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A new chromatographic response function for use in size-exclusion chromatography optimization strategies: Application to complex organic mixtures

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ABSTRACT

A new chromatographic response function (CRF) is presented aiming at designing an optimal chromatographic separation protocol for assessing the molecular size distribution of complex organic mixtures, such as those of natural organic matter from different sources (atmospheric, aqueous, and terrestrial). This CRF can be applied to mixtures of unknown solutes, being well suited for describing separation processes of pair of peaks of highly unequal area, and also for overlapping and asymmetric peaks. The performance of the developed CRF was compared to that of an existing response function, using simulated chromatograms. The capability of the new function to qualify the resolution degree that it is attained under different chromatographic conditions was further assessed through a size-exclusion chromatography study of a variety of different organic compounds, via a two-level full factorial design. It was proved that this function is a reliable alternative to optimize simultaneously the composition of the mobile phase (pH, ionic strength, and organic modifier concentration) and the instrumental variables (flow rate).

1. Introduction

The main goal in the development of every liquid chromatography (LC) separation protocol is to achieve the best chromatographic performance possible, in terms of resolution, sensitivity, and analysis time, through the adjustment of the experimental conditions (e.g. stationary phase characteristics, mobile phase composition, pH, ionic strength, flow rate, temperature). In the particular case of complex organic mixtures, such as those of natural organic matter (NOM), LC with multiple online detectors has become a widely used method for exploring the heterogeneity of the molecular structures in terms of molecular weight and/or size, polarity, and hydrophobicity. Size-exclusion chromatography (SEC) is the technique of choice for estimating the molecular size distribution of NOM, being also well-suited for a fingerprinting of this type of organic matter and for the comparison of samples from different sources [1]. Despite the widespread application of this technique for the analysis of NOM, the design of a suitable experimental procedure is still an enormous analytical challenge with no general consensus. The optimization of a SEC separation is frequently performed on the basis of the conventional one-factor-at-a-time method [2–5], which typically requires a relatively large number of experiments, being therefore time consuming. To date, the reported SEC optimization studies on the molecular properties of NOM have mainly generated qualitative information on the influence of the analytical conditions on the chromatographic profiles. A vast amount of experimental data has been gathered regarding the marked effects of mobile phase composition – mostly pH and ionic strength – on the separation results. However, the extent of the interaction between the analytical variables has been neglected by most of these studies, as well as how this interdependency may affect the estimative of the molecular size distribution of NOM.

Searching for a more practical and efficient method for designing optimal SEC separations is therefore an important requirement for those researchers who are interested in the analysis of NOM. Experiment-based interpretative methodologies, supported by mathematical models or algorithms, are currently considered the most efficient tools for finding the optimal conditions in chromatography, particularly in those related to the routine analyses of selected individual compound(s) [6]. These methodologies include two main steps: (1) build up of a retention model of the compounds to be separated (this requires running a limited number of experiments in order to fit equations or train algorithms that will allow the prediction of retention) [6,7] and (2) estimative of the separation quality, and therefore of the optimum conditions, through computer simulation [6]. One of the most important requirements for the success of these interpretative procedures is the selection of a proper mathematical equation that mimics the retention behaviour of the solutes [8]. This means that the analyst must have a comprehensive knowledge on the behaviour of the chro-

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matographic system including the stationary phase characteristics, eluents composition, flow and temperature effects, analyte–eluent interactions, and most importantly, the characteristics of the target analyte(s). While this requirement could be easily accomplished in the routine analysis of well-known compounds (e.g. drugs and amino acids), it is not feasible in the field of SEC of NOM. Due to its extremely diversity, with a broad range of molecular weights and functional groups, this type of organic matter cannot be described in unambiguous structural terms. Furthermore, in real SEC practice, and for the particular case of NOM, the occurrence of non-size exclusion effects (ionic exclusion and adsorption) is known to influence the separation of sample constituents [9]. The difficulties in applying a mathematical expression for describing such complex retention behaviour limits the simulation of real size-exclusion chromatograms, which in turns hinders the development of a SEC method based on interpretative optimization methodologies.

This paper suggests an alternative approach based on the use of a quality function for assessing the resolution level associated with the obtained SEC profile in order to find the best separation conditions. It should be mentioned, however, that measuring the quality of the chromatographic separation constitute itself the second step, and the main goal, of experiment-based interpretative methodologies. In these procedures, the global resolution is the most commonly used separation qualifier [6,10]. This criterion can be combined with other secondary aims, such as the analysis time, number of distinguishable peaks, and/or robustness [6,11]. These quality criteria can be optimized on a fully independent way through multicriteria decision-making functions [6,7,12,13], or they can be combined in a single mathematical expression, usually designated as chromatographic response function (CRF).

The vast majority of the composite CRFs described in the literature include a limited number of criteria (e.g. resolution, analysis times, number of detectable peaks, and/or information related to peaks shapes), and each is designed to quantify the resulting chromatograms based on the ultimate goal of the separation [6,11,14–19]. However, none of the existing CRFs is particularly suitable for mapping the quality of size-exclusion separation profiles of unknown samples. The existing CRFs usually require a previous knowledge on time constrains, desired peak resolution, and acceptable analysis time, and in most cases they rely on a model built on a theoretical basis [6,8]. When dealing with complex samples, such as those of NOM, the application of an objective measure that qualifies the separation degree without the need of *a priori* chromatographic information seems to be a better solution to assess the effects of the experimental variables adjustment on the chromatogram profiles and, therefore, to search for the best SEC conditions.

