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A new chromatographic response function for assessing the separation quality in comprehensive two-dimensional liquid chromatography

Regina M.B.O. Duarte*, João T.V. Matos, Armando C. Duarte

CESAM & Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

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ABSTRACT

A new chromatographic response function (CRF_{2D}) is proposed and tested for the estimation of the quality index of separation in comprehensive two-dimensional liquid chromatography (2D-LC) of complex organic mixtures. This objective function is based on the concept of peak purity for one-dimensional liquid chromatography, which has been redefined for 2D-LC. The new CRF_{2D} also includes other separation quality criteria, namely the number of 2D peaks appearing in the chromatogram and the analysis time. To compute the peak purity for a given 2D peak, three important steps have been tackled in this study: (a) the development of an alternative algorithm for detecting 2D peaks automatically from real experimental 2D-LC data; (b) the application of a mathematical model to fit the obtained chromatographic data; and (c) the estimation of the volume of the overlapping region between two or more 2D peaks. The performance of the developed CRF_{2D} was compared to that of an existing resolution measure, using simulated chromatograms. The capability of the new function to qualify the overall separation degree that it is attained under different chromatographic conditions was further assessed through a 2D-LC study of a mixture of four aromatic compounds.

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1. Introduction

Data handling and optimal extraction of information in two-dimensional (2D) chromatography, namely the strategies related to data treatment/resolution measurement and the design of optimal 2D separation conditions, still are one of the major drawbacks that have impaired a wider application of this analytical technique. In the particular case of comprehensive two-dimensional liquid chromatography (2D-LC), the design of a suitable experimental procedure is an enormous analytical challenge that still lacks the use of an important requirement in any chromatographic optimization procedure: a mathematical function to measure and map the 2D separation quality. In fact, no reference could be found in the literature that addresses the definition and use of a chromatographic response function (CRF) for qualifying the resolution level associated with any 2D peak arrangement. So far, the most known composite CRFs described in the literature were proposed for optimization purposes in one-dimensional liquid chromatography (1D-LC). Besides a quality index for the separation, the existing CRFs in 1D-LC comprise other important secondary criteria, namely analysis time, number of detectable peaks, and/or robustness [1–8]. Each of these CRF was designed to quantify the resulting chromatograms based on the ultimate goal of the separation, and they 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 [1,9]. Such requirements indicate that none of the existing CRFs is particularly suitable for mapping the quality of the chromatographic separation profiles of unknown samples. To solve this problem, Duarte and Duarte [10] have recently proposed an alternative approach for a CRF in 1D-LC. Eq. (1) was applied to complex mixtures of organic compounds, being well suited for describing the separation of peak pairs of highly unequal area, and also for overlapped and asymmetric peaks [10]. 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.

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

where θ_i is a normalized measurement of the peak resolution using the valley-to-peak ratio for adjacent peaks, N is the number of detected peaks, $t_{R,L}$ is the retention time of the last eluted peak, and t_0 is the elution time corresponding to the column void volume.

Introducing a new CRF for the comprehensive 2D-LC analysis of complex unknown mixtures is not, however, straightforward, and it requires the redefinition of the conventional 1D-LC quality

* Corresponding author. Tel.: +351 234370200; fax: +351 234 370084.
E-mail address: regina.duarte@ua.pt (R.M.B.O. Duarte).

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criteria. Using Eq. (1) as a starting point for this study, the concepts of peak resolution and analysis time in the 2D domain have to be revisited. In terms of a resolution measure in 2D separations, the existing concept has evolved from the classical definition of valley-to-peak ratio in 1D-LC and it has been applied to both Gaussian [11] and non-Gaussian-shaped 2D peaks [12]. Currently, the method proposed by Peters et al. [12] can be considered the most systematic procedure for establishing an overall quality criterion of a particular non-Gaussian 2D peak. This methodology, developed for 2D gas chromatography (GC), starts by defining the chromatographic peaks in the 2D plane using a peak-detection algorithm [13]. This algorithm aims at deciding which 1D peaks detected in the fast second-dimension chromatograms originate from the same compound, and therefore should be "merged" into a single 2D peak. To accomplish this, all the 1D peaks need to be identified beforehand taking into account different features of the peaks (second-dimension peak regions and second-dimension retention times) and different criteria (common peak regions, retention time differences, and unimodality in the first dimension). This algorithm is especially well suited for 2D-GC, and it allows dealing with second-dimension retention time variability. However, this "merging" step is a time consuming process, particularly when analysing large amounts of data. Furthermore, the application of this peak-detection algorithm exhibits some drawbacks, particularly when dealing with closely eluting peaks in both separation dimensions, as acknowledged by the authors [13].

Alternative approaches for peak-detection in 2D chromatography encompasses those based on the watershed algorithm [14–16], which was originally developed for image analysis. The watershed algorithm-based method, firstly adapted by Reichenbach et al. [14], also for 2D-GC, makes use of the 2D image generated in the 2D chromatogram. This concept differs from the two-step algorithm of Peters et al. [13], which is based on the analysis of the raw 1D signal detected in the second-dimension chromatogram. According to Vivó-Truyols and Janssen [17], the watershed algorithm is somewhat intolerant to retention time variability in the second-column separations, and it can split a true single peak in two when the retention times are not fully coincident. Very recently, however, Latha et al. [16] re-evaluated the performance of both peak-detection algorithms (watershed and two-step) for retention time variability in the secondary chromatogram when both methods employ shift correction prior to peak-detection. These authors demonstrated that the watershed algorithm performed better than the two-step approach when correction is applied for both the methods. Furthermore, as both the noise and peak widths increase, the two-step algorithm has more failed detections than the watershed algorithm [16].

At this point, and in spite of their popularity for peak detection, it is obvious that the methods of Peters et al. [13] and Reichenbach et al. [14] do not incorporate all the solutions for every 2D peak-detection problem. In the particular case of the algorithm developed by Peters et al. [13], the authors state that their method is applicable to both 2D-LC and 2D-GC. In practice, however, the acquisition of 2D-LC data is not prone to the same uncontrolled variations in the second-dimension retention times as the 2D-GC. As the application of 2D-LC for the analysis of complex unknown samples is still in its infancy, to find alternative and simpler approaches for peak-detection in 2D-LC is still of significant interest.

Once the peaks have been detected within the 2D domain, and prior to the calculation of the valley-to-peak ratio (i.e. resolution), Peters et al. [12] defined several criteria to determine which 2D peak pairs can be considered real "neighbours". Using trajectory profiles connecting the maxima of 2D peaks, those authors suggested that resolution between two peaks is a meaningful parameter only if no other peaks are located between the two peaks of interest, i.e., if the trajectory profiles are free of interference of a third 2D peak. One disadvantage of this "peak vicinity" concept is that it does not take into account how far the 2D peaks are separated, which means that two peaks can be considered neighbours even when located far apart from each other [12]. This feature may be viewed as a weakness of the proposed methodology, and the estimation of the resolution between these two peaks actually becomes redundant from a "data treatment" point of view.

The final step of the methodology developed by Peters et al. [12] entails the estimation of the valley-to-peak ratio, based on the saddle point concept, and the computation of the resolution (R_s) between the 2D peak pairs using the Schure [11] equation:

$$R_s = \sqrt{-\frac{1}{2} \cdot \ln\left[\frac{1-V}{2}\right]} \tag{2}$$

where V is the valley-to-peak ratio calculated using the intensities of the 2D peaks maxima and of the saddle point [12]. The definition of the saddle point is based on the concept of trajectory profiles in a 2D surface plot and corresponds to the minimum when following the 2D surface in one direction and the maximum when crossing it in a different direction [12]. As it will be demonstrated in this study, the saddle point concept may fail when applied to a 2D chromatogram containing a large amount of peaks with a poor resolution degree. In such situations, Eq. (2) cannot be applied for the estimation of the resolution between the 2D peak pairs. Nevertheless, establishing the best chromatographic conditions in such situations continues to be of especial interest, which means that finding new approaches to qualify the degree of chromatographic separation becomes fundamental, and it will constitute a major breakthrough for an effective widespread application of 2D-LC in the analysis of complex samples.

