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Advancement in optimization tactic achieved by newly developed chromatographic response function: Application to LC separation of raloxifene and its impurities

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ABSTRACT

In this paper a new chromatographic response function (CRF) is designed and proposed for utilization in the optimization strategies. The function capability to represent the overall quality of a experimentally obtained chromatograms was compared to the other two objective functions and proved to give more accurate and reliable results. The new CRF has improved concept of separation and time term estimation. It reflects all important defects of the chromatogram such as the appearance of asymmetrical or overlapping peaks and prolonged elution time and allows the appropriate weighting of each of them. The LC separation of raloxifene and its four impurities was evaluated through the central composite design experimental plan choosing the new CRF to be the only output of the system. The function demonstrated the ability to judge the impact of the complex interactions of the selected chromatographic parameters (acetonitrile content in the mobile phase, sodium dodecyl sulfate concentration in the water phase, pH of the mobile phase and column temperature) on the mixture behavior and led to the determination of the optimal separation conditions. The newly developed CRF proved to have the advanced performances and it presents the important step forward in the optimization of the chromatographic separation.

1. Introduction

Looking for the separation strategy in liquid chromatography (LC) an analyst can develop many different approaches, but the crucial aim is always the same – to obtain the best possible chromatogram. When it comes to the complex mixtures, set of chromatograms obtained by changing the chromatographic conditions is very complicated to evaluate since it is usually impossible to have all perfect performances (overall resolution, minimum analysis time, uniformed separation, etc.) at the same time. In fact, modifying the experimental conditions so that the better resolution is accomplished, the elution time is often prolonged, and vice versa. Additionally, slowing down the elution of structurally similar mixture compounds can provide better separation, but on the other hand can lead to peak deformation. The researcher is then facing the problem what is the prior criterion based on which the final optimum should be selected. Not even the great scientific experience is sufficient for making an objective conclusion and certain kind of mathematical solution for the problem is necessary. The objective functions enable the ranking of the chromatograms according to the numerical value calculated for each of them. That value represents overall estimation of the chromatographic separation and because of that, the function should be designed to consider all important quality criteria.

The use of many different objective functions in optimization process has been suggested up to now. Berridge [1] proposed the chromatographic response function (CRF), Schlabach and Excoffier [2] introduced the chromatographic resolution statistic (CRS), Morris et al. [3] recommended the chromatographic exponential function (CEF) and many other CRFs were also designed or applied [4–11], as well as some multicriteria decision – making functions [12–14]. The main reason for developing such a large number of objective functions is the fact that each of them has some shortcomings and can not be applied in every single optimization process.

This study presents a new chromatographic response function that is formulated here, in this paper, for the first time in order to overcome the defects of the previous functions appeared to have, and to provide correct determination of the global optimal conditions in different optimization procedures. The validity of the function was tested optimizing the LC separation of the new drug raloxifene hydrochloride ([6-hydroxy-2-(4-hydroxyphenil)benzo[b]thien-3-yl][4[2-(1-piperidinyl)etoxy]phenil]methanone HCl) and its four structurally related impurities: 2-(4-hydroxyphenyl)-1-benzotiophene-6-ol

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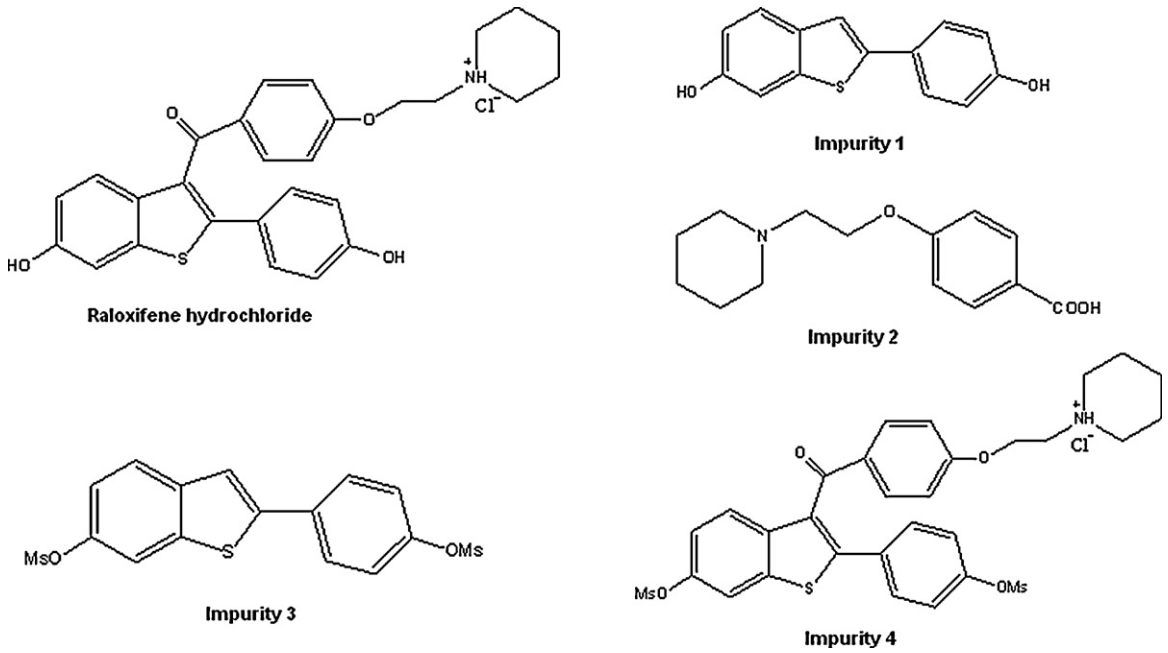

Fig. 1. Chemical structure of raloxifene and its impurities: (A) raloxifene hydrochloride; (B) raloxifene mesylate; (C) piperidyl ethoxy benzoic acid; (D) dimesyl benzotiophene and (E) 2-(4-hydroxyphenyl)-1-benzotiophene-6-ol.