Hence, this paper introduces a new CRF that comprises the most important goals of any chromatographic procedure: the quality of separation in terms of resolution between adjacent peaks, the total number of distinguishable peaks in the chromatogram, and the analysis time. To the best of authors' knowledge, no study has ever attempted to select and apply an optimization criterion in the field of SEC of NOM. The performance of the developed CRF was compared to that of Berridge [14], using simulated chromatograms. The new CRF was further used as a response variable in a series of SEC analyses of a variety of different organic compounds relevant to NOM from atmospheric and aquatic environments. This type of organic matter is likely to be composed of oxygenated compounds with functional groups such as COOH, COH, C=O, COC, $CONO_2$, and CNH_2, as well as aromatic structures [20–22]. The main purpose was to verify the adequacy of the CRF to study the influence of four different experimental variables (pH, salt concentration, organic modifier content, and flow rate) on the quality of the size-exclusion separation of such complex organic mixtures. These experiments were performed according to a two-level full factorial design, which allowed the identification of the main effects of each experimental variable, as well as the factors which do not significantly affect the quality of the chromatographic separation.

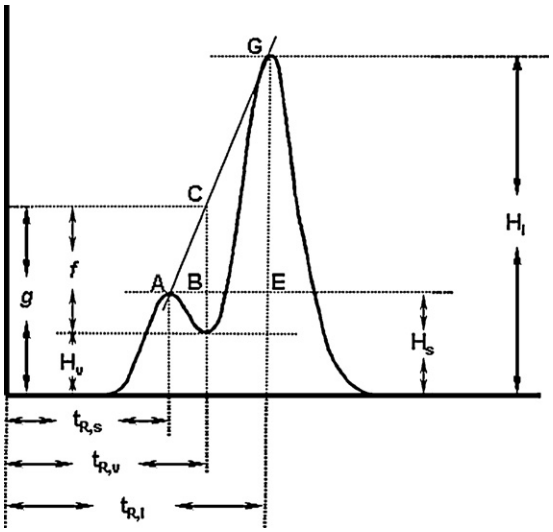

Fig. 1. Schematic chromatogram illustrating the parameters for the estimative of resolution between unresolved peaks (adapted from Carle [23]).

2. Chromatographic response function

In the field of chromatography, there are different outputs that can be considered for any separation problem: the resolution between adjacent peaks, total elution time for the whole sample, number of distinguishable peaks in the chromatogram, detection limit, and the robustness of the separation [6,7,11]. The resolution between two consecutive peaks is often selected as an elementary criterion when building response functions. The chromatographic resolution for two adjacent peaks is traditionally defined as a function of the width of each peak at the base (or at the half-height) and the difference in retention distances for the peaks of interest. Although this description can be easily applied for Gaussian shaped peaks, it is not generally applicable for overlapping and asymmetric peaks, which constitute the most common SEC profile of complex organic mixtures.

In 1972, Carle [23] presented a good alternative for determining the resolution between unresolved peaks whose heights differ greatly. The mathematical equation was derived using the Kaiser's definition for peak-to-valley ratio, $\theta = f/g$, which is a function of peak overlap, as shown in Fig. 1. The Kaiser's definition is one example of the various types of peak-to-valley ratios that can be applied to assess the separation quality of a solute in a mixture [10]. The term f in the Kaiser's definition represents the distance between the valley separating the two peaks and a line joining the apexes of the peaks, whereas g represents the distance from the baseline to the line joining both peaks maximum. When interpreting real complex chromatograms, where the sizes of the adjacent peaks are grossly different and disproportionate, the estimative of θ is not straightforward. In such cases, the line joining the peak apexes and the perpendicular through the valley becomes parallel to each other. This makes the intersection point of these lines difficult to locate, and therefore difficult to estimate both f and g in the peak-to-valley ratio.

To overcome this problem, Carle [23] suggested the replacement of f and g by their geometrical equivalents. According to this author, the dimensions f and g can be defined by the following terms, as

depicted in Fig. 1:

$$\theta_{s,1} = ((\overline{BC} + H_s - H_v)/(\overline{BC} + H_s)) = 1 - (H_v/(\overline{BC} + H_s)). \quad (1)$$

Since triangles ABC and AEG are similar, their respective sides are proportional, thus allowing the following equivalence:

$$\overline{BC}/\overline{AB} = \overline{EG}/\overline{AE} = (H_l - H_s)/(t_{R,l} - t_{R,s}). \quad (2)$$

To solve Eq. (2) for $\overline{BC}$, the dimension $\overline{AB}$ must also be substituted by its equivalent, i.e., by the difference $(t_{R,v} - t_{R,s})$, which correspond to the distance from the retention time of peak s to the valley between adjacent peaks. Substituting $\overline{AB}$ by this term in Eq. (2), $\overline{BC}$ becomes

$$\overline{BC} = (((t_{R,v} - t_{R,s}) \times (H_l - H_s))/(t_{R,l} - t_{R,s})). \quad (3)$$