Therefore, the aim of this study is to develop a CRF for the estimation of the quality of separation in 2D-LC of unknown complex organic mixtures, evolving from the 1D model, Eq. (1), recently proposed by Duarte and Duarte [10]. The estimation of the quality index of separation is now oriented to each individual 2D peak, instead of each 2D peak pair. For this reason, the peak purity concept, previously applied as a resolution measure in computer-assisted interpretive optimizations of 1D-LC methods [1], is now adapted to qualify a 2D chromatogram where no information obtained from standards of the analysed sample is available. The total number of distinguishable 2D peaks and the time needed to achieve a sufficiently resolved 2D chromatogram are also decisive for the global optimization process. On this regard, a new "time-economy" function is also proposed as a secondary criterion to assess the quality of separation. The performance of the developed CRF is illustrated by simulated 2D chromatograms and comprehensive 2D-LC analysis of a mixture of four aromatic compounds, using the acetonitrile content of the mobile phase of first-dimension as the experimental variable. The advantages and drawbacks of the proposed objective function for 2D-LC are also highlighted.

2. Development of a two-dimensional chromatographic response function (CRF_{2D})

The following three consecutive major steps were considered for the development of the two-dimensional chromatographic response function, CRF_{2D}: (a) 2D peak detection using the interpretation of the sum of the partial second-derivatives of the 2D chromatogram; (b) assessment of the quality of 2D peak separation using a novel 2D peak purity concept; and (c) set-up of the objective function incorporating all the criteria established in the previous steps. Additional details regarding each step are discussed in the following sub-sections.

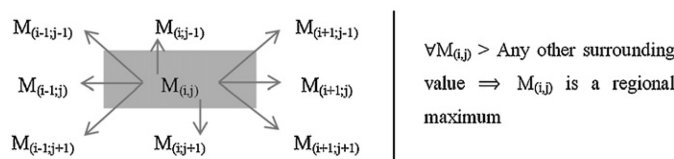

Fig. 1. Basis for definition of the algorithm for detecting 2D peaks as regional maxima.

2.1. Detection of 2D peaks in 2D-LC

Regardless the method used for the qualitative evaluation of a 2D chromatogram, the raw data obtained in a 2D-LC experiment must first be converted into a 2D matrix grid using the modulation period. For large modulation periods, it is advisable to interpolate between experimental values. The 2D peaks can be then detected using an algorithm based on the search for a regional maximum value. As shown in Fig. 1, $M_{(i,j)}$ represents the value under test of the 2D matrix, at the row i and column j. The basis of the algorithm is to perform a test on every and each of the matrix values, and finding out whether all the eight surrounding values are lower than the tested value. Once detected, all regional maxima contain data on the height of peaks as well as their respective 2D coordinates.

However, when dealing with overlapped peaks there can be peaks with its maximum hidden, and consequently they cannot be detected by an algorithm of the same type as mentioned above. In such cases, peak detection can still be achieved using the interpretation of the second derivative of the chromatogram. Fig. 2 exemplifies how the second-order derivative can be used to determine the retention time of strongly overlapping and shouldering 1D chromatographic peaks. The minimum values of the negative peaks of the second derivative (Fig. 2B) represent the retention time of the peaks appearing in the zero-order chromatogram (Fig. 2A). In the example of Fig. 2 there are a total of three peaks: one peak with only one identifiable maximum and two overlapping peaks with clearly visible peak maxima.

Computing the second-order derivative of a 2D chromatogram is not, however, straightforward as in the case of a 1D chromatogram. In a 1D function (one variable), the derivative process takes place only along the x axis dimension, whereas in a 2D problem (two variables) it is possible to compute partial derivatives in respect to both x (1st-dimension) and y (2nd-dimension) axes. The partial second-derivative of a 2D function usually generates the second-derivative in respect to x, $\partial z/\partial x^2$, the second-derivative in respect to y, $\partial z/\partial y^2$, and the second-derivative in respect to x and y, $\partial z/\partial x\partial y$. The detection of a 2D peak in cases of strongly overlapping peaks can be achieved by plotting the sum of the two partial second-derivatives, $\partial z/\partial x^2$ and $\partial z/\partial y^2$. The x and y coordinates of the minimum point of this 2D representation corresponds to the coordinates of the maximum point in the original 2D matrix. Fig. 3 illustrates the application of this methodology to a computer simulated 2D chromatogram, which is represented as a contour plot (Fig. 3A) and a three-dimensional (3D) surface plot (Fig. 3B) for a better visualization of the 2D peaks. The second derivative of the 2D chromatogram is shown in Fig. 3C and D, where the minimum values are represented by the dark-blue colour. The automatic search for the minima can then be implemented by applying an algorithm similar to that used to detect well defined 2D peaks as regional maxima (Fig. 1), although, in this case, it has been modified in order to detect regional minima. In the example of Fig. 3, the algorithm for peak detection was able to identify two strongly overlapping 2D peaks.

Finally, it should be mentioned that the numerical calculations for producing the second-derivatives can generate minor numerical instabilities, which are directly related to the original matrix size and grid spacing. A visual inspection of both the 3D raw data and second derivatives should provide clues on whether some degree of chromatogram smoothing or even peak modelling would be advisable.

2.2. Assessment of the quality of separation between peaks: the concept of peak purity revisited

After determining the number of 2D peaks and their respective retention times, the next step entails the evaluation of the resolution level associated to the peaks arrangement in the 2D chromatogram. The resolution metrics (Eq. (2)) for comprehensive 2D chromatography made available by Peters et al. [12] rely on the use of the saddle point concept. Although this concept is most suitable for expressing the degree of interaction in a system of two peaks, regardless their shape, it may fail when applied to a system containing a large amount of peaks, such as in the case of comprehensive 2D-LC of complex organic mixtures. The first problem emerges when attempting to apply the concept of saddle point as a measure of resolution between strongly overlapping and shouldering peaks. Fig. 4 shows a simulation of a 2D chromatogram where the proposed algorithm for peak detection identified three overlapping peaks (A, B, and C). Using the concept proposed by Peters et al.

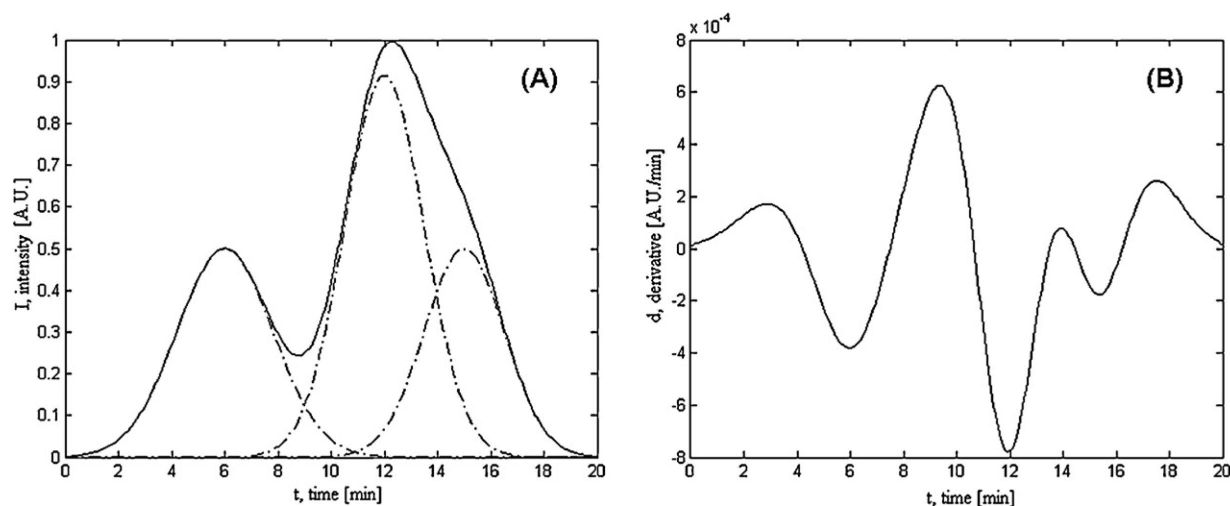

Fig. 2. Computer simulation of overlapping 1D chromatographic peaks: (A) composite chromatogram (solid line) and its deconvolution (dotted line) highlighting the existence of overlapped peaks; (B) second-order derivative of the composite chromatogram.