(impurity 1), piperidyl ethoxy benzoic acid (impurity 2), dimesyl benzotiophene (impurity 3), raloxifene mesylate (impurity 4) which are presented in the Fig. 1.

Up to now there have been developed only few methods for analysis of raloxifene hydrochloride – spectrophotometric determination in pharmaceutical formulations [15–20], LC determination in tablets [21–23], LC–MS–MS determination in human urine [24], LC determination in rat plasma [25] and rat tissue [26]. Also, glucuronide metabolites of raloxifene from biological simples were investigated by LC–MS–MS [27,28]. However, there are no paper dealing with the investigation of the raloxifene impurities, so as far as the authors know, this is the first study treating that separation problem.

The study commenced with selection of the most appropriate experimental design for experimental plan definition. The obtained chromatograms were estimated by a new CRF[1] (marked as N_{CRF}) but also by previously published, Morris's CEF and Duarte's CRF[2] (in this paper denoted as D_{CRF}). These two functions were selected since their shortages had not been emphasized yet. This is the first time that the simultaneous comparison of the functions effectiveness was done on the experimentally obtained chromatograms, while the authors in the previous papers [3,11] evaluated their function advantages on the simulated chromatograms. Finally, the chosen experimental design with the N_{CRF} as the only output let us judge the influence of the selected factors on the separation process and determination of the global optimum.

2. A new chromatographic response function

The traditional optimization strategies usually opted for one basic output whose value was then used to find the global optimal conditions. The most common choice was the resolution between adjacent peaks. However, a separation method developed for practical use has to consider the total elution time as a factor that has direct impact on the expenses of such procedure. This implies that improving the resolution, and decreasing the elution time are crucial goals that should be achieved and incorporated in the objective function. But the challenging task is to design the function correctly, so that it really can represent entire quality of the chromatogram.

The very first CRF was defined by Berridge [1] as:

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

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 factors whose values are selected depending on the accent the analyst wants to give them. This function failed to give correct results, as some authors have already noticed [3,11], because of the inadequacy of the resolution term which is only defined by a well resolved peak pairs.

Furthermore, Morris [3] offered improved version of objective function called CEF:

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

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 expected peaks. The lowest obtained CEF signifies the optimum. The problem with the CEF is that it is difficult to adjust the balance between the influence of the resolution and the time term on the function since the resolution term is defined by exponential function. Namely, exponential function will increase roughly as the $(R_{opt} - R_i)$ increases, comparing to the increasing of the linear function (by which the time term is defined) when the t_f/t_{max} increases.

[1] Our new CRF function is marked as N_{CRF}.
[2] Duarte's CRF is marked as D_{CRF}.

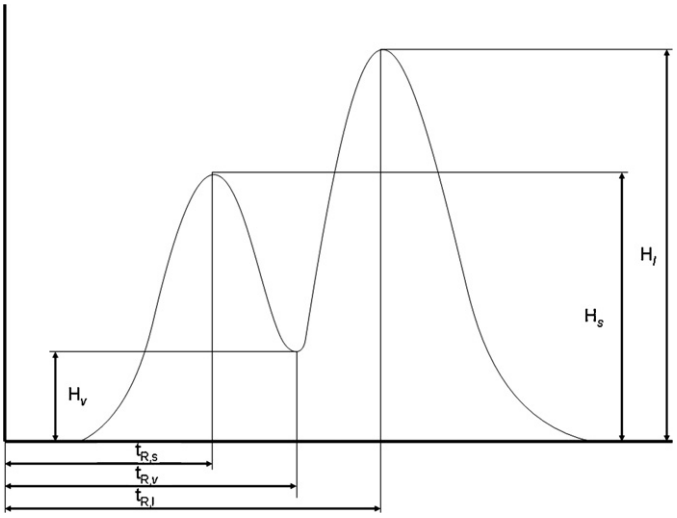

Fig. 2. Chromatogram scheme illustrating the parameters in Carle's θ criterion calculation [29].

Whereby, the separation is not always estimated correctly when the resolution term is the function of the R value:

$$R = \frac{\Delta t_R}{(1/2)(w_1 + w_2)} \tag{3}$$

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. Although R is a universal separation parameter, it can only be successfully applied for Gaussian shaped peaks. Unfortunately, the peak shape is often far more complex as it happened in our study. Then, evaluation of the separation of adjacent peaks by calculating R has serious drawbacks since the determination of the peak width demands the construction of the tangents and small variations in the slope implies large variations in the width. Additionally, the medium peak tailing does not affect the R, although it deteriorates the separation.

In order to overcome the problem of R in the resolution term of the objective function, Duarte [11] improved the CRF function. He started from Carle's [29] formula for the evaluation of the peak separation:

$$\theta_{s,l} = 1 - \left(\frac{H_v \times \left| t_{R,l} - t_{R,s} \right|}{\left(\left| t_{R,v} - t_{R,s} \right| \times (H_l - H_s) + H_s \times \left| t_{R,l} - t_{R,s} \right| \right)} \right) \tag{4}$$

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 it is presented in the Fig. 2.

