chemical structure and characteristics of the column. For this purpose, commercially available software was used. The ChromSword software contains a database of column characteristics of many commercially available reversed-phase columns including the a, b, and c coefficients in Eq. 1 at any concentration of MeCN and MeOH in the mobile phase [20,21]. Hence, it was used to calculate the compound parameters and simulate chromatograms for different linear gradient profiles using Eq.1 [13,14].

We predicted the retention times of the eluted compounds for 45 min of linear gradient from 0 to 100% of MeCN and MeOH in the mobile phase utilizing the “first guess” approach from the structural formulae of the phenylisothiocyanate derivatives of amino acids and physicochemical properties of the stationary and mobile phases. Retention time data after 45 min of linear gradient elution from 0 to 100% in both mobile phases were obtained. Some results of these experiments are shown in Table 2 for MeCN as the mobile phase and MeOH in Table 3. These tables show the difference between the predicted and experimental retention times (t_R) for every compound.

The difference between the predicted and the experimental gradient retention data for the 25 analytes are acceptable. In the case of the acetonitrile mobile phase, the differences between the calculated and experimental gradient retention times with the highest value is 3.5 min (Phe, YMC-Pack C4 column). This is less than those observed for the methanol-containing eluent with the highest value of 4.4 min (Orn, Alltima C8 column). The determination coefficient between the predicted and experimental gradient retention times are higher than R^2=0.9776 (YMC-Pack C4) for the acetonitrile mobile phase and higher than R^2=0.9792 (Zorbax CN) for the methanol eluent. The t_R and determination coefficient support suggest that this approach can be used to predict retention times for mixtures of phenylisothionate derivatives of amino acids. The determination coefficient for all columns are shown in Table 4.

Table 2. The difference between the predicted and experimentally obtained retention time of phenylisothiocyanate derivatives of amino acids for 45 min linear gradient (0.0 min 0% B, 45 min 100% B) of MeCN as the mobile phase

No.	Amino acids	SunFire C18	Alltima C8	Nucleosil 100 C8	Zorbax SB-C8	Zorbax CN	YMC-pack C4	Mixed-mode RP-C8/Catione	Mixed-mode RP-C18/Catione
		t_R, min	t_R, min	t_R, min	t_R, min	t_R, min	t_R, min	t_R, min	t_R, min
1	Aab	2.26	1.78	1.30	1.21	0.47	-0.75	-1.85	0.38
2	Aad	2.54	1.25	0.27	0.41	0.05	0.87	-1.87	-0.02
3	Ala	2.12	1.64	0.93	0.99	0.34	-0.47	-1.84	1.08
4	Arg	2.72	1.65	1.22	1.08	0.68	0.61	-1.71	-0.01
5	Asn	2.44	1.46	0.62	0.70	0.22	0.98	-1.66	-0.10
6	Asp	1.86	0.77	0.06	0.16	0.04	0.79	-1.69	0.05
7	βAib	2.42	1.73	1.08	1.08	0.43	0.27	-1.61	1.05
8	Cit	2.60	1.64	0.98	0.88	0.35	0.81	-1.57	-0.02
9	γAba	2.50	1.71	1.02	1.08	0.43	0.04	-1.53	0.14
10	Gln	2.73	1.58	0.81	0.79	0.30	0.91	-1.53	0.17
11	Glu	2.16	0.96	0.12	0.24	0.04	0.83	-1.41	0.13
12	Gly	1.80	1.41	0.59	0.69	0.21	0.69	-1.41	0.23
13	His	2.75	1.69	1.15	0.99	0.57	1.15	-1.41	-0.12
14	Ile	2.11	1.74	1.66	1.33	1.13	-3.02	-1.37	-0.95
15	Leu	2.08	1.73	1.67	1.35	1.12	-2.61	-1.20	-0.95
16	Met	2.25	1.82	1.61	1.37	0.91	-2.25	-1.05	-0.04
17	Orn	2.39	1.79	1.79	1.46	1.57	-1.62	-0.87	-0.73
18	Phe	2.11	1.72	1.69	1.35	1.35	-3.46	-0.55	-0.29
19	Pro	2.45	1.76	1.10	1.09	0.39	-0.30	-0.40	-0.01
20	Ser	2.16	1.44	0.55	0.68	0.19	1.01	-0.31	0.07
21	Tau	2.61	1.68	0.87	0.98	0.32	1.00	0.11	0.17
22	Thr	2.43	1.71	0.91	0.99	0.31	1.06	0.14	0.04
23	Trp	2.22	1.74	1.72	1.36	1.48	-1.89	0.52	-0.64
24	Tyr	2.68	1.87	1.60	1.37	1.03	-0.57	0.57	-0.22
25	Val	2.22	1.79	1.54	1.32	0.80	0.19	1.27	0.02