Fig. 3. Contour plot and three-dimensional surface plot of two 2D chromatographic peaks highly overlapped ((A) and (B), respectively) and the sum of their second partial derivatives ((C) and (D), respectively).

[12], it is possible to identify the saddle point between peaks A and B (S_{AB}) and between peaks B and C (S_{BC}), but no saddle point exist between the peaks A and C. This result suggest that the peak pair A–C must be excluded, meaning that the resolution between those two peaks is not calculated, which certainly hinders the assessment of the overall quality of separation of the 2D chromatogram.

The second problem that arise from the application of the algorithm proposed by Peters et al. [12] to comprehensive 2D-LC of complex mixtures, is related to the concept of peak vicinity. Those authors suggested that two peaks are neighbours if, after plotting the non-interpolated trajectory profiles between two sets of 2D peak clusters, there is no other peak region crossing that same trajectory. However, in a region with three interconnected consecutive 2D peaks, the tail of the first peak could be long enough that it may pass through the second peak and reach even the third peak. Since the vicinity is found by taking into consideration only the second peak, this 2D peak arrangement implies that the interaction between the first and the third peak is not accounted for and this combination is excluded from the computation of the overall resolution of the 2D chromatogram.

In order to overcome the above mentioned difficulties, an alternative approach is suggested based on the application of a peak purity concept as a measure of the quality of the 2D-LC separation. The peak purity criterion has been widely used in 1D-LC, and it quantifies the interference level for a given peak in the chromatogram [1,18]:

$$P_i = 1 - \left(\frac{A_i'}{A_i}\right) \quad (3)$$

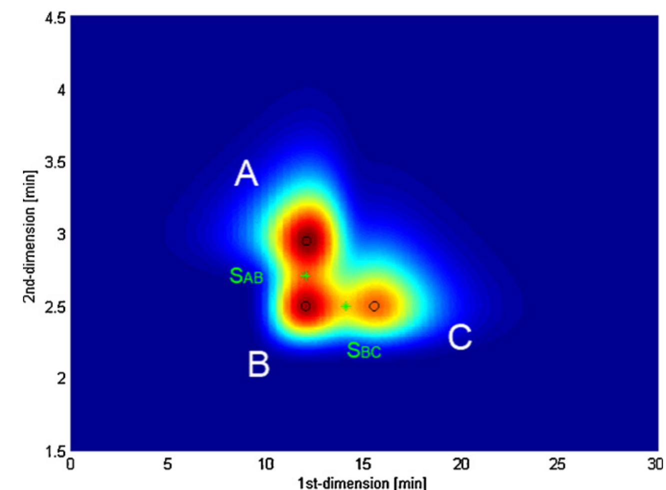

Fig. 4. Representation of a portion of a simulated 2D chromatogram exhibiting three peaks (A, B and C) and two saddle points (S_{AB} and S_{BC}).

where A'_i is the area under a given peak overlapped by the chromatogram yielded by the remaining peaks, and A_i is the total area of that peak. This criterion is a normalized measurement that ranges between 0 and 1, where the value 1 means that the peak is completely free of interference (i.e., completely resolved). In practical terms, the use of this concept in comprehensive 2D-LC is advantageous because its meaning is very intuitive: the obtained numerical value represents the exact portion of the interference-free 2D peak [1]. The most important feature of this figure of merit is that the evaluation of the resolution degree of a 2D chromatogram is now oriented to the estimation of the separation quality for each individual 2D peak, instead of each 2D peak pair (as in Eq. (2)). This is important not only for a better implementation of a general model for the CRF_{2D}, but also to avoid the problems of the multiple vicinities between several neighbouring peaks in a 2D liquid chromatogram. It should be further mentioned that it is possible to combine all the individual values of peak purity into a single global value. In the case of full resolution of all the 2D peaks, the maximum value for the sum of all individual values equals the number of 2D chromatographic peaks. This maximum value is important for applications in a CRF_{2D}, since it allows identifying the highest contribution of this term to the chromatographic function and also the deviation from the theoretical and maximum achievable value.

2.2.1. 2D peak fitting

In comprehensive 2D-LC, each chromatographic peak can be viewed as a surface on a 3D Cartesian plane, which means that the computation of the peak purity value of a 2D peak through Eq. (3) requires the replacement of the areas A'_i and A_i by the corresponding volume values. In 1D-LC, the computation of the overlapped peak area and, therefore, of peak purities, requires the prediction not only of the peak location but also of its profile. Usually, this is accomplished by using an asymmetrical peak model for peak simulation [18]. In 2D-LC, however, one is dealing with raw data obtained from real experimental procedures. In case of overlapping or shouldering 2D peaks, the original chromatographic data set do not give any information regarding the profile of the individual 2D peaks, which hinders any attempt to quantify the interference level for each of those 2D peaks and, consequently, their peak purity measure.

In order to overcome the above mentioned difficulties, and to obtain information on each individual 2D peak in the chromatogram, an alternative approach is suggested based on the use of a mathematical model to fit the original 2D chromatographic data. It should be mentioned, however, that the complexity of the 2D-LC process does not facilitate the proposal of a simple function to attain this goal. For the purpose of this study, the authors suggest the use of the sum of two 2D Log-normal functions to fit the overlapped peaks identified in a given section of the 2D chromatogram. The Log-normal function has been widely used for describing chromatographic peaks in 1D-LC. There are several formulae for this function reported in the literature and all are mathematically equivalent to each other [19]. The 2D Log-normal function used in this study for describing the profile of a single 2D peak is defined as:

$$h(t_{1D}, t_{2D}) = h_0 \exp\{-0.5\{[\ln(t_{1D}/t_{R,1D})/\sigma_{1D}]^2 + [\ln(t_{2D}/t_{R,2D})/\sigma_{2D}]^2\}\} \quad (4)$$

where $h(t_{1D},t_{2D})$ is the height at time t_{1D} and t_{2D} in the 1st and 2nd dimensions, respectively, h_0 is the maximal peak height, $t_{R,1D}$ and $t_{R,2D}$ is the retention time of the peak in the 1st and 2nd dimensions (i.e. peak coordinates), respectively, and σ_{1D} and σ_{2D} is the standard deviation of the peak in the 1st and 2nd dimensions, respectively. The fitting process will be implemented by a Trust-Region algorithm, with some pre-defined parameters, namely the peak coordinates ($t_{R,1D}$ and $t_{R,2D}$) and the range of values of the model parameters to be fitted, i.e. h_0, σ_{1D}, and σ_{2D}. The quality of the fitting can be assessed through the adjusted regression coefficient (R^2_{adj}), which represents the percentage of the variance accounted for by the fitted model. Once the best model is found for each of the 2D peaks, then it becomes possible to calculate the 2D peak purity based on the peak volume values (in the following sub-sections).

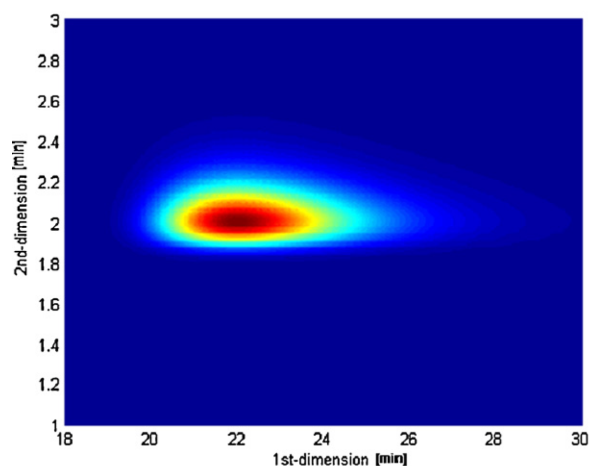

Fig. 5. Representation of a 2D peak simulated through an Extreme Value peak function, Eq. (5): h_0 = 0.5 (u.a.); $t_{R,1D}$ = 22.0 min and $t_{R,2D}$ = 2.0 min; σ_{1D} = 1.5 and σ_{2D} = 1.0; t_{1D} = 18.0 to 30.0 min and t_{2D} = 1.0 to 3.0 min; grid spacing of 0.15 in the 1st dimension and 0.05 in the 2nd dimension.

