Talanta 98 (2012) 54–61

Contents lists available at SciVerse ScienceDirect

Talanta

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

Improved chromatographic response function in HILIC analysis: Application to mixture of antidepressants

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ARTICLE INFO

Article history:
Received 13 March 2012
Received in revised form
11 June 2012
Accepted 15 June 2012
Available online 3 July 2012

Keywords:
Hydrophilic interaction liquid chromatography (HILIC)
Chromatographic response function (N_{CRF}*)
3^3 Experimental design
Antidepressants

ABSTRACT

This paper presents exploration of chromatographic behavior in HILIC system by experimental design and improved chromatographic response function denoted as N_{CRF}*. As a model mixture six antidepressants were chosen: selegiline, mianserine, sertraline, moclobemide, fluoxetine and maprotiline. Due to complexity of retention mechanisms in HILIC system, detailed examination of experimental space assessing the influence of important factors (acetonitrile content in the mobile phase, buffer concentration and pH of the mobile phase) and their interactions was done by applying 3^3 experimental design. N_{CRF}* is developed and designed to be the only output of the system which simultaneously measures the separation of all the examined substances, the chromatographic run duration and the quality of the obtained peaks shape. It allowed objective estimation of overall chromatogram quality and excluded the arbitrary judgment in ambiguous situations. The applied function highlighted the influence of investigated factors on entire mixture and enabled identification of experimental regions where the chromatographic behavior was satisfactory. Applied experimental design strategy combined with N_{CRF}* proved to be valuable assistance in HILIC separation of complex mixtures.

1. Introduction

Hydrophilic interaction liquid chromatography (HILIC) has recently obtained important position among the separation strategies applied for pharmaceutically active compounds [1–5]. HILIC overcomes the typical shortages of reversed phase liquid chromatography (RP-LC) such as the poor retention of polar substances. On the other hand, HILIC is superior comparing to the normal phase liquid chromatography (NP-LC) in cases when examined compounds are not soluble in non-polar solvents. Also, HILIC demonstrated to have many advantages in the analysis of polar basic compounds.

HILIC separation system consists of polar stationary phase and mobile phase with high polar organic solvent content (over 75%) and small portion of water phase. It is believed that the retention mechanism includes partition of analytes between water enriched layer of solvent near sorbent surface and more hydrophobic bulk eluent, i.e., partition in liquid–liquid separation system. Nevertheless, the retention phenomenon in HILIC is influenced by variety of intermolecular interactions between the solute and the stationary phase, the solute and the mobile phase, and the stationary and mobile phase including hydrogen bonding, donor–acceptor interactions, ion–dipole and dipole–dipole interactions, electrostatic interactions, hydrophobic interactions etc. [5]. The contribution of each of these interactions to the overall separation pattern depends on various factors such as the stationary phase selection, buffer concentration, pH of the mobile phase, type of organic solvent etc. Therefore, it is very difficult to predict in advance the exact degree in which each interaction will affect the retention behavior of particular mixture. Consequently, the detailed experimental investigation of different factors influence on HILIC system need to be performed in order to describe it well.

In literature, several papers explaining the dependence of HILIC retention mechanism on organic solvent content, pH of the mobile phase, buffer type or buffer concentration could be found [6–8]. However, the authors of those studies applied *one-variable-at the time* approach which explains the influence of a single factor while all the other factors are kept at fixed level. This approach is insufficient for thorough investigation of retention behavior since the factors interactions can play important role in HILIC separation. The problem can be solved by applying systematical experimental design strategy which was poorly used for HILIC method development, and only few papers can be found in literature up to now. In this way, Box–Behnken design was applied for optimization of separation of salicylic, acetyl salicylic and ascorbic acid [9]. Mixture design was used for the study of retention behavior of glycerol, urea and glycerol carbonate [10]. Effect of chromatographic parameters and detector settings on

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http://dx.doi.org/10.1016/j.talanta.2012.06.040

the response of HILIC – evaporative light – scattering detection system were investigated through 2^{7-4} fractional factorial design and fractional central composite design [11].

Generally, the chromatographic behavior analysis of complex mixtures is especially challenging task since the retention of particular substances can be completely differently affected by analyzed factors. Therefore, analyst experience is not sufficient for identification of chromatographic conditions that provide simultaneous separation of all mixture compounds. Additionally, if the other goals, apart from the separation, such as satisfactory peak symmetry, minimal total elution time etc., are set, the aid of mathematical tools is necessary for exploration of experimental space. Chromatographic response functions (CRF) can be useful choice allowing all the desired chromatogram qualities to be combined. Up to now, many CRFs have been developed for evaluation of RP-LC systems [12–15], but most of them appeared to have some shortages. Recently, the authors of this paper have developed new chromatographic response function (N_{CRF}) [16] which proved to have some advantages comparing to the previously developed ones. To our knowledge, there was no application of any CRF in HILIC separation system up to now.

The main objective of this study was the synergy of experimental design and chromatographic response function in evaluation of retention behavior of antidepressants mixtures in HILIC system. First, chromatographic response function was improved so that it measures the separation of all examined substances, the chromatographic run duration and the quality of the obtained peaks shape simultaneously. Then, the accuracy of the function was verified, and further on, the improved function was selected as the only response of the system to be followed. The applied strategy should provide identification of experimental regions where the analyzed mixture shows the best chromatographic behavior including simultaneous separation of all compounds, minimal total elution time and adequate peaks shape. As a model mixture six antidepressants were chosen: selegiline (substance I), mianserine (substance II), sertraline (substance III), moclobemide (substance IV), fluoxetine (substance V) and maprotiline (substance VI) (Fig. 1). Up to now, the analysis of antidepressants in HILIC system can be found only in papers dealing with investigation of paroxetin [17] and fluoxetin [18] in biological samples.