Table 3. The difference between the predicted and experimentally obtained retention time of phenylisothiocyanate derivatives of amino acids for 45 min linear gradient (0.0 min 0% B, 45 min 100% B) of MeOH as the mobile phase

No.	Amino acids	SunFire C18	Alltima C8	Nucleosil 100 C8	Zorbax SB-C8	Zorbax CN	YMC-pack C4	Mixed-mode RP-C8/Cationе	Mixed-mode RP-C18/Cationе
		t_R, min	t_R, min	t_R, min	t_R, min	t_R, min	t_R, min	t_R, min	t_R, min
1	Aab	0.88	1.83	0.93	0.98	-1.25	1.26	0.64	-0.60
2	Aad	1.37	1.05	0.63	0.69	-2.64	0.38	0.62	0.10
3	Ala	0.77	1.45	0.76	0.89	-1.50	0.67	0.12	-0.18
4	Arg	1.37	2.03	0.92	0.95	-1.43	0.92	1.17	-0.62
5	Asn	1.10	1.22	0.68	0.76	-2.12	0.45	0.34	0.12
6	Asp	0.60	0.28	0.35	0.33	-2.21	0.21	0.24	0.27
7	βAib	1.05	1.77	0.86	0.93	-1.60	0.84	0.49	-0.26
8	Cit	1.25	1.82	0.89	0.92	-2.16	0.81	0.67	-0.27
9	γAba	1.13	1.68	0.91	0.90	-1.61	0.70	0.48	-0.26
10	Gln	1.48	1.55	0.81	0.84	-2.38	0.49	0.60	0.10
11	Glu	0.91	0.54	0.45	0.46	-2.37	0.26	0.37	0.29
12	Gly	0.33	0.87	0.59	0.69	-1.37	0.40	-0.32	0.07
13	His	1.51	1.94	0.90	0.94	-1.99	0.93	1.14	-0.41
14	Ile	2.27	2.68	1.00	1.09	-0.21	2.06	1.72	-1.04
15	Leu	2.35	2.93	1.00	1.09	-0.21	2.05	1.75	-1.04
16	Met	1.17	2.53	1.00	1.00	-0.67	1.82	1.37	-0.29
17	Orn	1.94	4.37	1.00	1.00	-0.70	2.24	2.39	-1.34
18	Phe	2.56	2.47	1.00	1.00	0.28	2.12	2.09	-1.75
19	Pro	1.06	1.87	0.87	0.95	-1.70	0.75	0.56	-0.28
20	Ser	0.76	1.02	0.60	0.70	-1.78	0.45	0.02	-0.02
21	Tau	1.19	1.51	0.76	0.85	-1.91	0.60	0.38	-0.01
22	Thr	1.10	1.68	0.85	0.90	-1.95	0.93	0.41	-0.19
23	Trp	2.07	2.60	1.00	1.00	0.25	2.18	2.54	-1.68
24	Tyr	1.02	2.53	0.99	1.00	-1.37	1.67	1.78	-1.02
25	Val	1.22	2.55	0.98	1.00	-0.90	0.81	1.17	-0.77

Table 4. Determination of predicted and experimental results

Columns	Determination coefficient (R^2)	
	MeCN	MeOH
SunFire C18	0.9978	0.9986
Zorbax CN	0.9991	0.9792
Zorbax SB-C8	0.9982	0.9998
Nucleosil 100 C8	0.9975	0.9997
Alltima C8	0.9985	0.9981
YMC-Pack C4	0.9776	0.9988
Mixed Mode RP-C18/Cation	0.9959	0.9995
Mixed Mode RP-C8/Cation	0.9990	0.9969

The results of the first run can be used to fine-tune the model (Eq. 1). Hence, for the second experiment, the energy of the interaction term was used to correct the model in the first approximation step. The corrected model was used to predict the retention of the multi-step gradients for both organic modifiers. This can help us to separate the mixture. The multi-step gradients were created automatically utilizing ChromSword software. The multi-step gradients for all columns are presented in Figs. 1 (a-h).