2.2.2. Measurement of the volume of a 2D peak for assessing peak purity

If one considers a 2D peak as a surface described by a positive function of two variables, $z = f(x,y)$, the volume of the region between the surface and the plane which contains its domain can be calculated through the definite double integral of $z = f(x,y)$. Despite of the laborious computation associated to the numerical integration of a function of two variables, this is the most simple and easiest mathematical pathway to calculate the volume under a surface on a 3D plane. However, identifying the volume of an overlapping region between two or more 2D peaks and calculating its numerical integral is a dreadful task since no mathematical equation for computing such volume intersection is currently available.

A possible alternative for estimating the volume of a 2D peak from the obtained raw data entails the sum of all the values of the 2D matrix grid and multiplying the obtained result by the spacing of the grid in both dimensions. In order to demonstrate the feasibility and accuracy of this numerical approach, a 2D peak (illustrated in Fig. 5) was generated through a 2D Extreme Value type function:

$$h(t_{1D}, t_{2D}) = h_0 \exp\{-\exp\{-[(t_{1D} - t_{R,1D})/\sigma_{1D}]\} - [(t_{1D} - t_{R,1D})/\sigma_{1D}] + 1\} \times \exp\{-\exp\{-[(t_{2D} - t_{R,2D})/\sigma_{2D}]\} - [(t_{2D} - t_{R,2D})/\sigma_{2D}] + 1\} \quad (5)$$

where the parameters appearing in this mathematical equation have the same meaning of those described in Eq. (4) (additional details on the values of these parameters and variables are given in caption of Fig. 5).

The volume of the 2D peak in Fig. 5 was calculated by two different ways: (a) using the double integral of Eq. (5) over the region between the coordinates 18 and 30 in the 1st dimension, and coordinates 1 and 3 in the 2nd dimension (obtained result = 0.551484); and (b) using the numerical approach here suggested (obtained result = 0.551499). A relative error of 0.0025% in the prediction of the volume values by the two mathematical approaches suggests that the method here developed is highly acceptable and fit for the

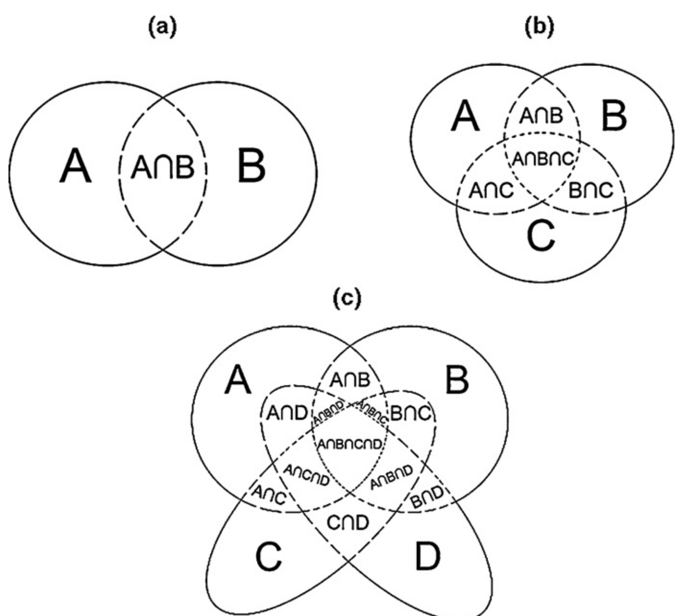

Fig. 6. Venn diagrams representing different cases of overlapping 2D peaks (A, B, C and D).

purpose of computing the volume of a 2D chromatographic peak. The relative error can be further improved by changing the grid spacing in both dimensions, which can be done at least at two different stages of the chromatographic process: data acquisition and data treatment.

2.2.3. Basis for calculation of the overlapped volume as a measure of peak purity

In the case of overlapping peaks, and after calculating the total volume of the 2D peaks, the following step entails the computation of the volume of the intersection region. In 2D-LC, however, this estimate is not an easy task because one single peak could be interacting with more than one neighbouring peaks. To illustrate and better understand the possible cases of overlapping peaks, one can make use of Venn diagrams, as depicted in Fig. 6, where the circles A, B, C and D represent the 2D chromatographic peaks.

In the first case, shown in the Fig. 6(a), the overlapping region is the same for peaks A and B, and it reflects the volume of the intersection between these two peaks ($V_{A\cap B}$). For this case, the purity of peak A, P_A, and the purity of peak B, P_B, can be calculated through Eqs. (6) and (7), respectively:

$$P_A = 1 - \left(\frac{V_{A\cap B}}{V_A}\right) \tag{6}$$

$$P_B = 1 - \left(\frac{V_{A\cap B}}{V_B}\right) \tag{7}$$

where V_A and V_B are the volume of peaks A and B, free from interferences, respectively. The volume of the intersection between these two peaks is calculated as $V_{A\cap B} = V_A + V_B - V_T$, with V_T being the total volume of the 2D peaks.

Fig. 6(b) exemplifies the case when there are interferences of second order; that is, when each 2D peak interacts with other two peaks, leading to the existence of an intersection region that is common to all the peaks ($V_{A\cap B\cap C}$). In this case, the value of $V_{A\cap B\cap C}$ should be removed from the computation of the overlapped volume of each peak (e.g. for peak A, the overlapped volume is calculated as $V_{A\cap B} + V_{A\cap C} - V_{A\cap B\cap C}$), and the purity of peaks A, B, and C can be calculated through Eqs. (8), (9) and (10), respectively:

$$P_A = 1 - \left[\frac{(V_{A\cap B} + V_{A\cap C} - V_{A\cap B\cap C})}{V_A}\right] \tag{8}$$

$$P_B = 1 - \left[\frac{(V_{A\cap B} + V_{B\cap C} - V_{A\cap B\cap C})}{V_B}\right] \tag{9}$$

$$P_C = 1 - \left[\frac{V_{A\cap C} + V_{B\cap C} - V_{A\cap B\cap C}}{V_C}\right] \tag{10}$$

Finally, Fig. 6(c) illustrates the case when there are interferences of second order (A∩B∩C, A∩B∩D, A∩C∩D, and B∩C∩D), which is an overlapped region common to three of four peaks, and an interference of third order (A∩B∩C∩D), which is an overlapped region common to all the four peaks. Using the same reasoning as above for the case of three overlapping peaks, the purity of peaks A, B, C, and D can be calculated as follows:

$$P_A = 1 - \left[\frac{V_{A\cap B} + V_{A\cap C} + V_{A\cap D} - V_{A\cap B\cap C} - V_{A\cap B\cap D} - V_{A\cap C\cap D} + V_{A\cap B\cap C\cap D}}{V_A}\right] \tag{11}$$

$$P_B = 1 - \left[\frac{V_{A\cap B} + V_{B\cap C} + V_{B\cap D} - V_{A\cap B\cap C} - V_{A\cap B\cap D} - V_{B\cap C\cap D} + V_{A\cap B\cap C\cap D}}{V_B}\right] \tag{12}$$

$$P_C = 1 - \left[\frac{V_{A\cap C} + V_{B\cap C} + V_{C\cap D} - V_{A\cap B\cap C} - V_{A\cap C\cap D} - V_{B\cap C\cap D} + V_{A\cap B\cap C\cap D}}{V_C}\right] \tag{13}$$

$$P_D = 1 - \left[\frac{V_{A\cap D} + V_{B\cap D} + V_{C\cap D} - V_{A\cap B\cap D} - V_{A\cap C\cap D} - V_{B\cap C\cap D} + V_{A\cap B\cap C\cap D}}{V_D}\right] \tag{14}$$

