Evaluation of Seven Chromatographic Response Functions on Simulated and Experimentally Obtained Chromatograms in Hydrophilic Interaction Liquid Chromatography System

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Abstract

This paper investigates the ability of seven chromatographic response functions to measure the quality of chromatograms obtained in hydrophilic interaction liquid chromatography (HILIC). Firstly, the functions were tested on a set of simulated chromatograms and differences in their mathematical design were discussed. Secondly, the functions were evaluated on the experimentally obtained chromatograms in HILIC analysis of model mixture consisted of beta agonists and antagonists. The ranking of chromatograms obtained by different functions was significantly different implying that the accuracy of the optimization procedure is strongly dependent on the function which was selected as an output. Investigation of potential drawbacks of each function was conducted and general recommendations concerning the use of chromatographic response functions in optimization strategies are proposed.

INTRODUCTION

Chromatographic response functions (CRF) are mathematical tools which enable objective numerical measure of chromatograms quality. When applied as a global optimization criteria, they should facilitate the development and optimization of chromatographic methods. This is especially important when the separation of complex mixtures, including several overlapping peak pairs, is required. The elementary criteria, such as critical resolution, is inapplicable in that kind of analytical problems. Another benefit of CRFs is their ability to estimate not only the separation, but also the other chromatograms characteristics such as total elution time, peak symmetry etc., thus providing multiobjective analysis. However, mathematical construction of a chromatographic response function is a difficult task and many CRFs have been developed so far (Berridge 1982, Schlabac 1988, Morris 1996, Morgan 1975, Dose 1987, Bylund 1997, Glajch 1980, Duarte 2010, Jancic–Stojanovic 2011, Rakic 2012). Significant differences in their mathematical design could lead to different estimation of the same set of chromatograms. This issue arises the question how to select a reliable CRF for a particular optimization problem. There are several papers presenting theoretical evaluation of differences between some CRFs (Siouffi 2000, Cela 1989). However, there are only few papers comparing two or three functions on experimentally obtained chromatograms in reversed phased liquid chromatography. Morris (Morris 1996) showed the advantages of chromatographic exponential function which he

proposed, over the chromatographic resolution statistics which Schlabach (Schlabach 1988) suggested before. Duarte (Duarte 2010) demonstrated the advantages of their CRF over Berridge's CRF. Our team has recently developed new chromatographic response function (N_{CRF}) which exhibited some preferences over Morris' and Duarte's functions (Jancic–Stojanovic 2011).

Nevertheless, chromatographic response functions are rarely used in hydrophilic interaction chromatography (HILIC) method development and the performances of different functions have not been studied in this type of chromatography before. The application of HILIC in analytical separation strategies is growing, especially in the analysis of uncharged basic compounds where the majority of pharmaceutically active compounds belong (Hemstrom 2006, Hsieh 2008, Dajaegher 2008, Dajaegher 2010, Busuzewski 2012).

The aim of this study was the evaluation and comparison of seven different chromatographic response functions on simulated and experimentally obtained chromatograms in HILIC system in order to examine their advantages and drawbacks, but also to define the precautions that must be considered when selecting the function for the particular optimization problem. The functions included in the study were Berridge's chromatographic response function (denoted as B_{CRF}) (Berridge 1982), Glajch's chromatographic optimization function (COF) (Glajch 1980), Dose's CRF (Do_{CRF}) (Dose 1987), Schlabach chromatographic resolution statistics (CRS) (Schlabach 1988), Morris' chromatographic exponential function (CEF) (Morris 1996), Duarte's chromatographic

response function (D_{CRF}) (Duarte 2010) and new chromatographic response function (N_{CRF}) developed by the authors of this paper (Jancic–Stojanovic 2011).

The functions are firstly compared on a set of simulated chromatograms. Further on, model mixture consisted of beta agonists and antagonists (atenolol, metoprolol, fenoterol, salbutamol and propranolol) was analyzed in HILIC system and functions were tested on the obtained real chromatograms. As far as the authors know, this is the first paper comparing these seven functions simultaneously on experimentally obtained data.