Replacing in Eq. (1) the terms of Eq. (3), the resolution between peaks s and l, $\theta_{s,l}$, can be written in the following final form

$$\theta_{s,l} = 1 - ((H_v \times (t_{R,l} - t_{R,s}))/((t_{R,l} - t_{R,s}) \times (H_l - H_s) + H_s \times (t_{R,l} - t_{R,s}))). \quad (4)$$

If the opposite configuration of the peaks illustrated in Fig. 1 is used to calculate θ, $\overline{AE}$ and $\overline{AB}$ in Eq. (2) becomes $(t_{R,s} - t_{R,l})$ and $(t_{R,s} - t_{R,v})$, respectively. Therefore, to retain absolute identity with the θ of Kaiser and to describe both configurations with one equation, both $\overline{AE}$ and $\overline{AB}$ can be replaced by their absolute values and the final mathematical equation would then become,

$$\theta_{s,l} = 1 - ((H_v \times \left|t_{R,l} - t_{R,s}\right|)/(\left|t_{R,v} - t_{R,s}\right| \times (H_l - H_s) + H_s \times \left|t_{R,l} - t_{R,s}\right|)). \quad (5)$$

Eq. (5) may be, therefore, applied to estimate the resolution for adjacent peaks of highly unequal area, and also for overlapping and asymmetric peaks. This method has no restrictions on the peaks quality, and only requires the definition of the peaks and valley heights and their respective retention times. It should also be noted that $\theta_{s,l}$ is a normalized measurement that varies between 0, when the peaks are fully overlapped, and 1.0, when the peaks are resolved at baseline. This boundary condition constitutes an advantage when dealing with SEC of highly complex systems, since it does not require the prior estimation of an optimum and/or minimum acceptable resolution.

The next step of the development of the CRF includes the definition of an overall resolution term for the evaluation of an entire chromatogram. This is usually accomplished through the summation of the resolution of all consecutive pairs of peaks in the chromatogram, and is defined as follows

$$\mathrm{CRF} = \sum_{i=1}^{N-1} \theta_{s,l}, \quad (6)$$

where N is the number of peaks. Eq. (6) constitutes a first mathematical approach for the assessment of chromatogram quality. Obviously, this overall resolution term should be maximized during the optimization process. However, for an efficient strategy for optimization of SEC of complex organic mixtures with unknown structures, additional criteria must be used, besides an overall resolution criterion. The total number of peaks appearing in the chromatogram, N, was also considered an important requirement for the size-exclusion analysis of complex systems. The reduction of the elution time, whilst retaining sufficient resolution, is also an important objective in any chromatographic procedure, particularly for those entailing the routine analysis of individual compounds. However, when dealing with SEC of NOM, changes in the retention behaviour of the sample have a crucial effect on the estimative of the molecular size distribution. Theoretically, the elution behaviour of a sample should be independent of the SEC conditions (e.g. pH, ionic strength, mobile phase composition, flow rate, and stationary phase characteristics). However, in practice, and for the particular case of NOM, the occurrence of non-size exclusion effects such as ionic exclusion and adsorption are likely also to influence the separation of the sample constituents [9]. While the occurrence of repulsion interactions between a partially charged stationary phase and the analytes possessing the same charge will result in the decrease of the elution time, the hydrophobic interactions slow down the analytes mobility and further down in a chromatogram the fraction will elute (in some cases close to the total pore volume of the column) [9]. Due to the effects of these phenomena on the retention behaviour of NOM, the information regarding the elution time was used in this study as a correction term, reducing the CRF if the elution time of the last peak approaches a rather large value.

The new CRF, developed for assessing the quality of size-exclusion chromatograms of complex samples, takes then the following final form:

$$\mathrm{CRF} = \sum_{i=1}^{N-1} \theta_{s,l} + N - ((t_{R,L} - t_0)/t_{R,L}), \quad (7)$$

where $t_{R,L}$ correspond to the retention time of the last eluted peak, and t_0 is the elution time corresponding to the column void volume. The CRF of Eq. (7) is designed to reach a maximum as the optimum is approached. It is also expected that the numerical value of the CRF significantly decreases if the eluting peaks exhibit a severe overlap, being also affected by the time window inside which all the peaks will appear in the chromatogram.

3. Experimental

3.1. Reagents and solutions

All the chemicals used in this work were of analytical reagent grade and obtained from commercial suppliers without further purification. All the solutions were prepared with high purity water (18 MΩ cm).

The effect of the organic solvent content was evaluated with HPLC grade acetonitrile (ACN). The pH of the mobile phase was adjusted with 2 mM phosphate buffer, prepared with di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) and sodium dihydrogen phosphate (NaH_2PO_4). To investigate the effects of salt concentration, sodium chloride (NaCl) was added to the mobile phase. The composition of the mobile phases was adjusted according to the experimental design as described in Section 4.2. Prior to use, the mobile phases were filtered through a membrane filter (PVDF, Gelman Sciences) of 0.22 μm pore size.