In the analysis of complex mixtures there are other possible scenarios of overlapping peaks, and the general mathematical model for computing the volume of the overlapped region (V_{O_i}) of a peak i can be written as follows:

$$\begin{aligned} V_{O_i} = &\sum_{i=1}^{n} V(p_i \cap p_{i+1}) + V(p_i \cap p_{i+2}) + \cdots + V(p_i \cap p_{i+n}) \\ &- V(p_i \cap p_{i+1} \cap p_{i+2}) - \cdots - V(p_i \cap p_{i+(n-1)} \cap p_{i+n}) + \cdots \\ &+(-1)^{n+1} V(p_i \cap p_{i+1} \cap \cdots \cap p_{i+n}) \end{aligned} \tag{15}$$

where p_i is the peak under study, and p_{i+n} are the remaining overlapped peaks. The purity of the peak (P_i) is then given by Eq. (16):

$$P_i = 1 - \left(\frac{V_{O_i}}{V_i}\right) \tag{16}$$

2.3. Global model of the CRF_{2D}

The definition of the new mathematical function for measuring and map the separation quality in comprehensive 2D-LC was performed on the basis of the objective function proposed by Duarte and Duarte [10] for assessing the quality of 1D liquid chromatograms of complex unknown samples (Eq. (1)). The new CRF2D is now expressed as:

$$\mathrm{CRF}_{2D} = \sum_{i=1}^{N} P_i + N - f(t) \tag{17}$$

where ΣP_i is the sum of all peak purity measures, N is the number of 2D peaks, and $f(t)$ is a "time-economy" function. This economy function is related to the time needed to complete the 2D separation process and it is given by the following equation:

$$f(t) = \left[\frac{(t_{R,L_{1D}} \times t_{R,L_{2D}}) - (t_{0_{1D}} \times t_{R,L_{2D}}) - (t_{0_{2D}} \times t_{R,L_{1D}}) + (t_{0_{1D}} \times t_{0_{2D}})}{(t_{R,L_{1D}} \times t_{R,L_{2D}})}\right] \tag{18}$$

where $t_{R,L_{1D}}$ and $t_{R,L_{2D}}$ are the retention times of the last eluted peaks in the 1st and 2nd dimensions, respectively, and $t_{0_{1D}}$ and $t_{0_{2D}}$ are the elution times corresponding to the void volumes of the columns of the 1st and 2nd dimensions, respectively. It should be further mentioned that when dealing with comprehensive 2D-LC analysis of complex organic mixtures, the use of the number of 2D

peaks as a secondary criterion for mapping the separation quality is useful and highly desirable. In an ideal situation where all the 2D peaks are well resolved, the maximum value of the sum of all peak purities equals the total number of peaks appearing in the chromatogram. However, in situations where the 2D chromatogram exhibit overlapping peaks, the value of the sum of all peak purities is lower than the total number of peaks. This feature allows to prospect the global quality of the peaks (Q, in percentage), which informs about the degree of completion of the separation for a set of 2D peaks:

$$Q \quad (\%) = \left(\frac{\sum_{i=1}^{n} P_i}{N} \right) \times 100 \qquad (19)$$

However, it is important to highlight that Q is a relative measure of the quality of the peaks and not a good replacement for the quantification of the separation process, since a 2D chromatogram can exhibit a Q close to 100% with a low number of well resolved peaks as compared to another 2D-LC profile, which has a better overall separation quality.

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).

Mobile phases for the 2D-LC experiments were prepared with HPLC grade acetonitrile (ACN) and methanol (MeOH), ammonium acetate (CH_3COONH_4), acetic acid (CH_3COOH), di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$), and sodium di-hydrogen phosphate (NaH_2PO_4). The composition of the mobile phases for both dimensions was adjusted according to the experimental conditions described in Section 3.2. Prior to use, the mobile phases were filtered through membrane filters (PVDF, Gelman Sciences) of 0.22 μm pore size.

The experiments were conducted using a mixture of four aromatic compounds: syringic acid ($HOC_6H_2(OCH_3)_2CO_2H$, Mw = 198.17 g mol^{-1}), ferulic acid ($HOC_6H_3(OCH_3)CH{=}CHCO_2H$, Mw = 194.18 g mol^{-1}), caffeic acid ($(HO)_2C_6H_3CH{=}CHCO_2H$, Mw = 180.16 g mol^{-1}), and 3-hydroxybenzoic acid ($HOC_6H_4CO_2H$, Mw = 138.12 g mol^{-1}). The concentration of each solute in the samples was in the range of 0.235–0.300 mg mL^{-1}, 0.215–0.369 mg mL^{-1}, 0.482–0.669 mg mL^{-1}, and 0.549–0.750 mg mL^{-1}, respectively. The samples were prepared by dissolving the appropriate amount of each compound in the mobile phase of the first dimension (Section 3.2).

3.2. Instrumentation and chromatographic conditions

The first-dimension (1st dimension) consisted of a JASCO semi-micro HPLC pump (model PU-2085 Plus), a Rheodyne injection valve (model 7725i) equipped with a 20 μL loop, and an Acclaim Mixed-Mode HILIC-1 column (Dionex, Sunnyvale, CA, USA; diameter 4.6 mm; length 150 mm; comprised of 5 μm high-purity, porous, spherical silica particles with 120 Å diameter pores bonded with alkyl diol functional groups). The 1st dimension was operated in isocratic mode using different mobile phase compositions comprising of 100 mM CH_3COONH_4/0.3% (v/v) CH_3COOH (pH 5) and different amounts of ACN: 20, 40, 60, and 80% (v/v). The flow rate was 0.025 mL min^{-1} and the temperature of the analytical column was maintained at 30 °C in a JASCO column oven (model CO-2065 Plus). In the second-dimension (2nd dimension), a JASCO quaternary low pressure gradient pump (model PU-2089 Plus) and a reversed-phase Kromasil® 100-5-C18 column (Eka Chemicals AB – Separation Products, Bohus, Sweden; diameter 4.6 mm; length 150 mm; particle size 5 μm; pore diameter 100 Å) were applied. The 2nd dimension was also operated in isocratic mode with a mobile phase composition consisting of 10 mM phosphate buffer (pH 6) and 8% (v/v) MeOH. The flow rate was 2.0 mL min^{-1} and the temperature of the analytical column was also maintained at 30 °C in a JASCO column oven. The outlet of the 2nd dimension column was connected to a JASCO fluorescence detector (model FP-2020 Plus) operating at emission/excitation wavelengths of 240/410 nm.

The 1st and 2nd dimensions were interfaced with an eight-port high pressure two-position interfacing valve (VICI® AG International, Schenkon, Switzerland) equipped with two identical 100 μL sampling loops. Modulation time was 240 s. The valve was controlled by the PSS WinGPC Unity software (Polymer Standards Service GmbH, Mainz, Germany) by receiving a start-up signal from a PSS Universal Data Center (model UDC 810).

The void volume of both 1st and 2nd dimension columns was measured by injecting HPLC grade ACN under the same instrumental operation conditions and with a mobile phase composition of 100 mM CH_3COONH_4/0.3% (v/v) CH_3COOH (pH 5) and 60% (v/v) ACN. The retention time of the columns void volume (also termed void time) was determined by the first baseline disturbance caused by the elution of the organic solvent. The void times were set at 62.5 min and 0.6 min for the 1st and 2nd dimension columns, respectively.

3.3. Software

The instrumentation was controlled and data set acquired with a PSS WinGPC Unity (Polymer Standards Service GmbH, Mainz, Germany) software. The algorithms developed in this study and data treatment were coded in MATLAB environment (The Mathworks Inc., Natick, MA, USA), in a computer with Intel(R) Core(TM)2 Duo CPU and 4.00GB RAM memory.