The final D_{CRF} is:

$$D_{\mathrm{CRF}} = \sum_{i=1}^{N-1} \theta_{s,l} + N - \left(\frac{t_{R,L} - t_0}{t_{R,L}} \right) \tag{5}$$

where $t_{R,l}$ is the elution time of the last peak, t_0 is the column void volume, and N is a peak number. The D_{CRF} reaches the maximum as the optimum is approached.

The main problem with D_{CRF} is that the resolution and time term are not adequately weighted. Namely, in the function designed by this formula, the influence of time is almost insignificant: when the resolution term is constant, and the total elution time changes, the differences between the D_{CRF} values are so small that we are in a serious risk to find the optimum in the region where the elution time is unacceptably long.

On the basis of already published functions, this paper introduces a new CRF that combines the advantages and overcomes the shortcomings of previously developed objective functions.

The N_{CRF} is formulated as:

$$N_{\mathrm{CRF}} = \left(a \left(1 - \frac{\sum_{i=1}^{N-1} \theta_{s,l}}{N} \right) + 1 \right) \left(1 + \left(\frac{t_f}{t_{\mathrm{opt}}} \right)^b \right) \tag{6}$$

where $\theta_{s,l}$ is the resolution criterion estimated by Eq. (4), 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 and 5 and b between 0 and 5). The N_{CRF} reaches the minimum as the global optimum is approached. The expectations from the N_{CRF} designed in this way are to evaluate both resolution and time impact adequately and to create appropriate balance between them. Estimating the separation by θ criterion, it should be able to evaluate the non Gaussian peaks, and to considerate possible peak asymmetry and tailings. The θ criterion has values in a range from 0 (for completely overlapped peaks) to 1 (for perfectly separated peaks), so each peak pair that is well separated will have the contribution 1 to the sum of θ and there will be no masking of poorly resolved peaks by high values of the resolution factor of the well resolved peaks. When the separation of all adjacent peaks is good enough, the sum of θ values is equal to N and the fraction of the resolution term in the parentheses is zero. That is why the addition of 1 in the resolution term was included - to ensure that the function is still defined by the value of the time term in the case of the perfect separation. The time term is a function of the chosen overall optimal elution time for the specific analysis. Additionally, coefficients a and b allow us exact weighting of the resolution term and the time term. That means that if the main goal is to obtain the chromatogram with perfect resolution and the time factor is not so important for the particular study, the analyst should opt for high value for a and small value for b. On the other hand, if one wants to emphasize the deviation of t_f from t_{opt}, the b factor should be increased.

3. Experimental

3.1. Chemicals

All used reagents were of an analytical grade. The mobile phase and the solvents were prepared of acetonitrile (Lab Scan, Ireland), orthophosphoric acid (Carlo Erba, Italy), sodium dodecyl sulfate – SDS (Sigma–Aldrich Chemie, GmbH, Germany) and HPLC grade water. Working standards of raloxifene hydrochloride and impurities were kindly donated from Solmag S.P.A (Italy, Milan).

3.2. Standard solutions

Stock solutions of raloxifene and the impurities were prepared by dissolving them into the acetonitrile–water phase (5 mM SDS, pH 2.8) mixture (45:55,v/v) to obtain the concentration of 100 μg mL^{-1} for raloxifene and 5 μg mL^{-1} for all impurities. The prepared stock solutions were stored at 4 °C.

3.3. Mobile phase

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

3.4. Chromatographic conditions

The experiments were performed on the chromatographic system Finnigan Surveyor Thermo Scientific consisted of HPLC Pump, Autosampler Plus and UV/VIS Plus Detector. ChromQuest was used for data collection. The analytical column was XBridge™, 100 mm × 3.0 mm, 3.5 μm particle size column. Flow rate was

Table 1
The CCD experimental plan.

Experiment	ACN	pH	SDS	*T*
1	43 (−1)[a]	2.5 (−1)	4.0 (−1)	25 (−1)
2	47 (+1)	2.5 (−1)	4.0 (−1)	25 (−1)
3	43 (−1)	3.5 (+1)	4.0 (−1)	25 (−1)
4	47 (+1)	3.5 (+1)	4.0 (−1)	25 (−1)
5	43 (−1)	2.5 (−1)	6.0 (+1)	25 (−1)
6	47 (+1)	2.5 (−1)	6.0 (+1)	25 (−1)
7	43 (−1)	3.5 (+1)	6.0 (+1)	25 (−1)
8	47 (+1)	3.5 (+1)	6.0 (+1)	25 (−1)
9	43 (−1)	2.5 (−1)	4.0 (−1)	35 (+1)
10	47 (+1)	2.5 (−1)	4.0 (−1)	35 (+1)
11	43 (−1)	3.5 (+1)	4.0 (−1)	35 (+1)
12	47 (+1)	3.5 (+1)	4.0 (−1)	35 (+1)
13	43 (−1)	2.5 (−1)	6.0 (+1)	35 (+1)
14	47 (+1)	2.5 (−1)	6.0 (+1)	35 (+1)
15	43 (−1)	3.5 (+1)	6.0 (+1)	35 (+1)
16	47 (+1)	3.5 (+1)	6.0 (+1)	35 (+1)
17	41 (−2)	3.0 (0)	5.0 (0)	30 (0)
18	49 (2)	3.0 (0)	5.0 (0)	30 (0)
19	45 (0)	2.0 (−2)	5.0 (0)	30 (0)
20	45 (0)	4.0 (+2)	5.0 (0)	30 (0)
21	45 (0)	3.0 (0)	3.7 (−2)	30 (0)
22	45 (0)	3.0 (0)	7.0 (2)	30 (0)
23	45 (0)	3.0 (0)	5.0 (0)	20 (−2)
24	45 (0)	3.0 (0)	5.0 (0)	40 (2)
25	45 (0)	3.0 (0)	5.0 (0)	30 (0)
26	45 (0)	3.0 (0)	5.0 (0)	30 (0)
27	45 (0)	3.0 (0)	5.0 (0)	30 (0)
28	45 (0)	3.0 (0)	5.0 (0)	30 (0)
29	45 (0)	3.0 (0)	5.0 (0)	30 (0)
30	45 (0)	3.0 (0)	5.0 (0)	30 (0)