EXPERIMENTAL

Simulated Chromatograms

The simulated chromatograms were generated by *Microsoft Office Excel 2003*. They are defined to possess different separation characteristics (well resolved peak pairs having high resolution factors, well resolved peak pairs having baseline separation but not high resolution factors, overlapped peaks) and different analysis duration.

Experimentally Obtained Chromatograms

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. Baker*, The Netherlands), glacial acid (*Zorka Pharma*, Serbia) and HPLC grade water.

Standard Solutions

Stock solutions were prepared by dissolving the substances into the acetonitrile−water phase (40 mM ammonium acetate, pH 4.5) 85:15 v/v in order to obtain the following concentration: 50 µg mL^{-1} for atenolol, metoprolol, fenoterol, salbutamol and 20 µg mL^{-1} for propranolol.

Mobile Phase

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

Chromatographic Conditions

The chromatographic system Waters Breeze was consisted of Waters 1525 Binary HPLC Pump, Waters 2487 UV/VIS dual absorbance detector and Breeze Software Windows XP for data collection. The analytical column was Betasil Silica-100 (100 mm x 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.

Software

Experimental design and data analysis were performed by using Design−Expert$^{®}$ 7.0.0. (Stat−Ease Inc., Minneapolis). The functions values were calculated in *Microsoft Office Excel*.

RESULTS AND DISCUSSION

Investigated Functions And Evaluation Of Their Mathematical Construction

The requirements for good chromatographic response function include two major demands: to effectively differentiate chromatograms quality and to provide reliable mathematical solution for quantitative scaling of chromatograms quality (Cela 1989, Cela 2003). Additionally, several minor requirements are set including the adaptability of CRF to the chromatographers objectives and the lack of mathematical incorrections. Despite the great number of CRFs proposed so far, none of them appears to be the perfect one, and each contains several restrictions that must be considered before selecting it as an optimization criteria. In this study the advantages and drawbacks of seven chromatographic response functions will be examined. In the following section the mathematical construction of the functions will be presented.

1. The first examined function is Berridge's chromatographic response function (Berridge 1982). It is formulated as:

$$B_{CRF} = \sum_{i=1}^{L} R_i + L^{w_1} - w_2 |T_A - T_L| - w_3 (T_1 - T_0) \tag{1}$$

where R_i is the resolution between i-th peak pair; L is the number of peak pairs; T_A, T_L, T_1 and T_0 are the maximum acceptable time, retention time of the final peak, retention time of the first peak and the minimum retention time of the first peak respectively; w_1, w_2 and w_3 are weighting coefficients chosen by the analysts.

2. The second investigated function is Glajch's chromatographic objective function (Glajch 1980). It is formulated as:

$$COF = \sum_{i=1}^{n} A_i \ln(R_i / R_{id}) + B(t_m - t_n) \tag{2}$$

where R_i and R_{id} are experimentally obtained and desired resolution factors between adjacent peaks, respectively; t_m and t_n are maximum desired and experimentally obtained retention times of last eluting peak, respectively and A_i and B weighting factors.

3. The third investigated function is Dose's chromatographic response function (Dose 1980). It is formulated as:

$$Do_{CRF} = \frac{t_{R,n}}{t_{R,cri}} + \sum_{i \neq j} e^{-R_{s,ij}/R_{s,crit}} \tag{3}$$

where $t_{R,n}$ and $t_{R,cri}$ are the retention time of the last eluting peak, and desired total elution time, respectively, $R_{si,j}$ is the experimentally obtained resolution factor between adjacent peaks while $R_{s,cri}$ is the desired resolution factor set by the chromatographer.

4. The fourth investigated function is Schlabach chromatographic resolution statistics (Schlabach 1988). It is formulated as:

$$CRS = \left\{ \sum_{i=1}^{n-1} \left[\frac{(R_i - R_{opt})^2}{R_i (R_i - R_{min})^2} \right] + \sum \frac{(R_i)^2}{(n-1)R_{av}^2} \right\} * \frac{t_f}{n} \tag{4}$$

where R_i, R_{opt} and R_{min} are experimentally obtained, optimal desired and minimal acceptable resolution factor, respectively. R_{av} stands for the average value of all experimentally obtained resolution factors, t_f is the elution time of the last eluting peak and n is the total number of peaks appeared on chromatogram.