4. Results and discussion

4.1. Application of the CRF_{2D} to simulated 2D chromatograms

The fitting and simulation of chromatograms is of great importance in the field of interpretative optimization procedures in 1D-LC. In the literature there are a variety of theoretical and empirical mathematical equations that describe the shape of 1D chromatographic peaks [19]. The most simple mathematical peak function simulates the Gaussian elution profile. However, in both 1D and 2D-LC practice, perfectly Gaussian peaks are not usually observed and most of them are characterized by a large asymmetry (either tailing or fronting). In this work, the rationale behind the simulation of 2D chromatograms was not the prediction of the retention for a subsequent optimization study, but rather to assess the sensitivity of the developed CRF_{2D} (Eq. (17)) towards different resolutions, number of 2D peaks appearing in the chromatogram, and analysis time. For this purpose, a set of 2D peaks were generated by the 2D Extreme Value function, expressed in Eq. (5), and then summated to create simulated 2D chromatograms with different peak arrangements.

The 2D chromatographic profiles depicted in Fig. 7 aim at reproducing the possible situations obtained in real 2D-LC experiments of complex samples. The separation quality of each simulated chromatogram was assessed by two different ways: (a) using the peak purity concept developed in this study (Eq. (16)); and (b) the resolution measure (Eq. (2)) proposed by Peters et al. [12]. These two resolution criteria were used to compute the values of the CRF_{2D} (Eq. (17)) for each simulated chromatogram in order to evaluate and compare their performance as separation qualifiers of an

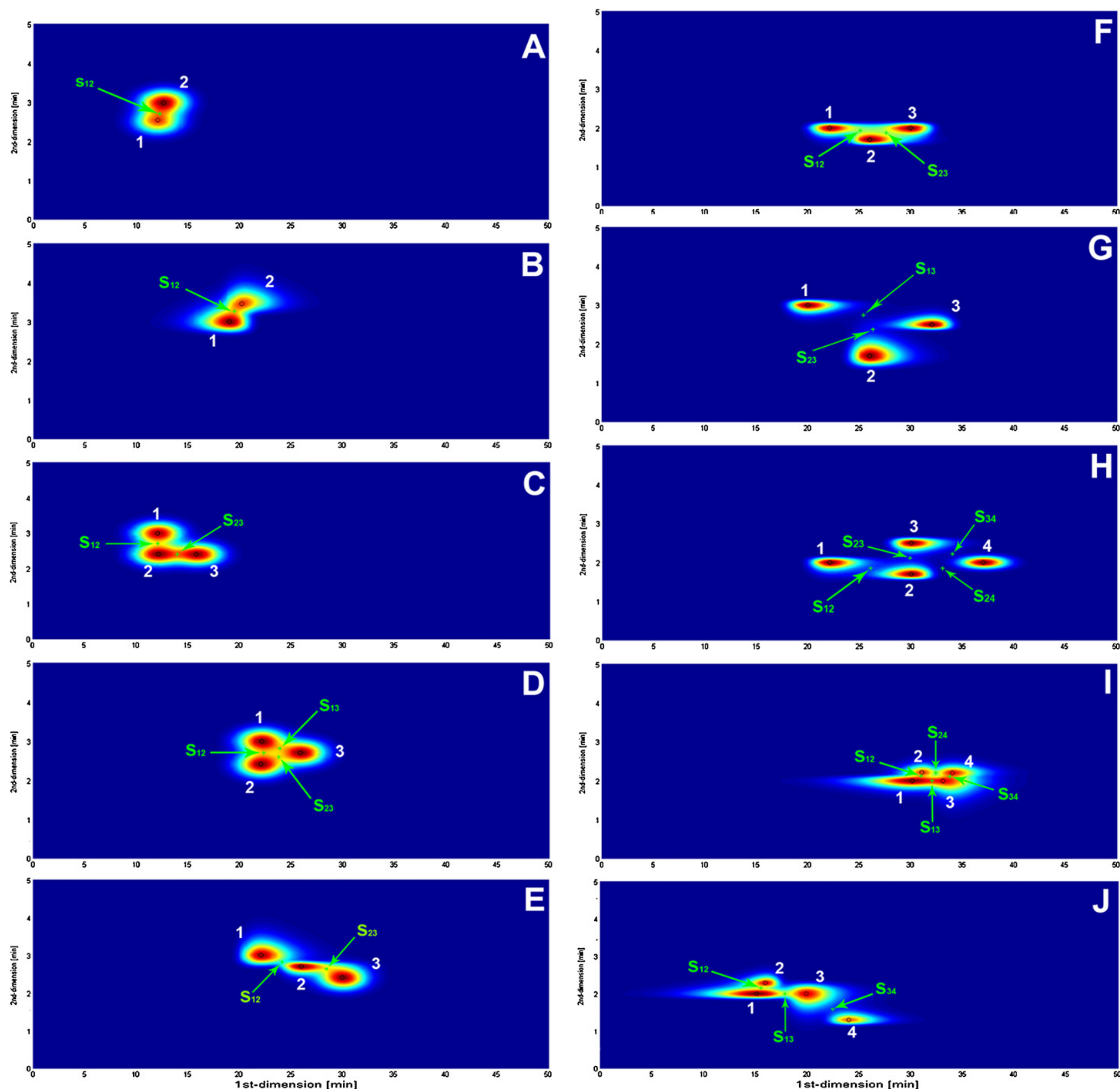

Fig. 7. Simulated 2D chromatograms used to demonstrate the performance of the developed CRF_{2D}. $S_{i,j}$ refers to the saddle point between peaks i and j according to the definition of Peters et al. [12]. Refer to Table 1 for further information on these simulations.

entire 2D chromatogram. Table 1 indicates, for each simulated chromatogram, the resolution values for each 2D peak pair, the peak purity values for each 2D peak, and the values of the CRF_{2D} estimated by means of the two resolution criteria. In both cases, the elution time corresponding to the column void volumes were set at 8 min for the 1st dimension and 0.5 min for the 2nd dimension. As can be seen, the resolution metric of Peters et al. [12] has a larger number of failures than the new peak purity measure proposed in this study. As depicted in chromatograms C and E to J (Fig. 7), there are peak pairs for which the detection of the saddle point fails, meaning that the resolution and, subsequently, the CRF_{2D} cannot be calculated for these 2D chromatograms. Peak pair 1–3 in chromatogram C is an example: intuitively, one may consider that peaks 1 and 3 are neighbours and, therefore, it is expected to have a saddle point between these two peaks. However, according to the definition of Peters et al. [12] for peak-vicinity, peaks 1 and 3 are not considered to be "neighbours" since the trajectory profile connecting the peak maxima of these two peaks pass through the peak region of peak 2. Therefore, computing the resolution between this 2D peak pair is not considered to be meaningful because this peak pair fails the peak-vicinity test. Furthermore, even if the 2D peaks 1 and 3 were recognized as "neighbours", the interference of peak 2 completely distort the surface region between those two peaks, which hinders the detection of a saddle point. On the other hand, for chromatograms A, B and D, all peaks pass the peak-vicinity test and, therefore, the resolution (Eq. (2)) can be calculated between

Table 1
Resolutions, peak purities, and CRF_{2D} values calculated for the simulated 2D chromatograms shown in Fig. 7.