Based on the multi-step gradient data, we next compared the experimental and predicted retention data. Determination between the observed and the predicted retention times for all HPLC columns studied are shown in Figs. 2 (a-h) for both organic modifiers.

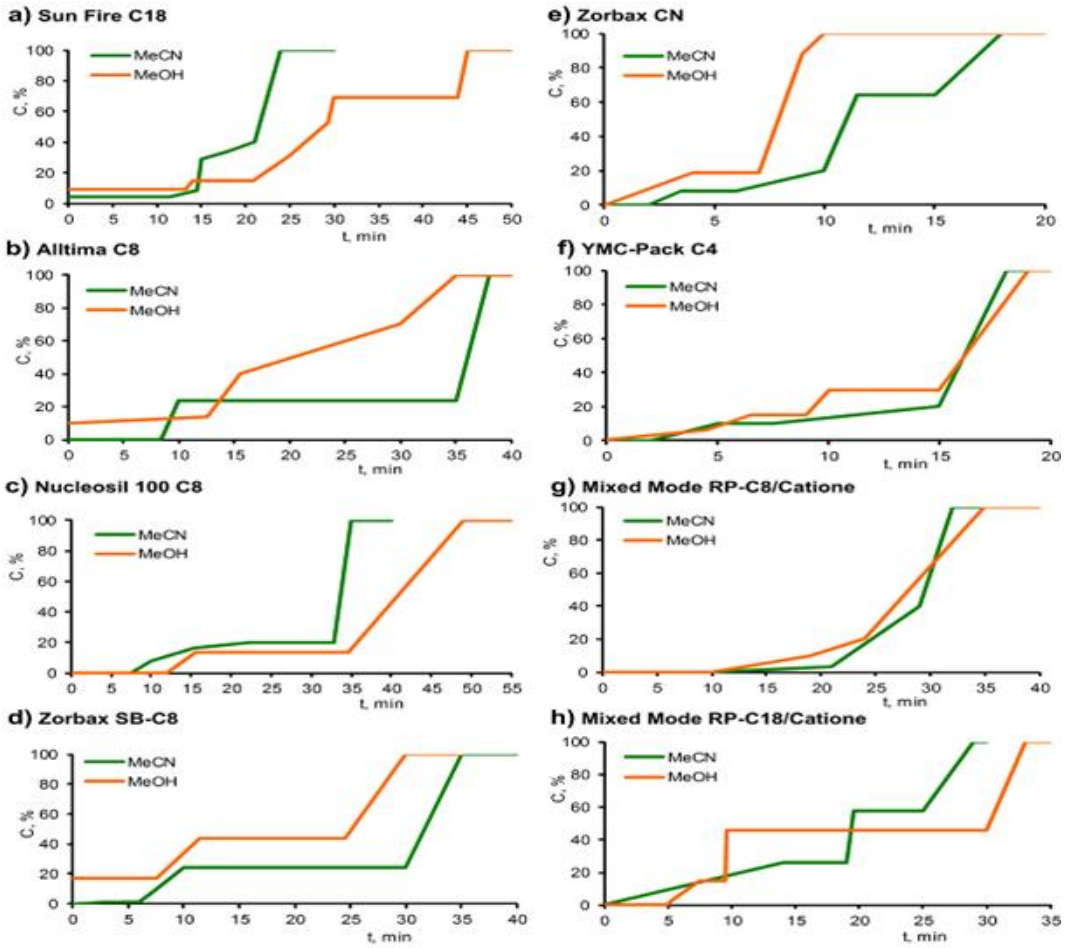

Fig. 1. The automatically optimized multi-step gradients for columns: (a) Sun Fire, (b) Alltima, (c) Nucleosil 100, (d) Zorbax SB-C8, (e) Zorbax CN, (f) YMC-Pack, (g) Mixed Mode RP-C8/Catione, and (h) Mixed Mode RP-C18/Catione