ACN, acetonitrile content in the mobile phase (%); pH, pH value of the mobile phase; SDS, molar concentration of sodium dodecyl sulfate in the water phase (mM); *T*, column temperature (°C).

[a] Coded values for the factor levels are given in the parentheses.

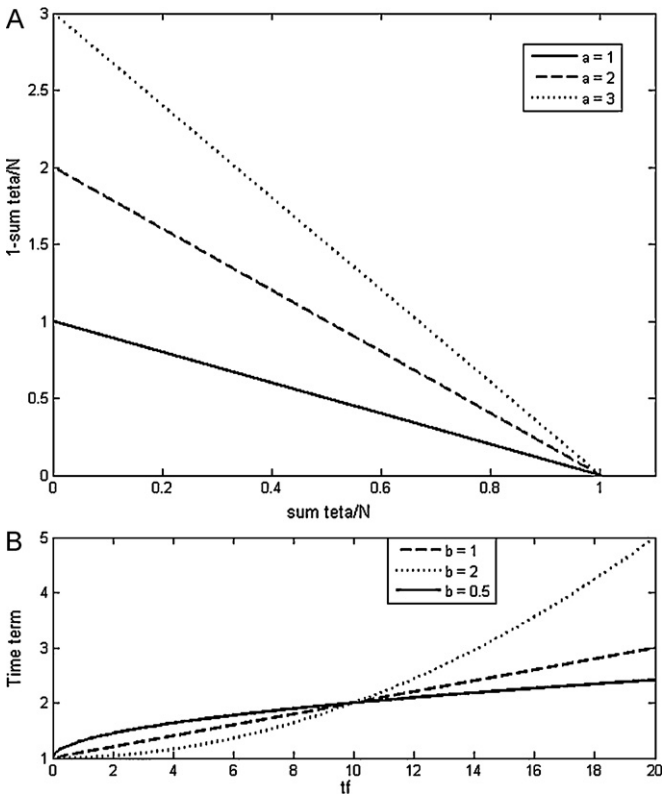

Fig. 3. (A) The influence of the coefficient *a* on the resolution term of the N_{CRF}; (B) the influence of the coefficient *b* on the time term of the N_{CRF} (t_{opt} = 10 min).

1 mL min^{-1} and column temperature was adjusted according to the experimental plan given in the Table 1. UV detection was carried out at 254 nm.

3.5. Software

The central composite design experimental plan was done by Design-Expert® 7.0.0. (Stat-Ease Inc., Minneapolis). The functions values were calculated in Microsoft Office Excel. Graphical presentations of the functions were prepared using MATLAB 7. 10. 0. Three-dimensional graphs were obtained by STATISTICA 7.

4. Results and discussion

In the pharmaceutical analysis, method development usually demands careful approach and application of the most suitable strategy. The development procedure depends on the method demands (whether it is designed for the evaluation of the active substances only, for the active substance and the impurities, for the stability study etc.), the nature of the investigated substance, the laboratory type and the analyst experience. It is always desirable to create a method which could be applicable in many different areas of substance analysis. Naturally, the most complex methods are used for the impurities profile evaluation since the content of the impurities in the analyzed sample is very low and the requirements for those methods are very strict. So, during the development phase of such method as many as possible situations must be considered. Careful preliminary study and method optimization could provide some useful data.

Taking into account the chemical structures of raloxifene hydrochloride and its impurities (Fig. 1), the set of experiments was conducted. During that phase, acetonitrile and methanol content was varied as well as the composition and pH of the water phase. The most appropriate results were obtained with acetonitrile in mobile phase and ion-pairing reagent in the water phase. The most acceptable chromatograms were obtained with SDS as ion-pair reagent. So, as the best starting point for optimization, factors related to the mobile phase (acetonitrile content, SDS content in the water phase, pH of the mobile phase) and the column temperature as the forth important factor were recognized. In the next step, central composite design (CCD) was chosen. The CCD was utilized for creating the experimental plan since it can estimate all parameters in a chosen model. The Table 1 presents factors and their levels for the full factorial design and the star design, as well as six central point replications that were added to the design. The runs were carried out randomized, and thirty chromatograms were obtained.

The next step was to define the global optimization strategy. Due to the complexity of this particular mixture presented with five structurally similar compounds it was decided to put strong accent on the separation between adjacent peaks and the run time of 10 min was chosen as the optimal elution time. Therefore, the plan was to find the chromatogram with the best value of the overall separation criterion while the run time is up to 10 min. When the elution time crosses the optimal value, the time factor has to be taken into consideration and it would decrease the quality of the chromatogram.