5. The fifth investigated function is Morris' chromatographic exponential function (Morris 1996). It is formulated as:

$$CEF = \left[\left(\sum_{i=1}^{n-1}\left(1-e^{a\left(R_{opt}-R_i\right)}\right)^2\right)+1\right]\left[1+\frac{t_f}{t_{max}}\right] \qquad (5)$$

where R_{opt} and R_i stand for the optimal resolution and the resolution of the i-th peak pair respectively, t_{max} and t_f are the maximum acceptable time and the elution time of the final peak respectively, a is the slope adjustment factor and n is the number of the expected peaks.

6. The sixth investigated function is Duarte's chromatographic response function (Duarte 2010). It is formulated as:

$$D_{CRF} = \sum_{i=1}^{N-1}\theta_{s,l} + N - \left(\left(t_{R,L}-t_0\right)/t_{R,L}\right) \qquad (6)$$

where $t_{R,l}$ is the elution time of the last peak, t_0 is the column void volume, N is the total number of peaks appearing in chromatogram and θ is calculated according to Carle's equation (Carle 1972) defined by the following expression:

$$\theta_{s,l} = 1 - \left(\left(H_v \times |t_{R,l}-t_{R,s}|\right)/\left(|t_{R,v}-t_{R,s}| \times \left(H_l-H_s\right)+H_s \times |t_{R,l}-t_{R,s}|\right)\right) \qquad (7)$$

where H_s and H_l are the heights of the adjacent 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 .

7. The seventh investigated function is new chromatographic response function (Jancic-Stojanovic 2011). It is formulated as:

$$N_{CRF} = \left(a\left(1-\frac{\sum_{i=1}^{N-1}\theta_{s,l}}{N-1}\right)+1\right)\left(1+\left(\frac{t_f}{t_{opt}}\right)^b\right) \qquad (8)$$

where $\theta_{s,l}$ is the resolution criterion estimated by Eq. 7, 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 weighting coefficients.

The differences in estimation of chromatograms by seven examined functions may arise

from different mathematical construction of separation and time terms. Functions B_{CRF},

COF, Do_{CRF}, CRS and CEF evaluate the separation quality by resolution factor. Although

resolution factor is considered to be universal separation parameter, its critical value that

presents baseline separation (in this paper selected as 1.5) is reliable only in case of

Gaussian shaped peaks (Duarte 2010, Jancic–Stojanovic 2011). For chromatograms with

asymmetrical peaks, containing fronting or tailing, it is hard to define optimal resolution

factor in advance, so we are in serious risk that the function will give falsely positive (if

we chose low optimal resolution), or falsely negative results (if we chose high optimal

resolution). Extra caution must be paid on masking poorly resolved by well resolved peak

pairs. Therefore, all resolution factors higher than defined optimal value should be

levelled to the optimal value before function calculation.

On the other hand, functions D_{CRF} and N_{CRF} estimate separation by θ criterion. This

criterion is suitable for both Gaussian and non-Gaussian peaks estimation. Also, it is

influenced by peak tailings and can indicate peak asymmetry. Its valuable advantage,

comparing to the resolution factor, is the possibility to measure baseline separation. The

contribution of each well resolved pair is always 1, so there is no masking of poorly

resolved peaks when summing θ criterion. Also, sum of θ is influenced by the total number of peaks appeared on chromatogram (Jancic–Stojanovic 2011).

As far as the time term is concerned, in functions B_{CRF}, COF, Do_{CRF}, CEF and N_{CRF} it is constructed to measure the deviation from the chosen optimal total elution time. CRS defines the time term only by the value of the experimentally obtained total run duration, which can lead to the overestimation of time term above the resolution term (Morris 1996). D_{CRF} on the other hand measures time term as the deviation of the obtained total run time from the time of column void volume appeared on chromatogram. This approach could lead to the underestimation of time term comparing to the resolution term (Jancic–Stojanovic 2011).