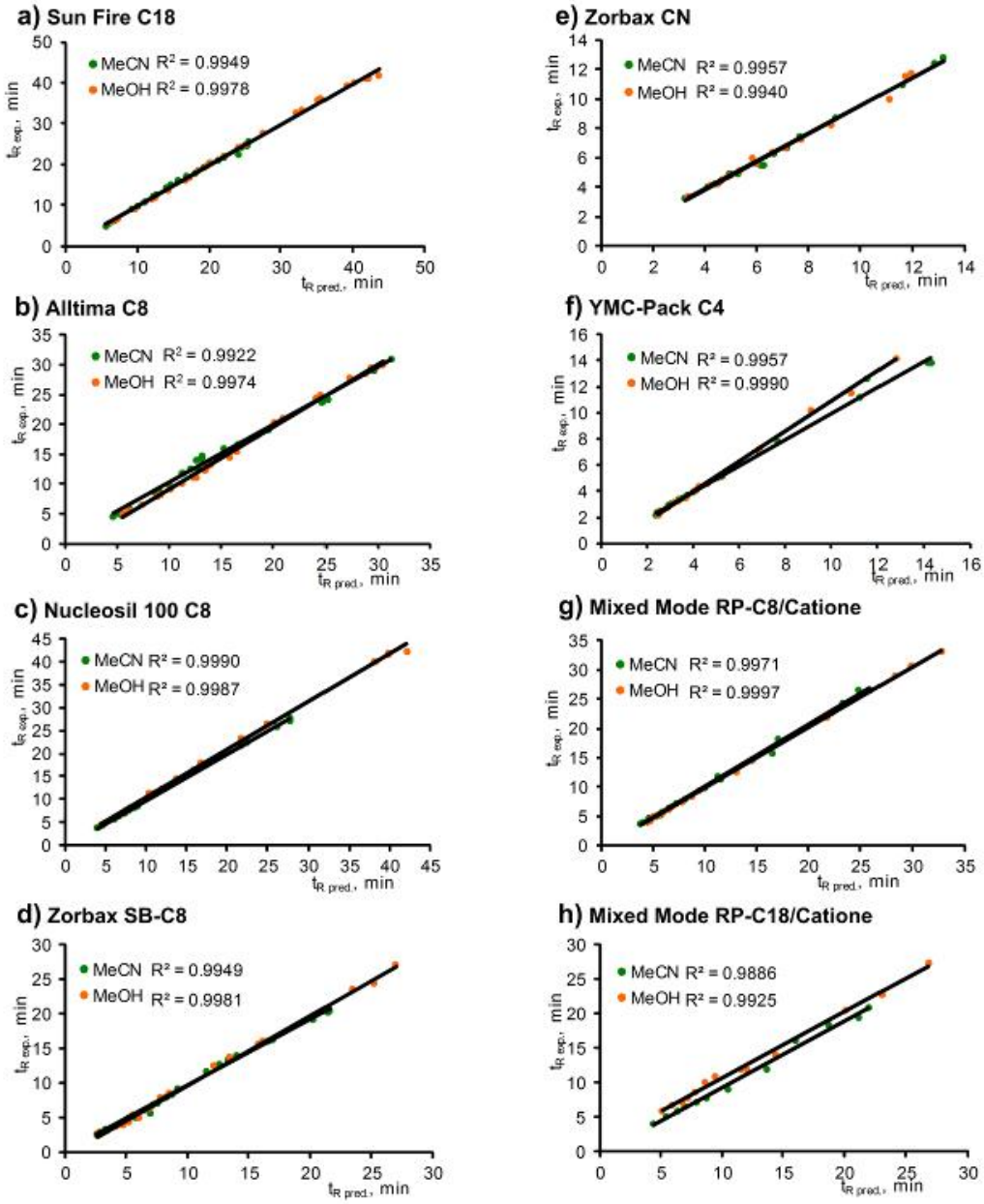

Fig. 2. Determination between the predicted and experimental multi-step gradient retention times obtained on: (a) Sun Fire C18, (b) Alltima C8, (c) Nucleosil 100 C8, (d) Zorbax SB-C8, (e) Zorbax CN, (f) YMC-Pack C4, (g) Mixed Mode RP-C8/Catione, and (h) Mixed Mode RP-C18/Catione for both organic modifiers

Very good retention time predictions are achieved through the use of the structural formulae of the compounds, physicochemical properties of the stationary and mobile phases, and experimental data of only one linear gradient during the development of multi-step gradient yields. The determination coefficient were not less than 0.993 for methanol-water and 0.989 for acetonitrile-water gradients (Mixed Mode RP-C18/Catione column). The error in the predicted values is less than 1.97 min for methanol-water and 1.82 min for acetonitrile-water gradients.