Development of liquid chromatographic method usually assumes seeking for conditions where all examined substances are resolved. Consequently, resolution between adjacent peaks is the most commonly selected output. When dealing with analysis of several substances, optimal resolving of critical peak pair can be defined as the leading goal. However, the existence of several critical peak pairs can make the chromatographic separation of complex mixtures more complicated. In that case, the overall separation quality must be achieved. Nevertheless, apart from the desired separation, the other important aspects of chromatographic analysis should be taken into consideration, such as the total run time, peak symmetry, robustness of the system etc.

Exploring the experimental space for a particular mixture, chromatograms with all perfect characteristics are rarely seen. It is more likely that majority of chromatograms will have some goals achieved (e.g., separation), but the other goals missed (e.g., prolonged total elution time). In such ambiguous cases the analysts arbitrary judgment of which chromatogram is better is not reliable. Chromatographic response functions offer the mathematical solution to this problem since they incorporate all desired chromatogram characteristics into single numerical value. The calculated CRF value for a set of chromatograms allows their accurate ranking and therefore identification of the chromatographic conditions where all desired aims are fulfilled.

The correct design of CRF is challenging task: first, it should describe appropriately the investigated parameters (resolution between adjacent peaks, peak symmetry, total elution time etc.) and second, it should make adequate weighting of each parameter. Our recently developed function, new chromatographic response function (N_{CRF}), is formulated as [16]:

$$N_{CRF} = \left(a \left(1 - \frac{\sum_{i=1}^{N-1} \theta_{s,l}}{N-1} \right) + 1 \right) \left(1 + \left(\frac{t_f}{t_{opt}} \right)^b \right) \quad (1)$$

where $\theta_{s,l}$ is the resolution criterion estimated by Eq. (2), N is the number of expected peaks, t_f is the elution time of the last peak, t_{opt} is the chosen optimal overall elution time, and a and b are coefficients that should be determined in advance (a is usually set between 1–5 and b between 0–5). N_{CRF} consists of separation term (first parentheses) and time term (second parentheses). Separation term is defined by Carle's θ criterion [19] estimated as:

$$\theta_{s,l} = 1 - ((H_v \times |t_{R,l} - t_{R,s}|)/(|t_{R,v} - t_{R,s}| \times (H_l - H_s) + H_s \times |t_{R,l} - t_{R,s}|)) \quad (2)$$

where H_s and H_l are the heights of the peaks, H_v is the valley height, $t_{R,s}$ and $t_{R,l}$ are the retention times of the peaks, and $t_{R,v}$ is the time position of the valley as presented in Fig. 2.

θ criterion has several advantages comparing to the most commonly applied separation parameter-resolution factor Rs

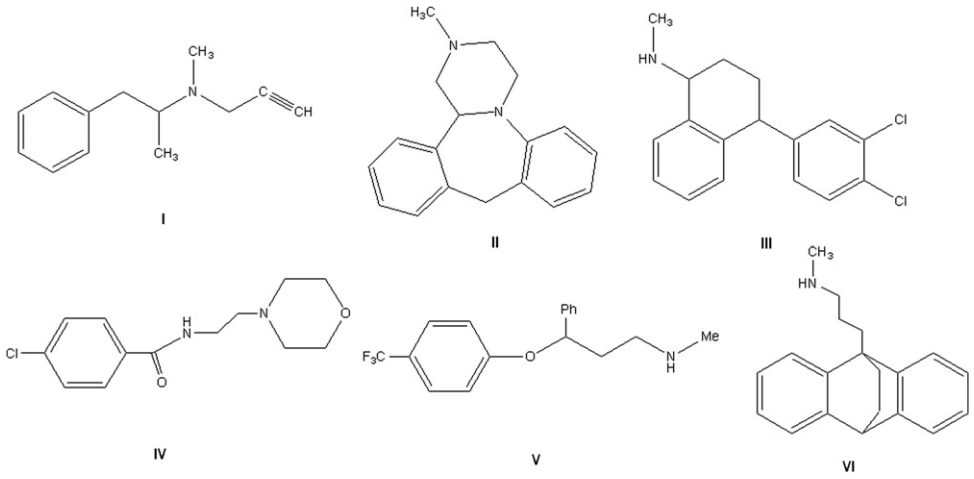

Fig. 1. Chemical structures of the analyzed antidepressants: (I) selegiline, (II) mianserine, (III) sertraline, (IV) moclobemide, (V) fluoxetine and (VI) maprotiline.

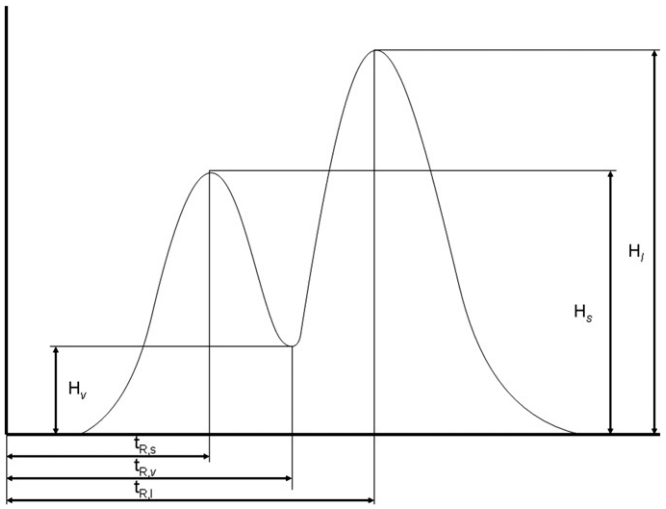

Fig. 2. Chromatogram scheme illustrating the parameters in Carle's θ criterion calculation [19].