The size-exclusion experiments were conducted using a mixture of six organic compounds representative of different chemical groups: α-ketoglutaric acid disodium salt dihydrate ($NaOOCCH_2CH_2COCOONa{\cdot}2H_2O$, Mw = 226.1 g mol^{-1}), syringic acid ($HOC_6H_2(OCH_3)_2CO_2H$, Mw = 198.2 g mol^{-1}), ferrulic acid ($HOC_6H_3(OCH_3)CH{=}CHCO_2H$, Mw = 194.2 g mol^{-1}), mandelic acid ($C_6H_5CH(OH)CO_2H$, Mw = 155.1 g mol^{-1}), thiamine monophosphate chloride dihydrate ($C_{12}H_{18}ClN_4O_4PS{\cdot}2H_2O$, Mw = 416.8 g mol^{-1}), and acetone (CH_3COCH_3, Mw = 58.1 g mol^{-1}). The concentration of each solute in the samples was in the range of 3.10–4.15 mg mL^{-1}, 0.24–0.73 mg mL^{-1}, 0.31–0.96 mg mL^{-1}, 2.32–4.38 mg mL^{-1}, 0.46–0.95 mg mL^{-1} and 2.5% (v/v), respectively. The column void volume (represented by t_0 in Eq. (7), Section 2) was determined with albumin fraction V (from bovine serum, Mw ≈ 66 kDa).

The generation of the experimental design and data analysis were performed using the Unscrambler Version 7.5 statistical software.

3.2. Chromatographic conditions

The SEC analyses were performed on a JASCO chromatographic system equipped with a quaternary low pressure gradient pump (model PU-2089) and a photodiode array (PDA) detector (model MD-2010). A PSS Suprema 30 Å analytical column (Polymer Standards Service GmbH, Mainz, Germany; diameter 8 mm; length 300 mm; particle size 10 μm; separation range 100–30,000 Da; stationary phase polyhydroxymethacrylate copolymer), protected by a PSS Suprema guard column (diameter 8 mm; length 50 mm; particle size 10 μm), was used for separation. The temperature of the analytical column was maintained at 30 °C in a Phenomenex column oven, and the injection volume was 20 μL. The PDA detector is a sensitive photometer, which can be operated over a wavelength range from 195 to 650 nm. The work reported here was based on a wavelength of 224 nm. The chromatographic system was conditioned by passing the mobile phase through the column until a stable baseline was observed. The effect of eluent flow rate was also evaluated by applying different values of this variable (see Section 4.2).

4. Results and discussion

4.1. Application of the CRF to theoretical chromatograms

The fitting and simulation of chromatograms is of great importance in the field of interpretative optimization procedures. In the literature there are a variety of mathematical equations that describe the shape of chromatographic profiles [24,25]. The most simple mathematical peak function simulates the Gaussian elution profile. However, in practice, this function is not suitable for describing peaks characterized by a large asymmetry. In such cases, other algorithms have been developed, being the exponentially modified Gaussian function one of the most popular algorithms for this purpose [25].

In this work, the rationale behind the simulation of chromatograms was not the prediction of the retention for a subsequent optimization study, but rather to assess the sensitivity of the developed CRF (Eq. (7)) towards different resolutions, number of peaks appearing in the chromatogram, and analysis time. For this purpose, the chromatograms were computer simulated through the generation of each peak by the Gaussian model:

$$H(t) = H_{\max} \times \mathrm{EXP}(-(t - t_R)^2/2\sigma^2), \quad (8)$$

where $H(t)$ represents the peak height at time t in the chromatogram, $H_{\max}$ is the peak height at maximum, t_R is the retention time of the peak, and σ is the standard deviation of the peak. In the case of Gaussian peaks, the standard deviation of the peak is related to the peak-width at baseline (w_b) by the following equation: $\sigma = w_b/4$. The simulated chromatograms were then created by summing the peaks that were generated separately through Eq. (8).

The profiles depicted in Fig. 2 aim to reproduce the possible situations obtained in real size-exclusion chromatograms of complex samples with unknown components. Typical SEC profiles of NOM may exhibit a unimodal distribution (not considered in this study) such as those of terrestrial origin [26], or they may reveal broader peaks with superimposed sharp peaks and/or shoulders such as those of NOM from aquatic and atmospheric (e.g. fine air particles) environments [3–5,27,28]. However, in a characterization study of aquatic humic substances, Nagao et al. [29] obtained different SEC elution profiles exhibiting both resolved (at baseline) and unresolved peaks. Simulating such a variety of experimental chromatograms is an advantage since it can provide an excellent basis for understanding how the different criteria affect the proposed CRF, and how this function behaves against other existing CRFs.

Table 1 indicates, for each simulated chromatogram, the resolution between the adjacent peaks ($\theta_{s,l}$) and the value of the CRF estimated through Eqs. (5) and (7), respectively. The elution time corresponding to the column void volume, t_0 in Eq. (7), was set at 2 min. The difference between chromatograms H, J, K, and L and the other nine simulated chromatograms is the number of peaks. This difference is reflected in the values of the CRF, which classifies the profiles H, J, K, and L as those exhibiting the best quality. Although H and L exhibit the best overall resolution ($\Sigma\theta_{s,l}$ is around 4.0), the CRF considers chromatogram H of less quality than chromatogram L, being this a direct consequence of its lower number of peaks. Chromatograms A to F all exhibit the same number of peaks. However, and due to the emphasis that it places on resolution, chromatograms C, D, E, and F with unresolved peak pairs are considered of worse quality than chromatograms A and B. The order by which the CRF rank the quality of these chromatograms depends only on the resolution of the individual peak pairs, since all chromatograms elute in the same time window (12 min). The importance of the resolution term is also reflected in the values of the CRF of chromatograms K and L; although exhibiting the same number of peaks, the maximum resolution of 1.0 for the first two and final peak pairs in chromatogram L contributes to rank this chromatogram as the most desirable one. As shown in Table 1, the CRF classifies chromatograms E, G and I as the less desirable chromatograms. While for chromatogram E this could be attributed to a poor overall resolution of 1.5, for chromatograms G and I this is likely to be a consequence of the lower number of peaks alongside a higher elution time of the final peak (14 and 10 min, respectively).