Despite the listed numerous difficulties in accurate design of resolution and time term within CRFs, probably the most challenging task present setting the adequate balance between resolution and time term. Namely, the majority of functions include in their construction weighting factors that should be selected by an analyst according to his expectations from the particular method. However, not all the functions are easy for balancing. For example, functions estimating the quality of separation by exponential function lead to inevitable overweighting of separation term when the quality of separation deteriorate.

Evaluation Of The Investigated Functions On Simulated Chromatograms

The selected functions are simultaneously analyzed on six simulated chromatograms presented in Figure 1.

Chromatograms are denoted by numbers from 1 to 6. They are selected to possess different separation characteristics as well as different total elution times. From Figure 1 it can be noted that chromatograms 2, 3 and 6 have baseline separation of all adjacent peaks, and total run time 7.9, 13.8 and 7.9, respectively. Chromatograms 2 and 6 differ by peaks distribution, the separation between peak pairs is satisfactory in each case, but the difference in retention times is greater in case of chromatogram 2. Chromatogram 1 has first peak pair partially overlapped and total elution time 11 minutes, while chromatograms 4 and 5 have several overlapping peak pairs and total duration of 10 and 7 minutes, respectively. If the goal of analysis is defined as achieving the baseline separation in minimal analysis duration the chromatograms order by decreasing quality would be ranked as: 2=6>3>1>5>4.

The important chromatographic parameters for each chromatogram are summarized in Table 2.

The adjustable parameters included in the investigated functions are set so that the greater emphasize is put on separation term than on the time term. Therefore, the following constants are defined for the functions: B_{CRF} (T_A = 10 min, T_1 = 3 min); COF (R_{id} = 1.5, t_m = 10 min, A = 3, B = 1); DoCRF ($t_{R,cri}$ = 10 min, $R_{s,cri}$ = 1.5); CRS (R_{opt} = 1.5, R_{min} =

0.5); CEF (R_{opt} = 1.5, t_{max} = 10 min, a = 3) and N_{CRF} (a = 5, b = 1). Functions are calculated and presented in Table 2, as well.

The functions B_{CRF}, COF and D_{CRF} are mathematically designed so that they increase as the quality of chromatogram increases. On the other hand, functions Do_{CRF}, CRS, CEF and N_{CRF} reach the minimum as the optimal chromatogram is approached. Therefore, analyzing Table 2 it can be seen that B_{CRF} found the following order of chromatograms starting from the best one: 1>4>3>2>5>6, COF ranked them as 2>1>3>5>4>6, Do_{CRF} presented the order 2>3>1>5>6>4, CRS ranked them as 6>2>1>3>4>5, CEF's order was 6>2>1>3>4>5, D_{CRF} presented the order: 2=6>3>1>5>4 and finally N_{CRF} ranked them as 2=6>3>1>5>4.

As far as B_{CRF} is concerned, its main drawback (already described in literature [8, 16]) is the estimation of separation quality by summing the resolution factors. This approach leads to the masking of poorly resolved peaks by high values of resolution factor of well resolved ones. Therefore, this function found chromatograms 1 and 4 to be the best although they contain overlapping peaks. On the other hand, chromatogram 6 is found to be the worst, although all peak pairs are well resolved and resolution factors are above 1.5.

The function COF presented slightly better order of chromatograms than B_{CRF} (chromatogram 2 is identified as the best one). This function measures the quality of separation summing the logarithm of obtained resolution divided by optimal resolution

for all peak pairs. Although the influence of well resolved peaks is less intense then in B_{CRF} calculation, it is still present. For example, it can be seen that this function found chromatogram 5 (with three overlapping peaks and total elution time of 7 minutes) to be better than chromatogram 6 (containing baseline separation of all the peaks and slightly longer total run time). Additionally, COF suffers from the lack of mathematical correction: when the resolution factor is equal to zero, function tends to minus infinity. Also, function is not influenced by missing peaks on chromatogram, on the contrary, it improves when the peaks are completely overlapped (Cela 1989).