Fig. 3 compares the simulated and experimental results in methanol-water with a Mixed Mode RP-C18/Catione column. However, the separation of

phenylisothionate derivatives of the amino acids is not satisfactory. The predicted parameters correspond to the experimentally-obtained parameters. We suppose that this stationary phase is not suitable for the separation of phenylisothionate derivatives of amino acids.

Good separation of the phenylisothionate derivatives was obtained with the Sun Fire C18 stationary phase and acetonitrile-water gradients. The predicted and experimental chromatograms are seen in Fig. 4.

Predicted retention times are very satisfactory for all stationary phases including MeOH and MeCN as organic modifiers. According to polarity (P'MeOH=5.1 and P'MeCN=5.8) and dielectric constant values (εMeOH=32.7 and εMeCN=37.5), the identified organic modifiers have rather similar elution. However, MeOH and MeCN belong to different selectivity groups. The partial polarities are obtained by multiplying P' by proton acceptor power (P'xeMeOH=2.448 and P'xeMeCN=1.798), proton donor power (P'xdMeOH=1.122 and P'xdMeCN=1.566) and strength of dipole-dipole interaction (P'xmMeOH=1.581 and P'xmMeCN=2.436) [22,23]. To use these values, we can declare that the effect of MeOH can be mainly attributed to proton-acceptor interactions, while MeCN corresponds to dipole–dipole interactions. In spite of the different effects of the organic modifiers, we note that prediction according to the solvatic sorption model described by Eq. 1 is very good provided there is data for both organic modifiers.