The performance of the new function was further compared to that developed by Berridge [14] (Eq. (9)). Although introduced back in 1982, the response function of Berridge ($\mathrm{CRF_{Berridge}}$) is still referred as a good example of an objective function that maps into a single scalar a set of quality criteria [6,7]. Besides the resolution between peaks and the analysis time, the $\mathrm{CRF_{Berridge}}$ also includes a term related exclusively to the number of peaks, being therefore comparable to the CRF (Eq. (7)) presented in this work.

$$\mathrm{CRF_{Berridge}} = \sum_{i=1}^{L} R_i + L^x - a\left|T_M - T_L\right| - b\left|T_0 - T_1\right|, \quad (9)$$

R_i is the resolution between adjacent pairs of peaks (in practice constrained to a maximum of 2), L is the number of peaks appearing in the chromatogram, T_M, T_L, T_1, and T_0 are the maximum acceptable time, the retention times of the last and first peaks, and the minimum retention time of the first peak, respectively, and x, a and b are arbitrary weighting parameters selected by the operator (usually set to values between 0 and 3). In this study, the weightings for the analysis time (a) and the total number of peaks (x) were those recommended by Berridge for an isocratic elution programme and were both set at 1.5 [14], while the values of T_M and T_0 were set at 15 and 2 min, respectively. The resolution between peaks was calculated through the traditional approach:

$$R_{i,i+1} = 2(t_{i+1} - t_i)/(w_i + w_{i+1}), \quad (10)$$

where t_i and t_{i+1} are the retention times of two consecutive peaks, and w_i and w_{i+1} are the peak widths at baseline.

Table 1 summarizes the resolution between the adjacent peaks and the value of the $\mathrm{CRF_{Berridge}}$ function estimated through Eqs. (10) and (9), respectively. The $\mathrm{CRF_{Berridge}}$ function also considers L as the best quality chromatogram. However, it seems that the unresolved peak pairs have little influence on the function value compared to the pairs with large resolution. This feature results in the quality of