($Rs=2\Delta t/(w_1+w_2)$, where Δt_R is the difference in the retention time of the peak maxima, and w_1 and w_2 are width of the peaks). First, this criterion can estimate both Gaussian and non-Gaussian shaped peaks, while *Rs* offers adequate results only for the first ones. Second, θ criterion has values in the range from 0 (for completely overlapped peaks) to one (for perfectly resolved peaks) which eliminates the possibility of masking poorly resolved peak pairs by high value of separation criterion of well resolved ones.

Another advantage of N_{CRF} is the appropriate balance of the resolution and time impact on function which can be achieved setting coefficients *a* and *b* according to the analysts expectations from the method. N_{CRF} reaches minimum as the separation increases and total elution time decreases [16]. Therefore, the best chromatographic behavior of the analysed system is characterized by the lowest N_{CRF} values.

Important aspect of chromatographic peak quality is its shape, since the non-Gaussian shape and extremely large width can make unable adequate quantification. When dealing with active pharmaceutical ingredients, the regulatory demands are very strict, and chromatographic methods developed in this field must guarantee reliable quantification. Therefore, in this paper the improvement of N_{CRF} is presented so that it incorporates the width term in the function. The adapted function is denoted as N_{CRF}^* and formulated as:

$$NCRF^* = \left(a\left(1-\frac{\sum_{i=1}^{N-1}\theta_{s,l}}{N-1}\right)+1\right)\left(1+\left(\frac{t_f}{t_{opt}}\right)^b\right)\left(1+\frac{\sum w_i}{N}\right)^c \qquad (3)$$

where w_i is peak width at baseline, and all the other symbols are explained previously for Eqs. (1) and (2).

The obtained N_{CRF}^* allows the selection of chromatogram where not only the best separation and minimum total elution time are achieved but also the adequate peak shape is achieved. The balance of newly introduced width term in N_{CRF}^* is set by coefficient *c*.

2. Material and methods

2.1. Chemicals

All used reagents were of the analytical grade. The mobile phase and the solvents were prepared of acetonitrile (*Lab Scan*, Ireland), ammonium acetate (*J. T. Backer*, The Netherlands), glacial acid (*Zorka Pharma*, Serbia) and HPLC grade water.

2.2. Standard solutions

Stock solutions were prepared by dissolving the substances into the acetonitrile–water phase (40 mM ammonium acetate, pH 4.0) 90:10 v/v in order to obtain the concentration of: 400 μg mL^{-1} for

Table 1
3^3 experimental plan and the order of elution of the substances.

Run	A[a]	B	C	Eluting order [d]
1	1[b] (86)[c]	1 (3.0)	1 (20)	I–V–III–II–IV–VI
2	1 (86)	1 (3.0)	2 (40)	I–V–III–II–IV–VI
3	1 (86)	1 (3.0)	3 (60)	I–V–III–II–IV–VI
4	1 (86)	2 (4.5)	1 (20)	I–II–III–V–IV–VI
5	1 (86)	2 (4.5)	2 (40)	I–II–III–V–IV–VI
6	1 (86)	2 (4.5)	3 (60)	I–II–III–V–IV–VI
7	1 (86)	3 (6.0)	1 (20)	I–II–III–IV–V–VI
8	1 (86)	3 (6.0)	2 (40)	I–II–III–IV–V–VI
9	1 (86)	3 (6.0)	3 (60)	I–II–III–IV–V–VI
10	2 (90)	1 (3.0)	1 (20)	I–II–V–III–IV–VI
11	2 (90)	1 (3.0)	2 (40)	I–II–V–III–IV–VI
12	2 (90)	1 (3.0)	3 (60)	I–II–V–III–IV–VI
13	2 (90)	2 (4.5)	1 (20)	I–II–III–IV–V–VI
14	2 (90)	2 (4.5)	2 (40)	I–II–III–IV–V–VI
15	2 (90)	2 (4.5)	3 (60)	I–II–III–IV–V–VI
16	2 (90)	3 (6.0)	1 (20)	I–II–III–IV–V–VI
17	2 (90)	3 (6.0)	2 (40)	I–II–III–IV–V–VI
18	2 (90)	3 (6.0)	3 (60)	I–II–III–IV–V–VI
19	3 (94)	1 (3.0)	1 (20)	I–II–III–IV–V–VI
20	3 (94)	1 (3.0)	2 (40)	I–II–III–IV–V–VI
21	3 (94)	1 (3.0)	3 (60)	I–II–IV–III–V–VI
22	3 (94)	2 (4.5)	1 (20)	I–II–III–IV–V–VI
23	3 (94)	2 (4.5)	2 (40)	I–II–III–IV–V–VI
24	3 (94)	2 (4.5)	3 (60)	I–II–III–IV–V–VI
25	3 (94)	3 (6.0)	1 (20)	I–II–III–IV–V–VI
26	3 (94)	3 (6.0)	2 (40)	I–II–III–IV–V–VI
27	3 (94)	3 (6.0)	3 (60)	I–II–III–IV–V–VI
28	2 (90)	2 (4.5)	2 (40)	I–II–III–IV–V–VI
29	2 (90)	2 (4.5)	2 (40)	I–II–III–IV–V–VI
30	2 (90)	2 (4.5)	2 (40)	I–II–III–IV–V–VI

[a] A—acetonitrile content in the mobile phase (%); B—pH of the mobile phase; C—concentration of ammonium acetate in the water phase (mM).
[b] Coded factor levels.
[c] Real factor levels.
[d] Eluting order of the substances; I=selegiline, II=mianserine, III=sertraline, IV=moclobemide, V=fluoxetine, VI=maprotiline.