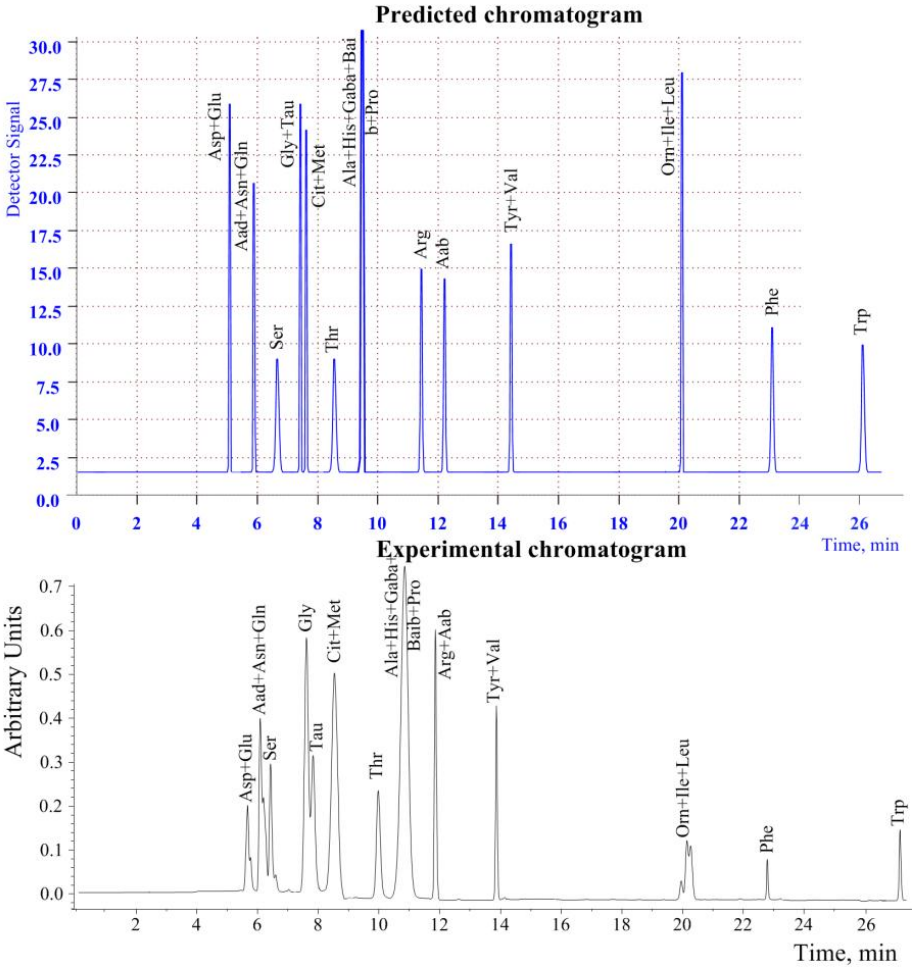

Fig. 3. Comparison between predicted and experimental chromatograms for mixed mode RP-C18/Catione columns using methanol-water organic modifiers

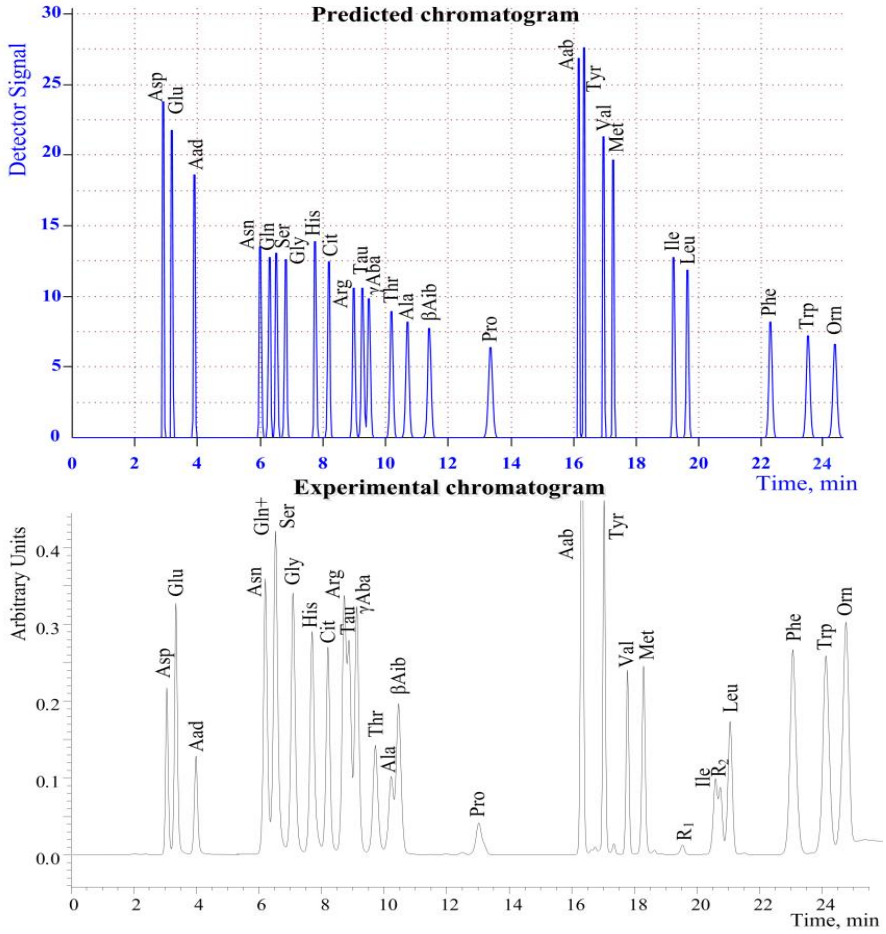

Fig. 4. The comparison between predicted and experimentally obtained chromatograms for SunFire C18 column using acetonitrile-water organic modifiers

4. CONCLUSIONS

Gradient elution is very powerful tool that enables chromatographers to obtain separations that are impossible with isocratic elution. The solvatic retention model in RP-LC facilitates prediction of retention time using the structural formulae of the analyte, column and mobile phase properties, and a "first guess". This is used to optimize not only linear elution, but also multi-step gradient elution. Based on these experimental data, we concluded that acceptable prediction of retention time values can be achieved in the first approximation step using only one linear run. The solvatic retention model in RP-LC operates effectively with methanol and acetonitrile aqueous mobile phases across a wide range of stationary phases. This approach might be of interest to those persons developing and optimizing the separation of various analytes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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