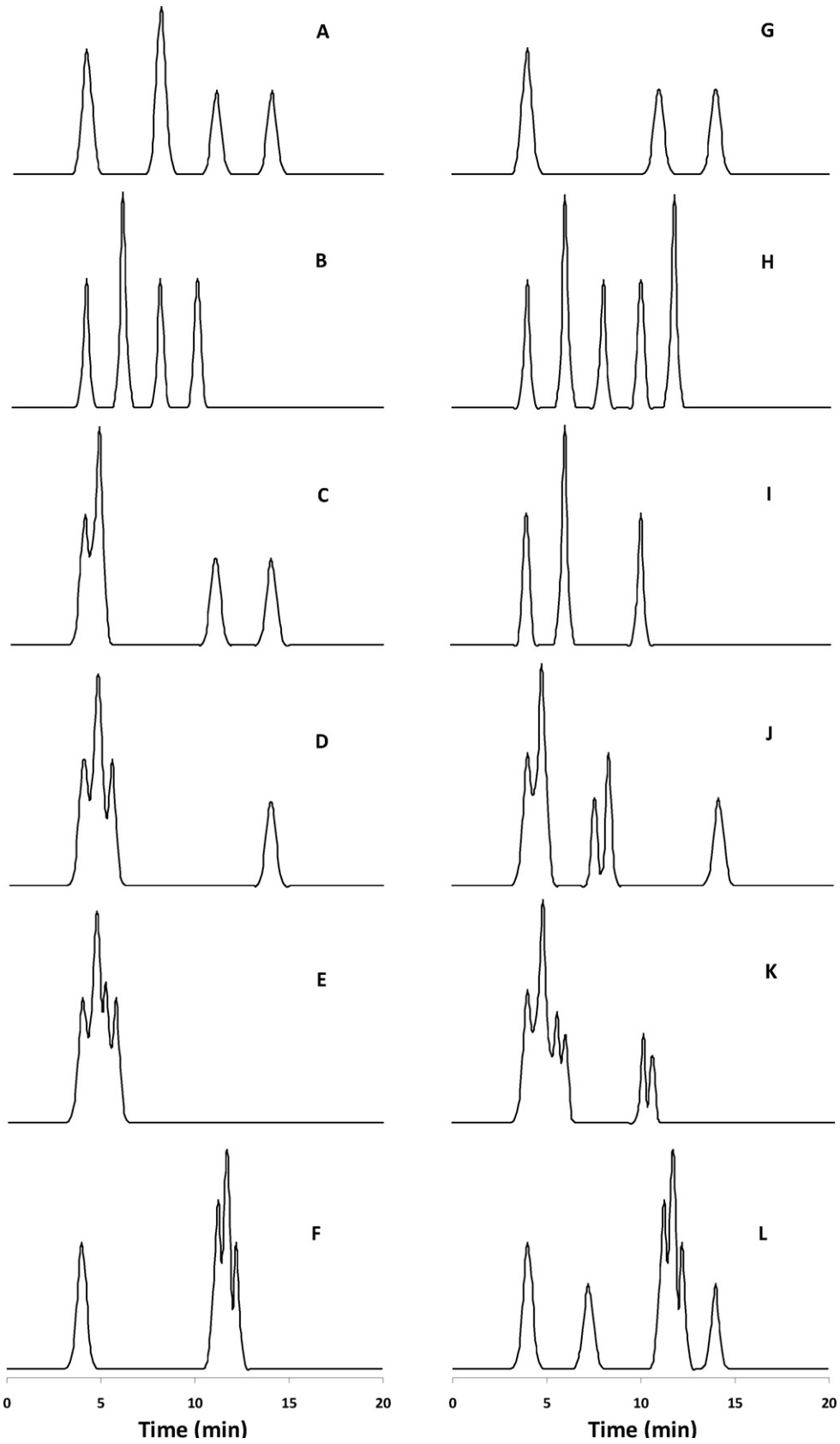

Fig. 2. Simulated chromatograms used to demonstrate the performance of the developed CRF. Refer to Table 1 for further information on these simulations.

Table 1
Resolutions and objective function values calculated for the simulated chromatograms shown in Fig. 2.

Simulation	Resolution ($\theta_{s,l}$)[a]					Resolution ($R_{i,i+1}$)[b]					New CRF[c]	CRF $_{Berridge}$[d]
	1,2	2,3	3,4	4,5	5,6	1,2	2,3	3,4	4,5	5,6		
A	1.0	1.0	1.0	–	–	2.0	2.0	2.0	–	–	6.1	15
B	1.0	1.0	1.0	–	–	2.0	2.0	2.0	–	–	6.2	8.5
C	0.32	1.0	1.0	–	–	0.83	2.0	2.0	–	–	5.5	13
D	0.45	0.57	1.0	–	–	0.83	0.94	2.0	–	–	5.2	12
E	0.45	0.64	0.44	–	–	0.83	0.77	0.77	–	–	4.9	−1.5
F	1.0	0.45	0.65	–	–	2.0	0.77	0.91	–	–	5.3	9.5
G	1.0	1.0	–	–	–	2.0	2.0	–	–	–	4.1	10
H	1.0	1.0	1.0	1.0	–	2.0	2.0	2.0	2.0	–	8.2	16
I	1.0	1.0	–	–	–	2.0	2.0	–	–	–	4.2	3.7
J	0.45	1.0	0.89	1.0	–	0.83	2.0	1.5	2.0	–	7.5	18
K	0.45	0.56	0.40	1.0	0.81	0.83	0.91	0.80	2.0	1.1	8.4	16
L	1.0	1.0	0.45	0.65	1.0	2.0	2.0	0.77	0.91	2.0	9.2	23

[a] Resolution ($\theta_{s,l}$) calculated through Eq. (5).
[b] Resolution ($R_{i,i+1}$) calculated through Eq. (10).
[c] CRF calculated through Eq. (7) (t_0 = 2 min).
[d] CRF calculated through Eq. (9) (T_0 = 2 min; T_1 = 4 min; T_M = 15 min; x = 1.5; a = 1.5; b = 1.0).

the chromatograms being determined by the presence of the well resolved peak pairs, regardless the presence of unresolved peaks. Chromatograms C, D, F, J, and K are good examples where this effect can be observed. Furthermore, the $CRF_{Berridge}$ function suggests that chromatogram B is less desirable than chromatogram A, which contradicts the ranking previously established by the new CRF. A similar trend is also verified for chromatograms G and I, and for chromatograms H, J and K. This result suggests that the $CRF_{Berridge}$ function places more emphasis on elution time over the resolution, leading to the conclusion that chromatograms with a higher final peak elution time are of better quality. Due to the shorter final peak elution time, chromatograms E and I are classified by the $CRF_{Berridge}$ function as the worst chromatographic separations.

Overall, the simulated chromatogram study indicates that the new CRF is able to provide an accurate description of the quality of the chromatograms, thus suggesting that it can be successfully used for SEC optimizations studies. In the particular case of chromatograms exhibiting the same number of peaks, the new CRF seems to qualify and rank more adequately the chromatographic resolution than the $CRF_{Berridge}$ function. Furthermore, neither the definition of an optimum resolution nor the maximum acceptable time of the chromatographic run nor the definition of weighting factors are needed for differentiating and ranking the quality of the chromatographic separations. The critical point of the definition of such criteria in different objective functions, such as that of Berridge, is that they can lead to different optimum chromatographic outputs, thus hindering any conclusion regarding the SEC analysis of unknown organic mixtures. A new approach that does not requires the scaling of the objective function terms can be an efficient way to overcome these limitations.