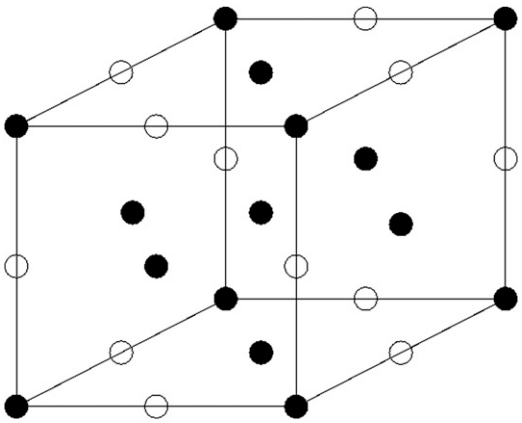

Fig. 3. Scheme of 3^3 experimental design: filled bullets represent points belonging to central composite design and transparent bullets represent points belonging to Box–Behnken experimental design.

fluoxetine and maprotiline, 100 μg mL^{-1} for mianserine, 50 μg mL^{-1} for moclobemide, 600 μg mL^{-1} for sertraline and 1 mg mL^{-1} for selegiline.

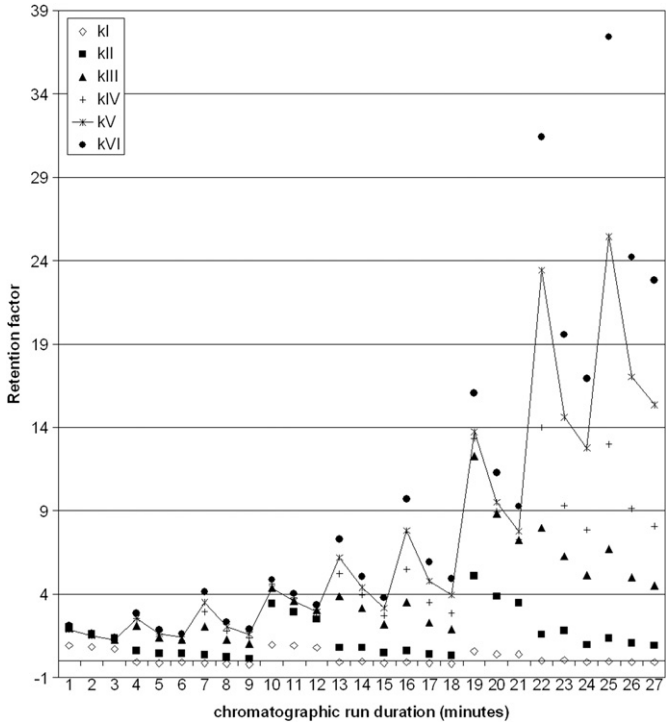

Fig. 4. Retention factors order of the analyzed substances in 27 examined chromatographic runs: k_1—selegiline, k_2—mianserine, k_3—sertraline, k_4—moclobemide, k_5—fluoxetine and k_6—maprotiline.

2.3. Mobile phase

The mobile phase composition was defined by the experimental plan given in Table 1.

2.4. Chromatographic conditions

The experiments were performed on chromatographic system *Finnigan Surveyor Thermo Scientific* consisted of HPLC Pump, Autosampler Plus and UV/VIS Plus Detector. *ChromQuest* was used for data collection. The injection volume was 5 μL. The analytical column was BETASIL Silica-100 (100 mm × 4.6 mm, 5 μm particle size). Flow rate was 1 mL min^{-1} and column temperature was 30 °C. UV detection was carried out at 254 nm.

2.5. Software

3^3 experimental plan, statistical analysis and three-dimensional response surfaces were obtained by STATISTICA 7. The functions values were calculated in *Microsoft Office Excel*.

3. Results and discussion

The HILIC analysis of the mixture selected in this paper started with preliminary investigations. As a stationary phase the bare silica column was chosen. Mobile phase consisted of high percentage of acetonitrile and small amount of water phase which comprised ammonium acetate buffer. It was noted that the analyzed mixture was strongly affected by mobile phase composition and that the mixture retention behaviour changed with variations of acetonitrile–water ratio, buffer concentration and pH of the mobile phase. Therefore, the goal was to identify the

Table 2
Resolution criterions, resolution factors, peak widths, total elution time and N^*_{CRF} values for the obtained chromatograms.