Do_{CRF} contains exponential function for separation assessment. Thus, chromatograms with poorly resolved peak pairs are penalized stronger than in the previous functions. It can be seen that chromatograms 2 and 3 are characterized as the best ones, while chromatograms 1, 4 and 5 have unsatisfactory function's values which is in accordance with separation quality of these chromatograms. However, chromatogram 6 is not estimated well again.

Functions CRS and CEF gave identical estimation of chromatograms. It is interesting that they found chromatogram 6 to be the best one (better than chromatogram 2 which has the same total elution time, and achieved baseline separation, as well). This happened due to mathematical construction of their separation terms which identifies good separation only in cases where resolution factor is equal to the chosen optimal resolution factor. Therefore, not only chromatograms with low values of resolution factors (chromatogram 5), but also those with high values of resolution factor (chromatogram 2) are penalized.

Morris emphasized that the problem with CRS function could be overweighting of time factor (Morris 1996), as well as the fact that the function is not defined when the obtained resolution is equal to the chosen minimal resolution.

Finally, the two remaining functions, D_{CRF} and N_{CRF} gave identical estimation of chromatograms quality. Their judgement seems to be the most accurate taking into consideration the defined goals at the beginning of investigation (separation is chosen as the goal with higher priority). Namely, these two functions were the only ones which recognized that baseline separation was achieved in both chromatogram 2 and 6 and selected these chromatograms as the best ones. Further on, chromatogram 3 with satisfactory separation and prolonged total elution time obtained the next best functions value. At the end, chromatograms 1, 4 and 5 are penalized due to poor separation quality. However, it should be noticed that D_{CRF} is poorly influenced by time factor. It can be seen that D_{CRF} value for chromatogram 2 is 8.05 and for chromatogram 3 it is 8.03 although they differ in total elution time for approximately 6 minutes.

Evaluation Of The Investigated Functions On Experimentally Obtained Data

The investigated functions are further on examined on real chromatograms obtained in HILIC mode. As a model mixture for chromatographic separation five beta agonists and antagonists are selected. There are some papers in the literature dealing with analysis of selected group of substances in HILIC (Quiming 2007, Quiming 2007, Quiming. 2008). Chromatographic system consisted of Betasil Silica-100 (100 mm x 4.6 mm, 5 μm particle size) column and acetonitrile: water solution of ammonium acetate (with pH

adjusted by glacial acetic acid) mobile phase. In the preliminary experiments three factors related to the mobile phase composition (acetonitrile content in the mobile phase, pH of the water phase and concentration of buffer in the water phase) showed significant impact on mixture's retention behaviour. The influence of these factors on the overall quality of separation was further on examined with the aid of experimental design while the quality of the obtained chromatograms was measured by seven investigated chromatographic response functions.

Experimental scheme, constructed according to the central composite design with four central points replications, is presented in Table 1 as well as the intervals within the factors were varied. Eighteen chromatograms are obtained. Important chromatographic parameters (Rs, θ, total run time) are assessed and presented in Table 3.

The experimentally obtained chromatograms varied in their separation quality as well as in the length of total run duration. We investigated the ability of seven selected chromatographic response functions to rank these chromatograms according to the following: the first goal is the baseline separation between adjacent peaks, and this is the goal of the highest priority, and the second goal is total elution time within 10 minutes.

The values of seven investigated functions are calculated and presented in Table 3. Unlike on the example of simulated chromatograms, the best chromatogram (achieved perfect separation within minimal total run time, Figure 2A, run 12) and the worst chromatogram (poor separation between all adjacent peaks or extremely prolonged total

elution time, Figure 2B, run 10) identified by different functions mostly match. However, the order of chromatograms between these extreme cases differs significantly. This is particularly important if CRF is selected as a response to be modelled during the optimization procedure. If CRF is not able to accurately estimate each obtained chromatogram than the response surface will be incorrect and would lead to the identification of wrong optimum.

Analysing the ranking of experimentally obtained chromatograms in Table 3 presented by investigated functions it can be noticed that the shortcomings of each function are similar to the ones spotted on simulated chromatograms. Further on, a brief discussion on some particular examples will be presented.