Simulation	Resolution (R_s)[a]						Peak purity (P_i)[b]				CRF_{2D} (R_s)[c]	CRF_{2D} (P_i)[d]
	1–2	1–3	1–4	2–3	2–4	3–4	1	2	3	4		
A	0.65	–	–	–	–	–	0.78	0.82	–	–	2.3	3.3
B	0.70	–	–	–	–	–	0.88	0.83	–	–	2.1	3.2
C	0.75	FAIL	–	0.68	–	–	0.86	0.73	0.81	–	FAIL	5.0
D	0.74	0.74	–	0.75	–	–	0.79	0.80	0.80	–	4.6	4.8
E	0.90	FAIL	–	0.75	–	–	0.96	0.67	0.84	–	FAIL	4.7
F	0.82	FAIL	–	0.81	–	–	0.82	0.66	0.70	–	FAIL	4.6
G	FAIL	1.4	–	1.2	–	–	1.0	0.98	0.96	–	FAIL	5.3
H	1.1	FAIL	FAIL	1.2	1.5	1.4	0.92	0.90	0.98	1.0	FAIL	7.2
I	0.66	0.63	FAIL	FAIL	0.76	0.65	0.85	0.81	0.73	0.63	FAIL	6.4
J	0.76	0.77	FAIL	FAIL	FAIL	1.1	0.85	0.68	0.91	0.91	FAIL	6.8

FAIL: saddle point not detected.
[a] Resolution measure (R_s, Eq. (2)) of Peters et al. [12] using the valley-to-peak ratio and saddle point concept.
[b] Peak purity (P_i) calculated through Eq. (16).
[c] CRF_{2D} calculated through Eq. (17) using the resolution measure (R_s, Eq. (2)) of Peters et al. [12] ($t_{0_{1,D}} = 8$ min; $t_{0_{2,D}} = 0.5$ min).
[d] CRF_{2D} calculated through Eq. (17) using the peak purity (P_i) concept of Eq. (16) ($t_{0_{1,D}} = 8$ min; $t_{0_{2,D}} = 0.5$ min).

them, allowing to compute and assess the overall quality of the 2D chromatographic separation. On this regard, the CRF_{2D} considers chromatograms A and B of less quality than chromatogram D, being this a direct consequence of their lower number of peaks.

When using the 2D peak purity concept (Eq. (16)) as the quality separation criterion, the CRF_{2D} function also classifies A and B as the less desirable chromatograms, which again reflects the importance of the number of distinguishable 2D peaks as a secondary requirement for ranking the quality of the chromatographic separations. Chromatograms C to G all exhibit the same number of 2D peaks. However, and due to the emphasis that it places on the purity of each peak, chromatogram F with a high degree of peak overlapping is considered of less quality than chromatogram G, which exhibits peak purities close to 1.0. The order by which the CRF_{2D} rank the quality of these chromatograms depends not only on the purity of each peak, but also on the total time of analysis. For example, chromatograms C and D exhibit similar overall resolution (ΣP_i is around 2.4). However, chromatogram C elute in a smaller time window, which contribute to rank this chromatogram as a more desirable chromatogram. The importance of the peak purity term is also reflected in the values of the CRF_{2D} of chromatograms H, I and J; although exhibiting the same number of peaks, values of peak purity close to 1.0 in chromatogram H contributes to rank this chromatogram as the most desirable one.

4.2. *Application of the CRF_{2D} for assessing the separation quality of complex mixtures*

The validity of the new CRF_{2D} for qualifying the separation degree attained under different 2D-LC conditions was further assessed through a set of experiments with a mixture of four aromatic compounds, and using the amount of ACN in the mobile phase of the 1st dimension as the experimental variable (see Section 3.2 for additional details on the chromatographic conditions). It must be emphasized, however, that the objective of this study was not to separate these organic compounds by polarity and/or hydrophobicity, but to demonstrate the efficiency of the new CRF_{2D} for measuring the separation quality of real comprehensive 2D liquid chromatograms. These experiments will also provide good indications on the feasibility of the CRF_{2D} as a valuable tool for the design of suitable comprehensive 2D-LC procedures.

Before assessing the overall separation quality of each 2D chromatogram, it is necessary to perform some data pre-treatment in order to reduce the effects of the experimental variations. Firstly, the experimental data points in the 1st dimension of the 2D matrix were interpolated using a spline algorithm. Secondly, this same algorithm was applied to the experimental data points of the 2nd dimension, but in this case to remove data points in order to smooth the experimental data set and to generate a square grid with spacing as close as possible of that used in the 1st dimension. Thirdly, spurious data points caused by low levels of random background noise were removed by considering a threshold for the minimal peak height acceptability. In this study, a peak was accepted as such when its height was higher than 10% of the height of the most intense peak in the chromatogram. After these data pre-treatment procedures, the methodology for 2D peak detection and CRF_{2D} calculation followed closely the steps described in Section 2.

Fig. 8 depicts the regions of interest of each 2D chromatogram of the mixture of four aromatic compounds obtained for the different amounts of ACN in the mobile phase of the 1st dimension. Before going any deeper into the application of the algorithm for peak detection suggested in Section 2.1, a visual inspection of the 2D chromatograms in Fig. 8A and C, and D indicates a number of peaks higher than would be expected from the analysis of the mixture of four organic compounds. These results are likely to be a consequence of the occurrence of unpredictable retention mechanisms (e.g. hydrophobic interactions) of the solutes into the column packing material. The apparent high number of peaks as a result of peak broadening in the time window between 2.5 and 5 min in the 2nd dimension is a clear example of such phenomena, i.e. strong adsorption between the solute (in this case, ferrulic acid) and the column stationary phase (reversed-phase C18). In light of these results, it is recommended to devote a special attention to the optimization of the chromatographic conditions in order to minimize the occurrence of unwanted interactions between the stationary phase responsible for poor peak shape and unpredictable retention, and the analytes.

At the completion of the peak detection algorithm, 10 peaks have been detected in the 2D chromatogram of Fig. 8A. These peaks were divided into two clusters of overlapping peaks: the cluster containing the peaks 1–6, and the cluster with peaks 7–10. The time windows of the chromatogram containing these two clusters were fitted using a mathematical model of 2D Log-normal functions (Eq. (4)). The quality of the fit procedure is supported by the values of the R^2_{adj}: 0.9186 and 0.9696, respectively. In the chromatogram of Fig. 8B, 4 peaks were recognized using the proposed algorithm: peaks 1–3 are overlapped, whilst peak 4 is isolated with no surrounding peaks. The time window containing peaks 1–3 was fitted with 2D Log-normal functions, resulting in a R^2_{adj} of 0.9383. In the chromatogram of Fig. 8C, the peak detection algorithm was able to