Run	$\theta_{1/2}$	$\theta_{2/3}$	$\theta_{3/4}$	$\theta_{4/5}$	$\theta_{5/6}$	Sum θ/N	$Rs_{1/2}$	$Rs_{2/3}$	$Rs_{3/4}$	$Rs_{4/5}$	$Rs_{5/6}$	Sum Rs/N	w_1	w_2	w_3	w_4	w_5	w_6	Sum w/N	t_f	N_{CRF}*
1	1	0.77	0	0.75	0	0.5	1.95	0.21	0.05	0.13	0.04	0.47	0.4	1.16	0.65	0.55	1.11	0.79	0.78	4.99	5.86
2	1	0.82	0.17	0.28	0.14	0.48	1.25	0.25	0.06	0.20	0.13	0.38	0.78	0.88	0.49	0.42	0.38	0.41	0.56	4.31	5.60
3	1	0.59	0.47	0.04	0.23	0.46	1.47	0.10	0.13	0.02	0.10	0.36	0.4	0.74	0.64	1.25	0.39	0.8	0.70	3.85	5.65
4	1	1	1	0	1	0.80	3.70	5.38	0.97	0.01	0.76	2.16	0.21	0.4	0.5	0.84	0.7	0.64	0.55	6.18	3.53
5	1	1	0.99	0	0.97	0.79	3.37	4.50	0.86	0.07	0.89	1.94	0.19	0.35	0.33	0.44	0.42	0.37	0.35	4.57	3.15
6	1	1	0.95	0.33	0.98	0.85	3.18	3.65	0.57	0.16	0.80	1.67	0.18	0.31	0.38	0.36	0.38	0.34	0.32	3.98	2.57
7	1	1	1	1	1	1	2.57	6.09	2.32	1.33	1.37	2.74	0.26	0.37	0.54	0.63	0.74	0.7	0.54	8.21	1.98
8	1	1	1	0.99	0.99	0.99	2.73	5.58	2.13	0.89	0.89	2.44	0.18	0.26	0.36	0.38	0.45	0.69	0.39	5.34	1.65
9	1	1	1	1	1	1	1.76	3.39	1.45	0.85	1.03	1.70	0.23	0.36	0.46	0.38	0.4	0.55	0.39	4.64	1.56
10	1	1	0.05	0.83	0.84	0.74	7.52	2.16	0.13	0.50	0.44	2.15	0.38	0.67	0.68	0.69	0.75	1.02	0.70	9.41	4.91
11	1	1	0.32	0.63	0.95	0.78	6.99	1.68	0.17	0.28	0.56	1.93	0.34	0.53	0.53	0.88	0.63	0.81	0.62	7.54	4.05
12	1	1	0.47	0.30	1	0.75	5.80	1.06	0.20	0.12	0.48	1.53	0.36	0.53	0.66	0.7	1.02	0.7	0.66	6.54	4.08
13	1	1	1	1	1	1	4.93	9.23	2.99	1.78	1.99	4.18	0.18	0.4	0.66	0.81	0.87	0.94	0.64	13.3	2.57
14	1	1	1	1	1	1	3.31	5.52	1.74	1.01	1.31	2.58	0.22	0.51	0.78	0.63	0.68	0.75	0.59	9.06	2.09
15	1	1	1	1	1	1	3.49	6.52	1.67	1.14	1.49	2.86	0.18	0.37	0.48	0.55	0.66	0.64	0.48	7.64	1.91
16	1	1	1	1	1	1	3.42	8.25	4.02	3.62	2.42	4.35	0.25	0.44	0.68	0.9	1.13	1.36	0.79	17.11	3.05
17	1	1	1	1	1	1	3.79	7.59	3.45	3.01	2.28	4.02	0.17	0.3	0.49	0.64	0.72	0.86	0.53	11.08	2.29
18	1	1	1	1	1	1	2.75	5.84	2.87	2.87	2.17	3.30	0.19	0.37	0.49	0.59	0.63	0.81	0.51	9.5	2.12
19	1	1	1	1	1	1	12.71	11.00	1.18	0.42	2.29	5.52	0.32	0.81	1.29	1.5	1.51	1.75	1.19	27.31	4.37
20	1	1	0.19	0.96	1	0.83	11.67	7.85	0.19	0.89	2.51	4.62	0.31	0.7	1.44	1.13	1.09	1.27	0.99	20.87	6.54
21	1	1	0	1	1	0.8	8.54	6.37	0.04	0.61	2.08	3.52	0.48	0.67	1.23	1.54	1.03	1.27	1.04	16.43	6.09
22	1	1	1	1	1	1	5.67	11.73	5.84	6.00	3.70	6.59	0.33	0.57	1.17	2.13	2.91	3.98	1.84	51.87	7.63
23	1	1	1	1	1	1	7.44	7.90	3.89	5.92	3.74	5.78	0.22	0.53	1.28	1.23	1.64	2.58	1.25	32.91	5.04
24	1	1	1	1	1	1	5.23	10.14	4.38	5.82	3.62	5.84	0.19	0.46	0.85	1.14	1.56	2.11	1.05	28.7	4.47
25	1	1	1	1	1	1	6.08	10.74	7.31	8.94	5.66	7.75	0.19	0.55	1.04	1.71	2.75	4.02	1.71	61.48	8.72
26	1	1	1	1	1	1	5.77	10.53	5.28	6.13	4.09	6.36	0.18	0.43	0.77	1.72	2.42	3.18	1.45	40.33	6.02
27	1	1	1	1	1	1	4.97	9.26	5.75	6.06	4.32	6.07	0.21	0.45	0.79	1.19	2.65	2.88	1.36	38.12	5.71
28	1	1	1	1	1	1	3.96	7.62	2.32	1.35	1.74	3.40	0.18	0.39	0.51	0.62	0.65	0.72	0.51	9.35	2.1
29	1	1	1	1	1	1	7.21	7.56	2.20	1.01	1.57	3.91	0.19	0.37	0.50	0.62	0.65	0.71	0.44	9.3	2.07
30	1	1	1	1	1	1	3.31	5.52	1.74	1.01	1.31	2.58	0.22	0.41	0.55	0.63	0.68	0.75	0.59	9.06	2.09

θs, l: resolution criterion of adjacent peaks calculated by Eq. (2); Rs_s, l—resolution factor between adjacent peaks; w_i—peak width; t_f: retention time of the final peak (total run time).

* *N*CRF:improved chromatographic response function; symbols 1–6 present the first –sixth peak eluted.