As far as function B_{CRF} is concerned, it can be seen that this function was predominantly affected by summing of a resolution factor. Namely, chromatograms 6 and 12 both achieved baseline separation between all adjacent peaks, and chromatogram 6 has approximately 2 minutes shorter total elution time. Yet, B_{CRF} found chromatogram 12 to be better (B_{CRF} for chromatogram 12 is 11.76, and for chromatogram 6 it is 9.81). Even more puzzling is the fact that chromatogram 11 is found to be better than chromatogram 7 although the latter one has better separation characteristics and shorter total elution time. This occurred due to masking of poorly resolved peak pairs by high value of resolution factor of well resolved ones. COF was similarly affected by resolution factor like B_{CRF}. On the other hand, COF provided better assessment of chromatograms avoiding masking of poorly resolved peak pairs (COF for chromatogram 11 is -0.84 and

for chromatogram 7 it is 0.38). Function Do_{CRF} showed somewhat low sensitivity to the

differences in separation characteristics if we compare chromatogram 1 (Do_{CRF} = 2.60

and $\theta_{1/2}$ = 0) and chromatogram 18 (Do_{CRF} = 2.63 and $\theta_{1/2}$ = 0.98). This may be due to

poor balance obtained between separation and time term in this function. Both functions

CRS and CEF exhibited ranking disorder since they estimated good separation only in

cases where resolution factor is equal to the chosen optimal resolution factor. Therefore

we can see that they found chromatogram 4 (baseline separation achieved and total

elution time 28.91 minutes) to be better than chromatogram 3 (baseline separation

achieved as well, and total elution time 8.36 minutes). Functions D_{CRF} and N_{CRF} were the

only ones that measured the baseline separation since their separation term is a function

of θ criterion. Therefore these functions presented the chromatograms order that mostly

corresponded to the defined goals. Additionally, since N_{CRF} allows adjustment of

weighting factors for separation and time term (unlike D_{CRF}) another variation of these

function is presented in Table 3 ($N_{CRF}2$) where the weighting factor b was defined as: if t_f

< 10 then b = 0, else b = 1. This variation put stronger stress on separation term while the

total run time is within 10 minutes, thus excludes the possibility that extremely short run

time will mask the poor peaks separation.

The presented results indicated that chromatographic response functions can be used for

chromatograms evaluation and consequent optimization strategy in hydrophilic

interaction liquid chromatography. However, the potential advantages and drawbacks of

each function must be considered prior to the selection of the one that will be applied in

the particular case.

CONCLUSIONS

This paper presents the evaluation of seven chromatographic response functions (Berrige's chromatographic response function (B_{CRF}), Glajch's chromatographic optimization function (COF), Dose's CRF (Do_{CRF}), Schlabach chromatographic resolution statistics (CRS), Morris' chromatographic exponential function (CEF), Duarte's chromatographic response function (D_{CRF}) and new chromatographic response function (N_{CRF}) developed by the authors of this paper, on simulated and experimentally obtained chromatograms in HILIC mode. The investigated functions appeared to give significantly different estimation of both simulated and real chromatograms. The main restrictions of functions are demonstrated and discussed. The functions which estimate separation by θ criterion (D_{CRF} and N_{CRF}) showed better ranking ability than the ones estimating separation by resolution factor. Furthermore, the functions in which the balance between separation and time term is easy to adjust are preferable. Although chromatographic response functions are valuable assistance in optimization strategies in HILIC mode, special attention on CRF selection must be paid, since they are not generally applicable in all the optimization procedures and may exhibit incorrect results.