The newly developed function N_{CRF} (explained in Section 1 of the paper) is flexible and allows the analyst to achieve the desired optimal conditions. The coefficient *a* let one to make the exact accent on the resolution term of the function as it can be seen at the Fig. 3 A. The greater value for *a* is chosen, the greater slope of the curve is achieved, meaning that the changes in the resolution will have the stronger influence on the functions value. In this particular case it

Table 2
Resolution criterions, total elution time and objective functions values.

Run	$\theta_{1.2}$	$\theta_{2.3}$	$\theta_{3.4}$	$\theta_{4.5}$	$R_{1.2}$	$R_{2.3}$	$R_{3.4}$	$R_{4.5}$	t_f	CEF	D_{CRF}	N_{CRF}
1	0.89	1	0.99	1	1.25	10.40	2.39	15.39	12.44	11.47	7.90	2.44
2	0.98	1	1	1	1.06	9.64	4.52	16.62	6.19	18.78	8.03	2.03
3	0.99	1	0	1	2.57	10.27	0.11	18.83	15.76	10397.21	6.01	4.53
4	0.97	1	0.92	1	2.00	7.94	1.15	11.95	7.31	12.27	7.94	2.16
5	0.98	1	0	1	2.67	13.62	0.08	20.22	14.79	12091.01	6.00	4.37
6	0.92	1	0.98	1	1.50	8.96	1.94	13.68	6.89	5.98	7.94	2.15
7	1	1	0.99	1	4.42	17.03	1.95	26.53	16.92	12.25	8.02	2.69
8	1	1	0.85	1	2.71	11.08	1.10	20.04	7.44	16.00	7.89	2.23
9	0.98	1	0.93	1	2.00	11.60	1.42	16.49	10.06	7.37	7.93	2.15
10	1	1	0.99	1	1.87	9.40	2.88	16.65	5.43	6.83	8.04	2.02
11	1	1	0.97	1	4.59	15.67	1.12	24.49	12.06	19.01	7.99	2.26
12	1	1	0	1	2.53	7.78	0.61	13.85	5.92	301.18	6.05	3.50
13	1	1	0.95	1	3.37	13.86	1.05	22.13	12.23	26.64	7.97	2.31
14	0.97	1	0.88	1	1.89	8.61	0.95	12.29	5.58	33.53	7.90	2.22
15	1	1	1	1	4.59	15.00	2.18	25.42	12.34	10.63	8.04	2.23
16	1	1	0	1	2.82	9.64	0.32	19.47	5.99	1796.93	6.05	3.50
17	1	1	0.96	1	3.12	12.52	0.94	16.83	18.79	65.19	7.98	2.96
18	0.98	1	0.96	1	2.12	8.30	2.00	14.81	4.83	6.41	8.01	2.08
19	0	1	1	1	0.30	9.21	3.10	12.31	7.3	2199.23	6.04	3.50
20	1	1	0	1	2.70	14.00	0.07	23.13	9.54	10336.70	6.04	3.50
21	0.96	1	1	1	2.00	9.59	2.10	14.74	8.16	7.81	8.00	2.06
22	1	1	0	1	2.50	8.63	1.00	17.06	4.66	23.49	6.06	3.50
23	1	1	0.83	1	2.50	12.94	0.88	15.27	12.28	73.15	7.86	2.50
24	1	1	0	1	2.63	9.04	0.24	16.29	6.97	3071.67	6.04	3.50
25	1	1	0	1	2.84	11.59	0.47	18.74	9.59	850.06	6.03	3.50
26	1	1	0	1	3.18	12.56	0.93	22.69	9.2	46.09	6.03	3.50
27	1	1	0	1	2.19	9.19	1.12	15.27	8.56	15.24	6.03	3.50
28	1	1	0	1	2.94	12.32	1.10	21.96	8.99	18.05	6.03	3.50
29	1	1	0	1	2.19	9.19	1.12	15.35	8.56	15.26	6.03	3.50
30	1	1	0	1	2.20	10.13	1.02	15.67	8.74	25.84	6.03	3.50

$\theta_{s,l}$, resolution criterion calculated by Eq. (4); $R_{i,j}$, resolution factor calculated by Eq. (3); t_f retention time of the final peak; CEF: Morris' [3] objective function; D_{CRF}, Duarte's [12] objective function; N_{CRF}, our new objective function.

was decided to triple the influence of the resolution term by setting $a = 3$.

The time term of N_{CRF} is defined first by selecting the t_{opt} (in this paper $t_{opt} = 10$ min) and then by setting the coefficient b whose impact on the function value is presented in Fig. 3B. It can be noticed that increasing the b value the variations of the total run time will have more significant influence on the functions value.

According to the optimization goal, b was defined as: $b = 0$ while $t_f \leq t_{opt}$ else $b = 1$.

The forth step was the evaluation of the chromatograms by N_{CRF}, D_{CRF} [11] and CEF [3] (important constants for CEF were set as $R_{opt} = 1.5$, $a = 3$ and $t_{max} = 10$ min). The calculated θ criterions and R values for each peak pair, the total elution time as well as the obtained results for the three chosen functions are presented in the Table 2.

The CEF and N_{CRF} converge to the minimum as the optimum is reached, while the D_{CRF} converge to the maximum. It is obvious that the ranking of chromatograms by these three functions is significantly different due to their different formulation.