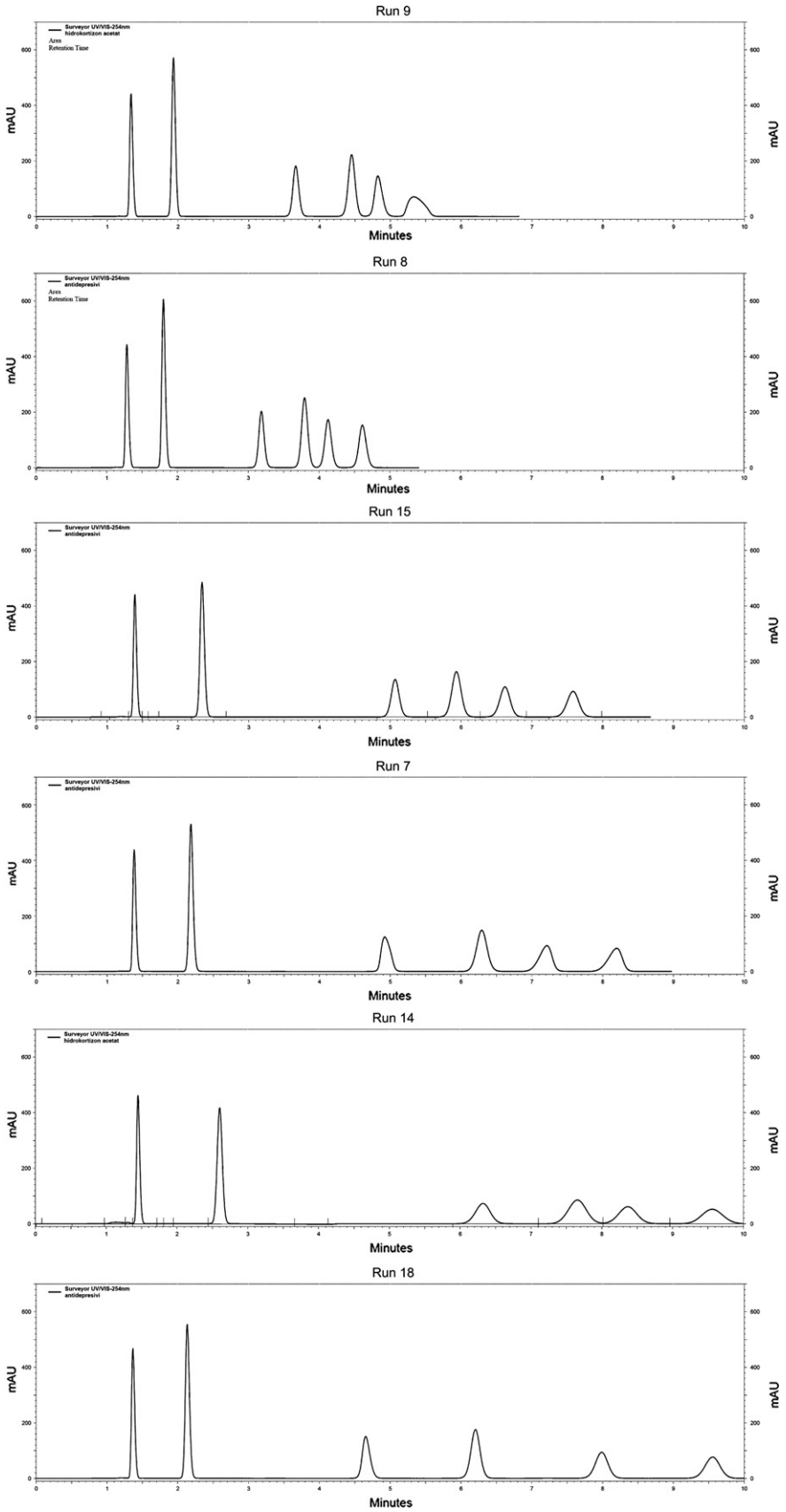

Fig. 5. Chromatograms obtained for: (1) run 9 ($N_{CRF}^{*}=1.56$); (2) run 8 ($N_{CRF}^{*}=1.65$); (3) run 15 ($N_{CRF}^{*}=1.91$), (4) run 7 ($N_{CRF}^{*}=1.98$), (5) run 14 ($N_{CRF}^{*}=2.09$) and (6) run 18 ($N_{CRF}^{*}=2.18$).

experimental regions where the adequate combination of these three factors would lead to the best chromatographic behavior of the analyzed mixture measured as the best N_{CRF}^* value.

The experimental scheme was designed with the aid of experimental design. The two most widely applied response surface designs are central composite design and Box–Behnken design. However, in order to cover the experimental space thoroughly, 3^3 full factorial design which combines both of these designs was applied. As it is presented in Fig. 3, this design investigates three factors on three levels and consists of all points included in central composite and Box–Behnken design.

The experimental plan defined by 3^3 design with three central point replications is presented in Table 1. Fig. 4 shows the map of retention factors of analyzed substances obtained in each chromatographic run.

Fig. 4 demonstrates the complexity of the chromatographic behavior of the analyzed mixture. It was not possible to identify one critical peak pair since there were experimental conditions where five out of six peaks were overlapped (runs 1, 2 and 3). The retention of each of the substances was differently affected by investigated factors. While substance I eluted always as the first one, and substance VI as the last one, substance V's position varied from second to fifth place. The chromatographic runs where indubitable separation of all substances was achieved had prolonged total elution time and inadequate peaks shapes. Identification of experimental regions which correspond to the most favorable mixture behavior in terms of adequate separation, total run time and peaks shape was challenging task. Improved chromatographic response function N_{CRF}^* should allow this kind of multiobjective problem to be solved.