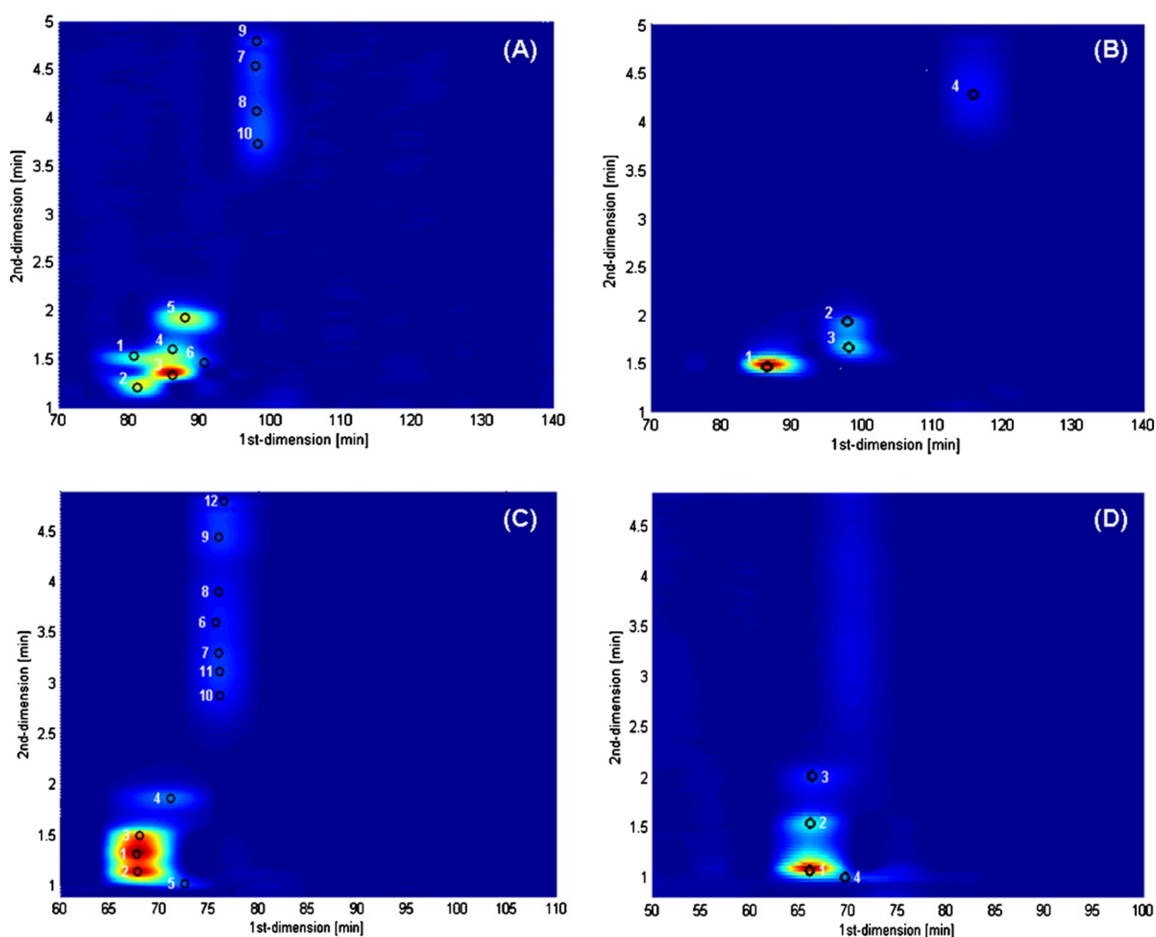

Fig. 8. 2D chromatograms of mixtures of four aromatic compounds obtained with different amounts of ACN in the mobile phase of the 1st dimension: 20% (A), 40% (B), 60% (C) and 80% (D). The retention time axes were expanded for a better visualization of each 2D chromatogram, and the detected peaks are identified by a number.

detect 12 peaks, which were divided into two clusters: one cluster containing peaks 1–5, and a second cluster containing peaks 6–12. The R^2_{adj} for the fit of the time windows of these two clusters were 0.9805 and 0.9699, respectively. Finally, the peak detection algorithm has identified only 4 peaks, all overlapped in one cluster, in the chromatogram depicted in Fig. 8D. The fitting procedure of the time window of this cluster exhibited an R^2_{adj} of 0.9060. The adequacy of the fitted mathematical model in chromatogram D seems to be relatively low. This result is likely to be a consequence of the low level of the detector response (below the stipulated 10% threshold), which is confounded with the background noise of the 2D chromatogram. Nevertheless, these results highlight the success of both peak detection algorithm and fitting procedure in determining the coordinates of the 2D peaks automatically from data obtained from real experimental 2D chromatograms. These results also illustrate the importance of selecting appropriate peak detection thresholds and also the difficulties in identifying the 2D peaks just by visual inspection of the chromatograms.

Once the peaks have been detected and fitted in each 2D chromatogram, the purity of each peak can be computed, and the assessment of the overall separation quality using the new CRF_{2D} can be completed. The values of the peak purities and of the CRF_{2D} obtained for each experiment are shown in Table 2. It can be observed that the highest values of the CRF_{2D} were obtained for experiments A and C, whereas the lowest CRF_{2D} value was obtained for experiment D. The reason why the new CRF_{2D} ranks chromatogram C as the most desirable one in comparison with chromatogram A, relies on the number of detected peaks, which are higher for chromatogram C. In fact, the time needed to complete the analysis is similar in both chromatograms A and C, as well as the global quality of the peaks (Q is equal to 52 and 53% for chromatograms A and C, respectively). On the other hand, chromatograms B and D exhibit the same the number of detected peaks, although the CRF_{2D} classifies chromatogram D as being of less quality. This result is likely to be explained by the lower chromatographic separation degree exhibited by chromatogram D, being this reflected in the values of the global quality of the peaks (Q is equal to 89% and 67% for chromatograms B and D, respectively). These results demonstrate that the new CRF_{2D} efficiently discriminate between chromatograms with different separation quality, in terms of the number of 2D peaks and degree of the separation reached for each 2D peak. Nevertheless, and disregarding the fact that one is dealing with a mixture of well-known compounds, the qualitative grading of the 2D chromatograms using the CRF_{2D} (Table 2) parallels the chromatographic data, i.e., the CRF_{2D} values are in line with the intuitive choice of an analyst. It should be mentioned, however, that when dealing with complex mixtures of organic compounds, the advantage of using a CRF_{2D} for measuring and map the quality of the 2D chromatographic separation only holds if a careful attention is devoted to the development of reliable chromatographic conditions, including the selection of appropriate columns in both dimensions (i.e. type of stationary phases and column sizes), mobile phases (i.e. organic modifier and buffer), elution modes (i.e. isocratic or gradient elution), flow rates, and volume of the transferred

Table 2
Individual peak purity and CRF_{2D} values for the four 2D chromatograms presented in Fig. 8.

Experiment	Peak purity (P_i)[a]												CRF_{2D} [b]
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.620	0.816	0.647	0.391	0.760	0.433	0.328	0.293	0.479	0.421	–	–	14.9
B	0.995	0.728	0.855	1.00	–	–	–	–	–	–	–	–	7.18
C	0.538	0.728	0.693	0.941	0.665	0.606	0.315	0.430	0.491	0.542	0.033	0.345	18.2
D	0.922	0.878	0.785	0.106	–	–	–	–	–	–	–	–	6.62

[a] Peak purity (P_i) calculated through Eq. (16).
[b] CRF_{2D} calculated through Eq. (17).

fractions and frequency of the sample transfer between the 1st and 2nd dimension.

It should also be mentioned that the time-economy function (Eq. (18)) does not exert a major influence on the values of the CRF_{2D}, since it can only discriminate between chromatograms bearing the same number of peaks and with similar peak purity values. In the case of the 2D chromatograms depicted in Fig. 8, the only situation where the time-economy function had some effect on the values of the CRF_{2D} was when comparing experiments B and D, but though not enough to change the qualitative grading of these 2D chromatograms on the basis of the CRF_{2D} values.

5. Conclusions

A new CRF_{2D} has been proposed for the estimation of the quality index of separation in 2D-LC of complex organic mixtures. This function has evolved from the objective function proposed by Duarte and Duarte [10] for assessing the quality of 1D liquid chromatograms of complex unknown samples. The new CRF2D also takes into account the most important criteria for measuring and rank the separation quality of any chromatographic procedure: overall resolution of the 2D chromatogram, number of distinguishable 2D peaks in the chromatogram, and time needed to complete the analysis. One important outcome of this study was the development of an algorithm for detecting 2D peaks automatically from real experimental 2D-LC data, followed by the application of a mathematical model to fit the obtained chromatographic data. The other important problem tackled in this study was the estimation of the quality of the 2D chromatographic separation for complex organic mixtures, which was accomplished by adapting the peak purity concept to 2D separations. The use of this concept changed the paradigm of the classical resolution metric for 2D-LC, which is now oriented to each individual 2D peak instead of each 2D peak pair. Furthermore, this 2D peak purity concept is a normalized measure that does not require the prior definition of a minimum acceptable threshold, thus facilitating the combination of each elementary 2D peak purity into a single global value. The theoretical study with simulated 2D chromatograms indicated that the CRF_{2D} places more emphasis on the purity of each 2D peak over total time of analysis. The number of distinguishable 2D peaks was also found to be an important secondary requirement for ranking the quality of the chromatographic separations.

The validity of the new function for qualifying the 2D separation degree attained under different chromatographic conditions was further assessed using a mixture of four organic compounds. It was demonstrated that the new CRF_{2D} gives indicative values for discriminating between 2D chromatograms with different separation quality. Nevertheless, the use of an algorithm that detects all 2D peaks may provide false information about the sample, which means that an adjustable threshold must be defined for the peak detection. The results from this study also provide information on the importance of developing optimum chromatographic conditions in order to minimize the occurrence of unpredictable retention phenomena between the analytes and the column packing material. Future publications will illustrate the applicability of the developed objective function to build chromatographic fingerprints of different complex samples.

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