The D_{CRF}, for example, treats the chromatograms 10 and 15 as equally good (D_{CRF} is 8.04 in both cases) although the total elution time for chromatogram 10 is 5.43 min, and for the chromatogram 15 is 12.34 min. On the other hand, the N_{CRF} ranks them considerably different (N_{CRF} is 2.02 and 2.23, respectively). This is because of the inappropriate weighting of the time term in the D_{CRF} which was already explained in Section 1 of the paper. Similarly, D_{CRF} ranks the chromatograms 11 and 17 as almost equivalent while the difference between their elution times is over 6 min. Fig. 4 compares the influence of the time term on the CRF value, while the resolution term is constant, between the D_{CRF} and N_{CRF}.

The further analysis of the Table 2 showed us that the D_{CRF} rashly decreased when the single θ value was equal to zero (the chromatograms 24–30), i.e. when certain peak did not appear on the chromatogram due to the co-elution. This happened because the influence of the lack of the peak on the function value is unnecessarily doubled: by reduction of the sum of θ, and by reduction of the N term value. Namely, the sum of θ already reflects the number of the eluted peaks, since there will be no contribution of the single θ value of the non-existent peak to the sum ($\theta = 0$ when co-elution occurred), so the addition of the N term to the function construction is completely useless.

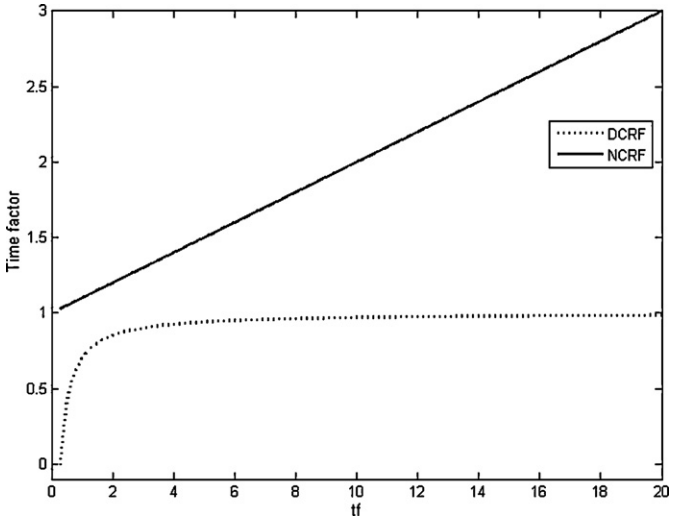

Fig. 4. The influence of the prolonged elution time on the D_{CRF} and N_{CRF} time factor.

Table 2 also shows the differences between CEF and N_{CRF} ranking of the chromatograms which come from the different design of these two functions. First of all, the CEF can get falsely good results of the resolution term for the Non-Gaussian shaped peaks since the evaluation of these peaks separation is not accurate enough when

Table 3
The effects of the experimental factors on the N_{CRF}.

Experimental variable	Effect	*p*-value
ACN	−0.21	0.104
pH	+0.14	0.249
SDS	+0.15	0.239
T	−0.017	0.887
ACN × pH	+0.16	0.293
ACN × SDS	+0.011	0.943
ACN × pH	+0.48	0.005
pH × SDS	−0.26	0.090
pH × *T*	+0.14	0.365
SDS × *T*	+0.003	0.983
ACN × ACN	−0.29	0.019
pH × pH	−0.046	0.685
SDS × SDS	−0.23	0.060
T × *T*	−0.17	0.144

ACN, acetonitrile content in the mobile phase (%); pH, pH value of the mobile phase; SDS, molar concentration of sodium dodecyl sulfate in the water phase (mM); *T*, column temperature (°C).

it is estimated by the *R* value as it was already explained in Section 1 of the paper. This is demonstrated on the example of the chromatogram 6. The *R* value calculated for the first and second peak is 1.58, and for the third and forth peak 1.94 which should suggest optimal separation of both peak pairs. But, these peaks are not perfectly separated as it was demonstrated calculating the resolution criterion θ which was 0.92 for the first peak pair and 0.98 for the second peak pair.

Moreover, the resolution term in the CEF is designed so that it has the best (minimum) value only when the *R* is equal to R_{opt} which was 1.5 in this case. This means that not only the poorly separated peaks but also the peaks with the *R* value bigger than 1.5 have negative influence on the overall quality evaluation. Because of this, the estimation of some chromatograms could be completely wrong as it happened with the chromatograms 6 and 10. It can be seen that chromatogram 10 has lower elution time and higher *R* values for all peak pairs and yet CEF characterizes it as worse one.

Finally, analyzing the N_{CRF} ranking we can notice that the best ranked are the chromatograms 10, 2 and 21. These chromatograms have the best separation criterion values and the total elution time within the defined optimal time. The deterioration of the N_{CRF} among the chromatograms with average separation and total run time over 10 min corresponds to the increase of the total elution time as it can be seen comparing the chromatograms 13 and 17. An excessive increase of the N_{CRF} happened in the cases of the poor separation between the adjacent peaks even though the run time was optimal (chromatograms 12 and 16) which is in the accordance with our desired optimization plan. The worst N_{CRF} values belong to the chromatograms with poor separation and very long elution time such as the chromatogram 3.