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Table 1. Central composite design experimental plan

Run	x_1	x_2	x_3
1	-1^a $(80)^b$	-1 (3.5)	-1 (20)
2	1 (90)	-1 (3.5)	-1 (20)
3	-1 (80)	1 (5.5)	-1 (20)
4	1 (90)	1 (5.5)	-1 (20)
5	-1 (80)	-1 (3.5)	1 (60)
6	1 (90)	-1 (3.5)	1 (60)
7	-1 (80)	1(5.5)	1 (60)
8	1 (90)	1 (5.5)	1 (60)
9	-1.68 (76.6)	0 (4.5)	0(40)
10	1.68 (93.4)	0 (4.5)	0 (40)
11	0 (90)	-1.68 (2.82)	0 (40)
12	0 (85)	1.68 (6.18)	0 (40)
13	0 (85)	0 (4.5)	-1.68 (6.36)
14	0 (85)	0 (4.5)	1.68 (73.64)
15	0 (85)	0 (4.5)	0 (40)
16	0 (85)	0 (4.5)	0 (40)
17	0 (85)	0 (4.5)	0 (40)
18	0 (85)	0 (4.5)	0 (40)

x_1 - acetonitrile content in the mobile phase (vol %); x_2 – pH of the mobile phase; x_3 –

concentration of ammonium acetate in the water phase (mM);

^{a} – coded factor levels; ^{b} – real factor levels

Table 2. The important chromatographic parametres of the simulated chromatograms and the values of seven investigated functions

Run	$\theta_{1/2}$	$\theta_{2/3}$	$\theta_{3/4}$	$\theta_{4/5}$	$Rs_{1/2}$	$Rs_{2/3}$	$Rs_{3/4}$	$Rs_{4/5}$	t_1	t_f	B_{CRF}	COF	Do_{CRF}	C_{RS}	CEF	D_{CRF}	N_{CRF}
1	0.89	1	1	1	1.26	10.39	11.24	5.28	2	11	31.18	14.11	1.56	3.68	10.62	7.92	2.40
2		1	1	1	6.39	4.92	2.94	5.72	2.4	7.9	22.27	16.04	1.00	2.42	8.90	8.05	1.79
3		1	1	1	5.36	11.5	5.14	5.06	2.7	13.8	27.95	13.47	1.47	4.40	11.90	8.03	2.38
4	0.36	1	0.61	1	0.79	13.61	0.96	9.83	2.1	10	29.29	8.97	2.12	22.56	152.14	6.02	4.56
5	1	0.76	0.15	1	11.8	1.07	0.67	4.93	2.3	7	19.7	9.36	1.86	50.31	218.86	6.97	4.02
6	1	1	1	1	1.86	1.68	1.76	1.78	5.2	7.9	7.79	4.09	2.02	1.75	4.00	8.05	1.79

$\theta_{s.1}$: resolution criterion of adjacent peaks calculated by Eq. 7; t_1 – retention time of the first eluting peak; t_f – retention time of the last eluting peak; $Rs_{s.1}$ – resolution factor between adjacent peaks; B_{CRF} - Berrige's chromatographic response function, COF - Glajch's chromatographic optimization function; CRS - Schlabach chromatographic resolution statistics, Do_{CRF} - Dose's CRF; CEF - Morris' chromatographic exponential function, D_{CRF} - Duarte's chromatographic response function and N_{CRF} - new chromatographic response function developed by the authors of this paper

Table 3. The important chromatographic paramtres of the experimentally obtained chromatograms and the values of seven investigated functions
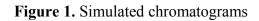
Run	$\theta_{1/2}$	$\theta_{2/3}$	$\theta_{3/4}$	$\theta_{4/5}$	$RS_{1/2}$	$RS_{2/3}$	$RS_{3/4}$	$RS_{4/5}$	t_1	t_f	$B_{C\,RF}$	COF	Do_{CRF}	CRS	CEF	D_{CRF}	N_{CRF}	$N_{C\,RF2}$
1	0.10	1	1	1	0.17	1.24	1.02	2.03	2.92	4.87	3.25	−2.16	2.60	24.73	4141.06	6.31	3.35	3.50
2	1	1	1	1	1.05	1.82	1.65	3.54	6.84	17.61	1.60	−5.24	2.98	4.96	28.82	8.08	2.76	2.76
3	1	1	1	1	0.98	1.46	1.59	2.18	3.19	8.36	9.40	1.59	2.31	8.34	29.50	8.18	1.84	2.00
4	1	1	1	1	3.47	2.51	2.62	4.18	7.07	28.91	−5.20	−10.10	3.41	6.71	18.81	8.05	3.89	3.89
5	0.09	1	1	1	0.17	0.98	0.97	1.47	2.31	3.85	0.75	−2.92	2.69	19.72	3816.19	6.48	2.96	3.37
6	1	1	1	1	0.57	1.45	1.51	2.94	4.03	9.35	9.81	−0.28	2.50	139.69	450.94	8.16	1.93	2.00