First phase of N_{CRF}^* application consists of setting up the appropriate weighting of resolution, time and width terms. For this particular mixture, resolution is selected as a response of high priority and therefore coefficient a is set to be 5. Coefficients b is set as 1, coefficient c as 0.2 and the value of optimal total run time (t_{opt}) is chosen to be 10 min.

For all obtained chromatograms the quality of the separation was measured by sum of θ criterions divided by the total number of peak pairs. For a comparison, traditionally applied separation parameter-resolution factor Rs is calculated as well. Further on, time and width terms of improved function were estimated, and finally, the obtained results for N_{CRF}^* are given in Table 2.

N_{CRF}^* reaches minimum as the defined goal is approached. Therefore, the lowest function values are obtained for chromatograms with good separation, relatively short total elution time and adequate peaks, as in the case of chromatograms 9 ($N_{CRF}^*=1.56$), 8 ($N_{CRF}^*=1.65$), 15 ($N_{CRF}^*=1.91$), 7 ($N_{CRF}^*=1.98$), 14 ($N_{CRF}^*=2.09$) and 18 ($N_{CRF}^*=2.12$). These chromatograms are presented in Fig. 5. Chromatograms 28, 29 and 30 which are replications of chromatogram 14 have satisfactory N_{CRF}^* values, as well.

The order of chromatograms presented in Fig. 5 is in the accordance with their total elution time and estimated peaks widths since the separation quality was satisfactory in each case. Chromatogram 18 have slightly better width term than chromatograms 7 and 14, but its N_{CRF}^* value is higher due to the longer total elution time.

Since the separation was chosen to be the goal of high priority (the baseline separation between adjacent peaks as achieved when θ value is 1), chromatograms with several overlapped peaks were punished with deterioration of N_{CRF}^* value. That is why chromatograms 1 ($N_{CRF}^*=5.86$), 2 ($N_{CRF}^*=5.60$) and 3 ($N_{CRF}^*=5.65$) are characterized with unsatisfactory N_{CRF}^* values although total run time is shorter than 5 min. Similarly, comparing chromatograms 20 ($N_{CRF}^*=6.54$) and 26 ($N_{CRF}^*=6.09$) it can be seen that the total run time of the first one is half of the total run time of the second one (40.33 and 20.87, respectively). Yet, chromatogram 20 is characterized as worse one due to the poor separation (two out of six θ criterions were unacceptable having values 0.19 and 0.83).

Extremely prolonged elution and peak deformation (extensive peak width) influenced the function as well. The worst function values are obtained for chromatograms 25 ($N_{CRF}^*=8.72$) and 22 ($N_{CRF}^*=7.63$) where total elution time was excessively long: 61.48 and 51.87 min, respectively. Also, these two chromatograms had the greatest sum of peaks width.

The advantage of estimating separation by θ criterion instead of resolution factor can be noted by examining the chromatograms 22 to 27. The sum of θ criterions for all these chromatograms is 1 since the baseline separation between all adjacent peaks is achieved. Therefore, the influence of resolution term will not mask the other important chromatogram characteristics, and the rank obtained by N_{CRF}^* ($24<23<27<26<22<25$) follows the order of their total elution times allowing the chromatogram with shortest run time to be the best one. On the contrary, resolution factor increases as the difference between retention times of adjacent peaks increases. Therefore, the sum of resolution factors divided by the number of peak pairs increases as the total elution time increases leading to the overestimation of separation term. In such case, the influence of prolonged total elution time or extensive peaks widths would be masked, and chromatogram 25 would be characterized as the best one, while N_{CRF}^* characterizes this chromatogram as the worst one.

Further on, the principle of estimating separation by θ criterion avoids masking of poorly resolved peak pairs by high value of resolution factor of well resolved ones. Analyzing chromatogram 20, it can be seen that the sum of resolution factors divided by total number of peak pairs is 4.62 while for the chromatogram 9 it is 1.70. However, in the case of chromatogram 9 all peak pairs are well separated, while in chromatogram 20 third and fourth peaks are overlapped. Therefore, estimating separation by resolution factor falsely positive results can occur, since some resolution factors are high enough to mask the poor values of other resolution factors. On the other hand, sum of θ criterions will judge accurately the separation, revealing all peak pairs that are overlapped.

N_{CRF}^* provided multiobjective evaluation of the obtained chromatograms in efficient and accurate way. Therefore, response surface methodology can be applied following N_{CRF}^* as a response to evaluate the chromatographic behavior of the entire mixture.

Prior to the response surface methodology, the influence of investigated factors on the retention of individual substances was evaluated. Increasing the acetonitrile content in mobile phase, the retention of polar substances is generally considered to increase.

Table 3
Coefficients of quadratic models for N_{CRF}^* and their statistical significance.

	coefficients	p value
Intercept	2.22	
A[a]	**1.28**[b]	0.000
A^2	**1.82**	0.000
B	**−0.78**	0.000
B^2	**0.84**	0.004
C	**−0.47**	0.009
C^2	0.26	0.325
AB	**1.28**	0.000
AC	−0.24	0.242
BC	**−0.42**	0.046

[a] A—acetonitrile content in the mobile phase (%); B—pH of the mobile phase; C—concentration of ammonium acetate in the water phase (mM).
[b] Bold values represent the factors that are significant for $\alpha=0.05$.