4.2. Application of the CRF for assessing the separation quality in SEC

The validity of the new CRF for qualifying the resolution degree attained under different SEC conditions was further assessed through a two-level full factorial design incorporating the mobile phase composition (pH, volume fraction of organic solvent, and salt concentration) and the eluent flow rate as the design variables. The choice of mobile phase is a key factor in obtaining a good sensitivity and resolution in the fractionation of NOM by SEC. The composition of the mobile phase is often modified to avoid the effects of electrostatic repulsion and specific adsorption between the analytes and the column stationary phase [2,26]. The ion-exclusion effects are typically suppressed through the use of buffers or low molecular-weight electrolytes, whereas the hydrophobic interactions are minimized by adding organic solvents (e.g. ACN or methanol) which occupy the hydrophobic sites of the stationary phase [9]. To account for these limitations of SEC, especially when dealing with unknown samples, the effect of pH, ACN content, and NaCl concentration of the mobile phase and eluent flow rate on the retention behaviour of six organic compounds was studied. These organic compounds were selected on the basis of their chemical groups (monocarboxylic acids, carbonyls, amines, phenols and aromatics), which are recognized to be relevant to NOM from atmospheric and aquatic environments [20–22]. It must be emphasised, however, that the objective of this study was not to separate these organic compounds by functional group, but to demonstrate the efficiency of the new CRF for measuring the chromatographic quality and, therefore, to achieve a more rigorous decision regarding the optimization of the SEC conditions.

Table 2 shows the experimental variables and the corresponding levels used in the full factorial design. The experimental range of the factors was selected on the basis of the current state-of-the-art regarding SEC of NOM [2,4,26], and of the physico-chemical properties of the column's stationary phase (Section 3.2). One of the major concerns of using factorial designs is its weakness in introducing enough curvature into the response surface. In factorial designs, the interaction terms twist the response surface only slightly, thus suggesting that the linear model may not be sufficient to represent the experimental data adequately [30]. In such cases, a quadratic model may be appropriate, and it can be used to predict factor levels that produce maximum or minimum response values [30]. In this study, however, the two-level full factorial design was applied to investigate the capability of the new CRF for identifying the main experimental variables affecting the quality of the chromatographic resolution rather than the interaction effects. Accordingly, the full factorial design for four experimental variables and two levels required 16 experiments, which were performed on a random order (Table 3). The values of the CRF obtained for each experiment are also shown in Table 3.

Table 2
Experimental variables and levels applied in the full factorial experimental design.

Experimental variables	Applied level	
	− (Low)	+ (High)
pH	7.0	9.0
ACN (%)	0.0	15.0
NaCl (mM)	0.0	10.0
Flow rate (mL min^{-1})	0.6	1.0

Table 3
Experimental design and CRF values obtained through the two-level full factorial design.

Experiment	pH	ACN (%)	NaCl (mM)	Flow rate (mL min^{-1})	CRF
1	9.0	15.0	10.0	0.6	9.62
2	7.0	15.0	10.0	1.0	8.33
3	9.0	0.0	0.0	1.0	7.71
4	7.0	0.0	0.0	0.6	7.74
5	9.0	15.0	0.0	1.0	6.81
6	7.0	15.0	0.0	0.6	5.74
7	9.0	0.0	10.0	0.6	8.43
8	7.0	0.0	10.0	1.0	7.88
9	9.0	0.0	10.0	1.0	8.43
10	9.0	15.0	0.0	0.6	7.03
11	7.0	15.0	0.0	1.0	6.91
12	7.0	0.0	10.0	0.6	7.71
13	9.0	0.0	0.0	0.6	8.91
14	7.0	0.0	0.0	1.0	7.61
15	7.0	15.0	10.0	0.6	8.39
16	9.0	15.0	10.0	1.0	9.40

Table 4
Effects of the experimental variables calculated for the SEC of the organic mixtures from the full factorial design.

Experimental variables	Effect	*F*-ratio	*P*-value
pH	0.753	13.5	0.014
ACN (%)	−0.273	1.77	0.240
NaCl (mM)	1.22	35.2	0.002
Flow rate (mL min^{-1})	−0.059	0.083	0.785
pH × ACN (%)	0.116	0.318	0.597
pH × NaCl (mM)	0.137	0.448	0.533
pH × Flow rate (mL min^{-1})	−0.351	2.94	0.147
ACN (%) × NaCl (mM)	1.10	28.6	0.003
ACN (%) × Flow rate (mL min^{-1})	0.226	1.21	0.321
NaCl (mM) × Flow rate (mL min^{-1})	0.034	0.028	0.874

It can be observed that the highest values of the CRF were obtained for experiments 1 and 16 (Fig. 3A and B), whereas the lowest CRF value was obtained for experiment 6 (Fig. 3D). The medium values (between 8.0 and 9.0) were obtained for the chromatograms with relatively good separation but with a lower number of distinguishable peaks (for example, experiment 7, CRF = 8.43, Fig. 3C). These results demonstrate that the new CRF efficiently discriminate between chromatograms with different separation quality, in terms of the number of peaks and resolution between the consecutive peaks. Furthermore, the qualitative scaling of the chromatograms using the CRF parallels the chromatographic data, i.e., for most chromatograms, the CRF values are in line with the intuitive choice of an analyst.