7	0.94	1	1	1	0.42	1.03	1.63	1.71	2.44	5.29	4.53	0.38	2.45	112.22	947.88	8.22	1.65	2.09
8		1	1	1	2.32	2.31	3.19	4.25	5.28	22.45	2.34	−4.45	2.85	5.28	15.11	8.07	3.25	3.24
9	0.18	1	1	1	0.17	1.04	0.91	1.60	2.31	3.87	1.91	−2.70	2.66	19.92	3826.35	7.56	2.81	3.23
10		1	1	1	1.72	2.12	2.38	3.17	14.03	59.21	−45.84	−44.11	6.80	13.34	26.33	8.02	6.92	6.92
11	0.78	1	1	1	0.34	1.08	1.02	2.18	3.01	5.35	4.96	−0.84	2.56	42.33	1572.19	8.06	1.95	2.33
12		1	1	1	1.69	2.08	1.87	2.82	3.67	11.03	11.76	2.86	2.12	2.43	6.91	8.13	2.1	2.10
13	0.90	1	1	1	0.38	1.37	1.25	2.45	6.60	13.06	3.80	−6.52	3.11	153.63	1785.93	8.01	2.59	2.59
14	0.6	1	1	1	0.2	1.0	1.2	1.8	2.67	5.41	4.58	−1.39	2.58	29.62	2446.1	7.9	2.2	2.54

	4				6	9	9	4								3	2	3	
15					0.60	1.61	1.71	2.64	3.41	7.59	8.74	1.99	2.26	46.68	330.53	8.20	1.76	2.00
	1	1	1	1														
16					0.59	1.47	1.42	2.30	3.41	7.54	7.90	0.70	2.41	70.43	368.02	8.20	1.75	2.00
	1	1	1	1														
17					0.59	1.29	1.38	2.15	3.33	7.21	7.29	0.39	2.45	60.69	353.07	8.21	1.72	2.00
	1	1	1	1														
18	0.98				0.63	1.08	1.11	2.49	3.51	8.16	7.95	−1.13	2.63	31.09	311.09	8.17	1.85	2.02
	1	1	1	1														

* the meaning of symbols are in given in the legend of Table 2

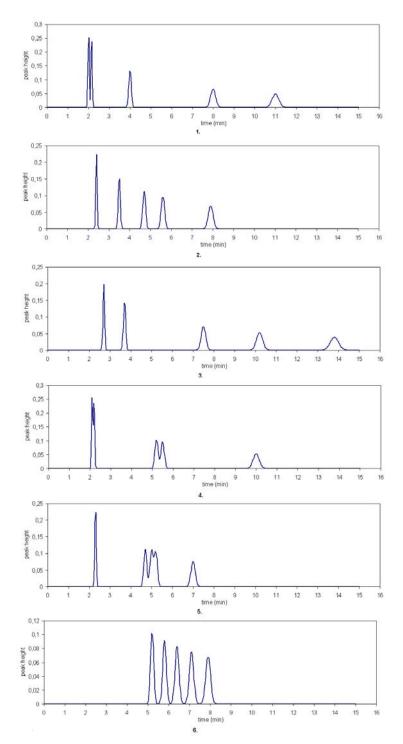**Figure 1.** Simulated chromatograms

Figure 2. Experimentally obtained chromatograms: A) the best chromatogram

(acetonitrile concentration = 85%, pH = 6.18, ammonium acetate concentration in water

phase = 40 mM); B) the worst chromatogram (acetonitrile concentration = 93.4%, pH =

4.5, ammonium acetate concentration in water phase = 40 mM)