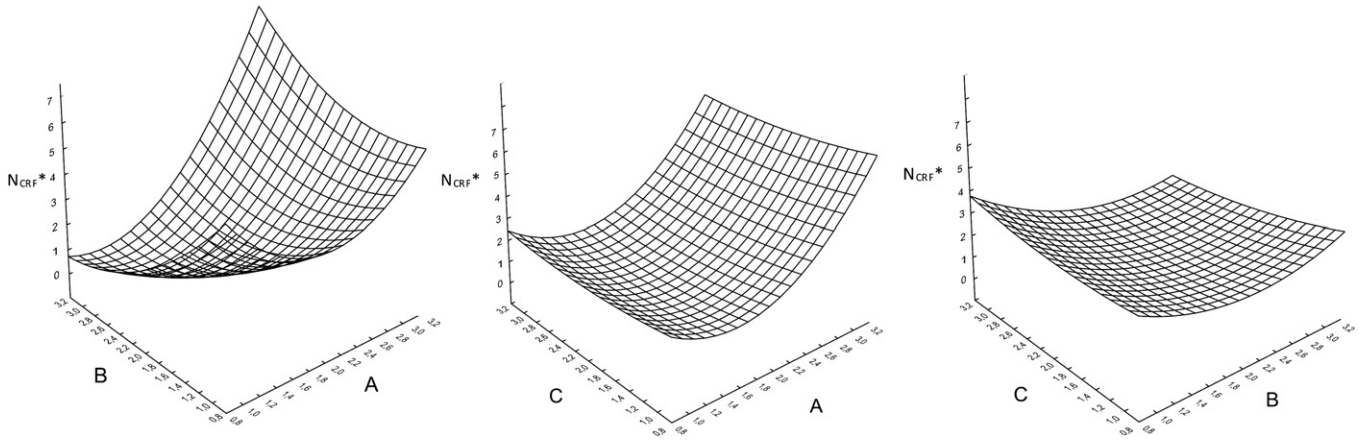

Fig. 6. Three-dimensional response surface plots: $N_{CRF}^{*}=f(A)$, (B) while $C=40$ mM; $N_{CRF}^{*}=f(A)$, (C) while $B=4.0$; $N_{CRF}^{*}=f(B)$, (C) while $A=90\%$; A—acetonitrile content in mobile phase, B—pH of the water phase and C—buffer concentration.

It can be seen that the last eluting substances (III, IV, V, and VI) were the most affected ones by this phenomena, while substances I and II were only slightly shifted to the longer retention time even in the experiments where ACN content was 94%.

The influence of pH variations in range from 3.0 to 6.0 had complex impact on mixtures chromatographic behavior. Namely, the strongest basic compounds such as substances V and VI were ionised within all range, and their retention was prolonged as the pH increased. This happened because the ionisation of free silanol groups on column surface was increased and electrostatic interactions between surface and substances were stronger. On the other hand, the retention of substances I, II, III and IV decreased as pH of water phase increased because their ionisation was suppressed and consequently their polarity, as well.

Increased buffer concentration lead to stronger competitive binding of buffer to free silanol groups on column surface. Therefore, the retention of substances generally decreased with buffer content increasing.

Finally, N_{CRF}^{*} was applied as the output of the system. The obtained mathematical model is presented in Table 3.

It can be seen that statistical analysis found all three investigated factors and interactions of factors A and B (acetonitrile content in the mobile phase and pH of water phase) and A and C (acetonitrile content in the mobile phase and buffer concentration in the water phase) to be important for the level of significance $\alpha=0.05$. Corresponding 3D response surface plots are presented in Fig. 6.

Inspection of 3D graphs shows that acetonitrile concentration (Fig. 6A) and pH of the mobile phase (Fig. 6B) strongly affected the chromatographic behavior of analyzed mixture, while the impact of buffer concentration (Fig. 6C) had slightly lower impact. From Fig. 6A, it can be noted that the interaction between acetonitrile and pH was particularly significant. Decreasing acetonitrile content improves the time term of the function since it leads to the faster elution of substances in HILIC. However, this takes the risk of deterioration of the separation term, as well. On the other hand, increasing the pH of mobile phase improves the separation of analyzed compounds. Therefore, the lowest N_{CRF}^{*} values are obtained in regions of low ACN content (approaching 86%) and increased pH (up to 6.0).

Fig. 6C displays the interaction of pH and buffer concentration. The lowest N_{CRF}^{*} values are obtained in regions where both factors were at their highest level (pH value 6.0; buffer concentration of 60 mM). Increased pH allowed improvement of function separation term, while increased buffer concentration improved time and width term.

Despite the complexity of the investigated factors influence, N_{CRF}^{*} succeeded to describe the retention behavior of entire mixture simultaneously. Consequently, it enabled identification of regions of experimental space where adequate combination of all examined factors provides good separation, minimal total elution time and adequate peak width for all analyzed substances.

4. Conclusion

This paper presented the evaluation of antidepressants mixture separation in HILIC system applying improved chromatographic response function. The first part of the study included application of 3^3 experimental design for examination of the influence of important factors related to the mobile phase composition (acetonitrile content in the mobile phase, pH of water phase and buffer concentration) on mixture retention behavior. Further on, recently developed chromatographic response function was improved (and denoted as N_{CRF}^{*}) so that it simultaneously estimates the substances separation, total chromatographic run duration and peak shape. N_{CRF}^{*} proved to accurately measure the overall chromatograms quality and thus, it was chosen to be the only output of the system. Finally, the applied function allowed identification of experimental regions where examined mixture showed optimal behavior in HILIC system.

Acknowledgments

The authors thank to Ministry of Education and Science of Republic of Serbia for supporting these investigations in Project 172052.

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