The full factorial design also allowed a first estimative of the main effects of each experimental variable on the quality of separation. According to Table 4, the most important effects on the CRF value were due to the NaCl concentration and pH of the mobile phase. Both variables influenced positively the response function, and the CRF value was found to increase as pH or NaCl concentration increases. The other two main factors, ACN content and flow rate, showed only minor negative effects on the CRF values. Surprisingly, the statistic model underlying this analysis indicates that the second order interaction between NaCl and ACN contents is significant and characterized by a positive value. Such a result emphasises the need to look further on the effects of these two main experimental variables on the CRF value. Currently, an assessment of the optimum experimental conditions for a high CRF value is being carried

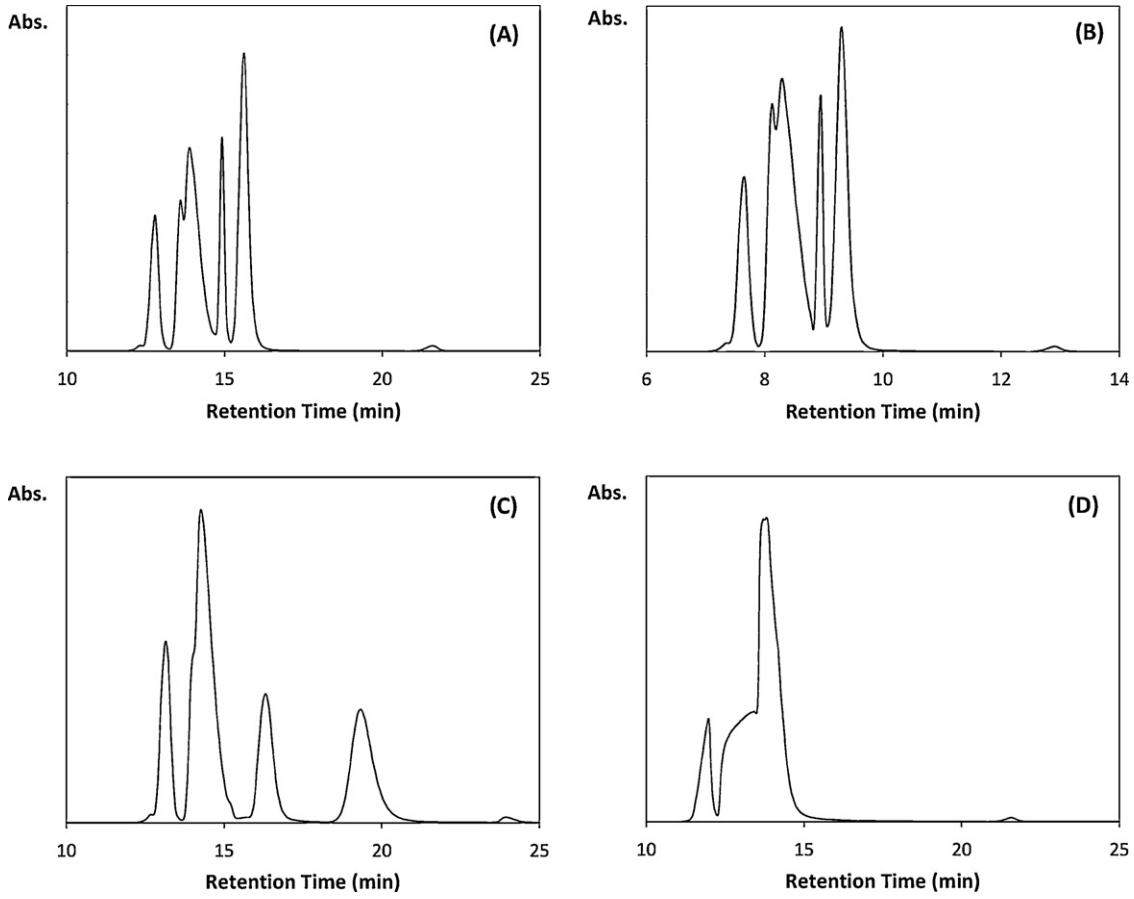

Fig. 3. Chromatograms with CRF of 9.62 (A), 9.40 (B), 8.43 (C), and 5.74 (D) obtained under the analytical conditions of experiments 1, 16, 7 and 6 (Table 3), respectively. The retention time axis was expanded for a better visualization of each chromatogram.

out through a central composite design. The flow rate was excluded from the experimental variables, and the upper and lower levels of the remaining variables (pH, NaCl and ACN contents) were redefined according to the results obtained through the two-level full factorial design.

5. Conclusions

A new CRF has been introduced for the optimization of size-exclusion separations of complex organic mixtures. This function has no restrictions on peak quality, being well suited for describing separation processes of pair of peaks of highly unequal area, and also for overlapping and asymmetric peaks. Furthermore, it does not require the prior definition of an optimum and/or minimum acceptable resolution, which limits the application of any objective function to the separation of highly complex systems. The new mathematical equation takes into account the most important criteria of a size-exclusion separation procedure: overall resolution of the chromatogram, number of distinguishable peaks in the chromatogram, and total time for the elution of the whole sample. The theoretical study with simulated chromatograms indicated that the new developed function places more emphasis on peak resolution over total elution time. This theoretical study also demonstrated that the CRF can provide an accurate description of the quality of the chromatograms, thus suggesting that it can be successfully applied to any particular size-exclusion separation problem.

The CRF along with a two-level full factorial design were applied to assess the validity of the new function for qualifying the resolution degree attained under different SEC conditions, and to study the influence of four experimental variables (pH, NaCl concentration, ACN content, and flow rate) on the quality of the SEC of a mixture of six organic compounds. It was demonstrated that the new CRF represents an efficient and easily accomplishable approach in discriminating between chromatograms with different separation quality, while simultaneously searches for the most favourable chromatographic conditions. The results of the full factorial experimental design also allowed a preliminary evaluation of the experimental variables that are important for the optimization of an SEC procedure.

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