The final step of this study was to define the optimal separation conditions for the analyzed mixture. Since the N_{CRF} proved to give accurate and reliable quantitative characterization of the chromatograms, it was selected as the only output to be followed. So, the influence of the selected independent variables i.e. inputs, reflected in the decreasing or increasing of the N_{CRF}. The calculated coefficients of the response model for coded factor levels (the effects) as well as the *p*-values are given in the Table 3.

The acetonitrile content had the greatest influence on the N_{CRF} and this influence was negative, meaning that with the increase of the ACN percentage in the mobile phase, the N_{CRF} approached the minimum. On the other hand, pH value and SDS content in the water phase had similar positive influence on the N_{CRF}, while the influence of the temperature factor was almost insignificant. But when changing these factors simultaneously, their interactions became more complex as we can see at the 3D graphs presented in

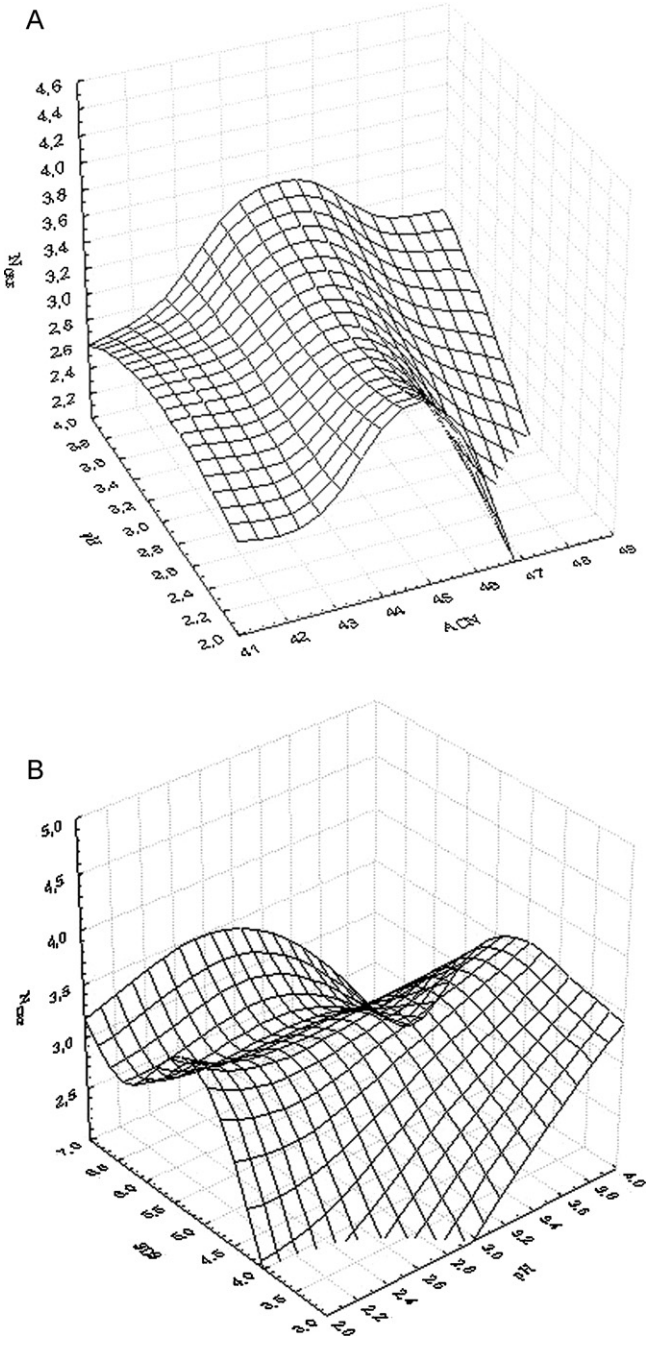

Fig. 5. Three-dimensional graphs: (A) $N_{CRF} = f$ (%ACN, pH) while SDS = 5 mM, $T = 30$ °C; (B) $N_{CRF} = f$ (pH, mM SDS) while ACN = 45%, $T = 30$ °C.

Fig. 5. Namely, it is obvious that the N_{CRF} did not decrease constantly as the ACN content increased, nor while the SDS content and pH value decreased, but it was the overall interaction of these factors which lead to the final shape of the function.

Global optimal conditions were defined: acetonitrile 4 mM SDS (47:53, v/v), pH of the mobile phase adjusted to 2.5, temperature of 35 °C with the $N_{CRF} = 2.02$. The chromatogram of the analyzed mixture under these conditions is presented in the Fig. 6A.

Finally, Fig. 6 also presents the optimal chromatograms according to the D_{CRF} and CEF, respectively, so the comparison between the identification of optimal conditions can be made. D_{CRF} considers both chromatograms A and B as optimal (ignoring the obvious difference in the total elution time) and CEF considers chromatogram C as the optimal one.

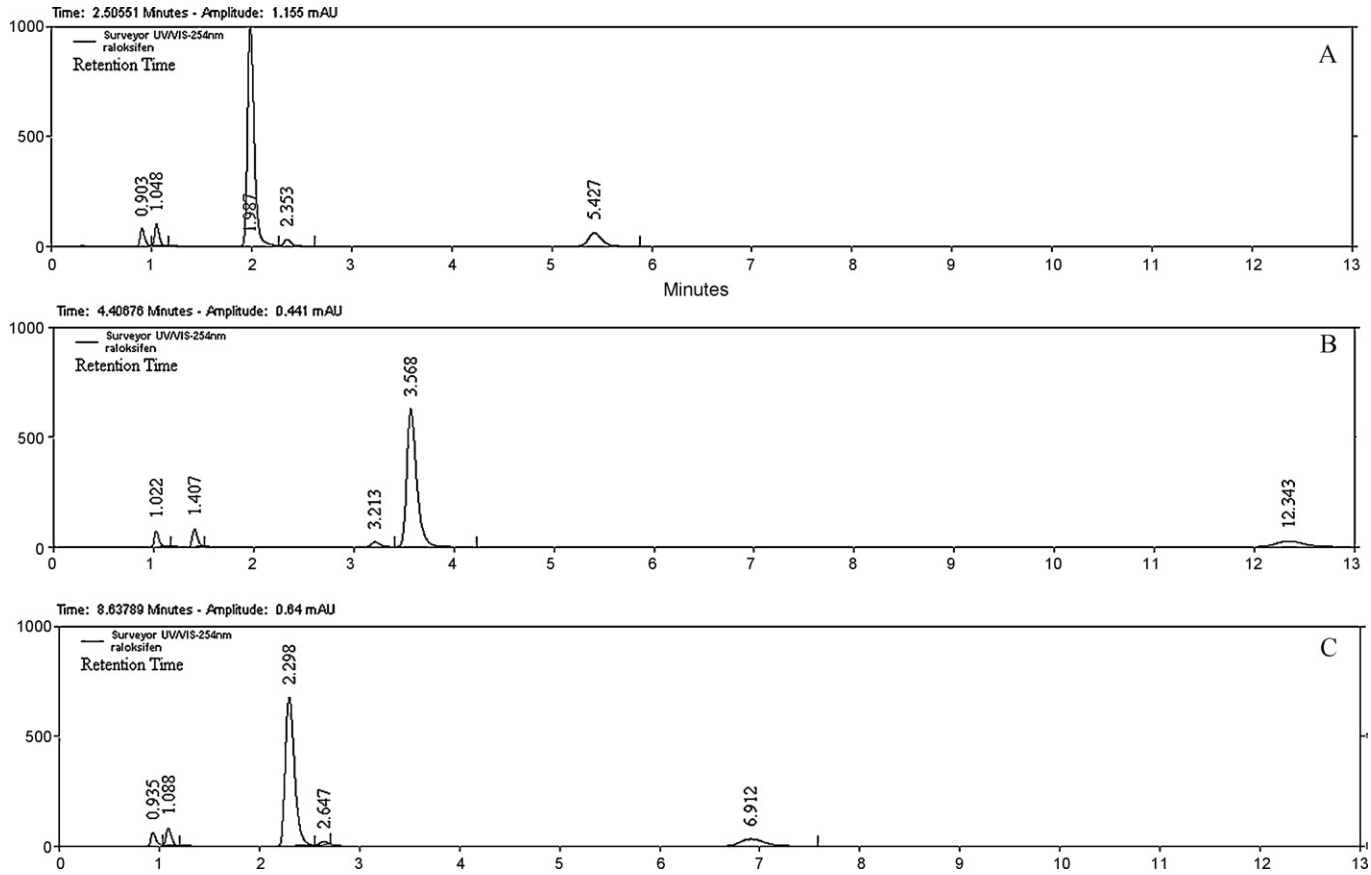

Fig. 6. (A) The chromatogram of raloxifene (t_r = 1.99 min) and its impurities (t_{i1} = 0.9 min; t_{i2} = 1.05 min; t_{i3} = 2.35 min and t_{i4} = 5.43 min) obtained with the optimal mobile phase obtained by N_{CRF}: acetonitrile - 4 mM SDS (47:53, v/v), pH of the mobile phase adjusted to 2.5, column temperature was 35 °C, detection wavelength λ = 254 nm and flow rate 1 mL min^{-1}. (B) The chromatogram of raloxifene (t_r = 3.57 min) and its impurities (t_{i1} = 1.02 min; t_{i2} = 1.41 min; t_{i3} = 3.21 min and t_{i4} = 12.34 min) obtained with the optimal mobile phase obtained by D_{CRF}. (C) The chromatogram of raloxifene (t_r = 2.3 min) and its impurities (t_{i1} = 0.93 min; t_{i2} = 1.09 min; t_{i3} = 2.65 min and t_{i4} = 6.91 min) obtained with the optimal mobile phase obtained by CEF.

It is proved that the evaluation of the chromatograms by the new chromatographic response function is significantly better comparing to the CEF and D_{CRF}.

5. Conclusion

A new chromatographic response function for use in optimization strategies has been proposed. The performances of the function were compared to the previously developed CEF and Duarte's CRF analyzing experimentally obtained chromatograms. The crucial advantages of the new function are the proper evaluation of the resolution and time term and the accurate weighting of both of them. The resolution term is designed to estimate the separation of peaks regardless of their shape, potential fronting or tailing. Also, each pair of adjacent peaks makes the equal contribution to the function value, so there is no possibility of masking poorly resolved peaks. The function allows the analyst to choose the accent he wants to put on the resolution or time term i.e. it is adaptable to the particular separation problem.

The optimization of raloxifene and its four impurities separation was presented for the first time following the central composite design experimental plan and the new CRF was selected as the output. The influence of the most important factors on the chromatographic behavior of the analyzed mixture was estimated and the optimal separation conditions were defined.

The new CRF proved to be good marker of the chromatogram quality and therefore enables reliable ranking of series of chromatograms with different characteristics so it can be successfully applied in optimization procedures of analytical methods.

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