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Criteria for optimizing the separation of target analytes in complex chromatograms

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Abstract

Optimization procedures require adequate criteria to assess the quality of the separation. So far, the vast majority of reports on the subject deal with the situation in which all peaks need to be separated ('complete optimization'). In practice one of the most important requirements for optimization criteria is their ability to deal with limited optimization, i.e. with situations in which only a limited number of solutes in a sample is relevant. In this paper, the adaptation of the most widely used optimization criteria to limited optimization is discussed. Among the most useful criteria are the minimum resolution and the calibrated normalized resolution product. The characteristics and objectives of these two criteria are discussed in detail. The case of non-ideal separations (featuring asymmetrical peaks and/or peaks of vastly different areas) is also investigated. The discussion and evaluation of optimization criteria are based on simulated and experimental data obtained during HPLC optimization studies.

Keywords: Chromatography; Optimization

1. Introduction

The development and optimization of chromatographic separations will rely increasingly on computer-assisted procedures because of (1) time saving, (2) better quality of the results and (3) accessibility and flexibility of efficient optimization software. An essential part of any optimization strategy is a clear definition of the goal(s) of the process. A mathematical description of such a goal is called an optimization criterion. The quality and flexibility of the criteria are among the essential factors determining the applicability of selectivity-optimization procedures. Indeed, the result of an optimization process depends on the selected optimization criterion, so that the latter has to be defined in the context of the objectives of the separation. In our opinion, the main reason for the modest advent of commercial optimization software is that most of them are too simplistic to deal with the complex problems encountered in the real chromatographic world (e.g. presence of asymmetrical peaks or exis-

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tence of irrelevant peaks). Improved optimization criteria are required that rigorously deal with this complexity and yet can be implemented in such a manner that is simple to use by the chromatographer in practice.

Many different criteria (or 'objective functions') have been suggested for describing the quality of chromatographic separations [1-3]. Most of these have been defined for the general situation in which all the solutes must be separated. However, analysts are often interested in the separation of a limited number of solutes in a complex matrix. Obvious examples are the monitoring of drug levels in body fluids and of environmental pollutants. In our opinion, one of the most important requirements for adequate optimization criteria is their ability to deal with limited optimization, i.e. with situations in which only a limited number of solutes in a sample is relevant. Relevant peaks need to be separated from all other peaks, but irrelevant peaks do not need to be separated from each other. A special case of a usually irrelevant peak is a signal around the dead time of the column (t_0).

The concept of limited optimization has been addressed in the literature [1,2,4-6], but a rigorous discussion of the potential of various criteria in this context has not yet been presented. The objective of this paper is to discuss the adaptation of the most recommended optimization criteria (see Refs. [7-9]) to deal with limited optimization. All criteria discussed are calculated from the usual chromatographic parameters (retention times, peak heights, peak areas and asymmetry factors), without relying on complex algorithms [3] or impractical deconvolution equations [10]. Practical, non-ideal separations (featuring asymmetrical peaks or peaks of very different areas) are covered for the first time. Specifically, the discussion will focus on normalized resolution product criteria adapted to limited optimization. The major advantage of this type of criteria is to favor the separation of solutes into groups (ideally all relevant peaks separated from all irrelevant ones; all of the latter are ideally confounded). This behavior opens the way towards the optimization of multi-column and, possibly, group-type separations.

The discussion and evaluation of optimization criteria are based on simulated and experimental data obtained during HPLC optimization studies.

2. Theory

There are fundamentally two types of criteria, namely elementary criteria, describing the separation between two adjacent peaks, and overall criteria, that can be used to describe the quality of an entire chromatogram. First we will briefly review elementary and overall criteria used in complete optimization (all peaks are considered to be relevant). Thereafter, criteria adapted to limited optimization will be introduced.

2.1. Elementary criteria

Three elementary criteria are considered in this study. These are listed in Table 1. The separation factor, S, is independent of the column efficiency and its calculation requires only retention times (t_R). In addition, the resolution, R_S, takes into account the efficiency of the separation. In our case, we use the average number of theoretical plates for the two individual peaks involved. Use of R_S rather than S is necessary if the number of theoretical plates may vary substantially (e.g. in pH optimization) or when different columns are being compared. The third possible elementary criterion is the effective resolution, R_1, which was developed by Schoenmakers et al. [11]. This resolution function is especially applicable in non-ideal situations (chromatograms containing asymmetrical peaks and/or peaks of vastly different areas). It takes into account the individual widths of the two peaks, the asymmetry factors, and the peak heights. When we consider the separation of a pair of peaks, two values of the effective resolution exist. The first one, ${}^{i}R_{n}$, describes the extent to which peak i is separated from the next peak (j) and the second value, ${}^{j}R_{p}$, reflects the extent to which peak j is separated from the previous one (i). Generally, in complete optimization, the lowest value characterizing a particular separation (i.e. ${}^{i}R_{n}$ or ${}^{j}R_{p}$) is kept. Another approach to deal with non-ideal separations was suggested by Sekulic and Haddad [12,13]. However, the equations derived by Schoenmakers (effective resolution) are more convenient in interpre-

Table 1
Elementary criteria

Symbol	Name and mathematical description
S	separation factor $S_{ji} = \frac{t_{R,j} - t_{R,i}}{t_{R,j} + t_{R,i}} = \frac{k_j - k_i}{k_j + k_i + 2}$ (1)
R_S	resolution $R_{S,ji} = S_{ji} \frac{\sqrt{\frac{1}{2}(N_i + N_j)}}{2} = \frac{S_{ji}\sqrt{N}}{2}$ (2)
R_1	effective resolution (lower of following two values) ${}^{i}R_{n} = \frac{(t_{R,j} - t_{R,i})(1 + A_{S,i})(1 + A_{S,j})\sqrt{N_i N_j}}{4A_{S,i}t_{R,i}(1 + A_{S,j})\sqrt{N_j} + 4t_{R,j}(1 + A_{S,i})\sqrt{N_i}\sqrt{1 + 0.5\ln(h_j/h_i)}}$ (3a) ${}^{J}R_{p} = \frac{(t_{R,j} - t_{R,i})(1 + A_{S,i})(1 + A_{S,j})\sqrt{N_i N_j}}{4A_{S,i}t_{R,i}(1 + A_{S,j})\sqrt{N_j}\sqrt{1 + 0.5\ln(h_i/h_j)} + 4t_{R,j}(1 + A_{S,i})\sqrt{N_i}}$ (3b)

t_R = retention time; k = capacity factor; N = number of theoretical plates; A_S = asymmetry factor; h = peak height.

tive optimization procedures as resolutions can be calculated by modelling the characteristics of the individual solutes. For the same reason, peak–valley ratios are not discussed in this paper. Typically, these empirical criteria can be obtained from a chromatogram but they cannot easily be predicted from the modelled chromatographic data of the individual solutes.

2.2. Overall criteria

The eight overall criteria considered in this work are listed in Table 2. Any one of these has to be maximized during the optimization process. We select these eight criteria for several reasons:

1. They correspond to the most usual objectives of the chromatographer.
2. Their use is recommended by the existing expert systems [7–9].
3. If they combine several factors, such as resolution and analysis time, then this is done in a correct fashion. Criteria based on some kind of *arbitrary* combination of resolution and time will in principle guide the chromatographer to an *arbitrary* optimum. Many such criteria (often called CRF or COF) have been suggested [2], but their use is not recommended.

The most classical and obvious overall criterion is the minimum resolution (based on S, R_S or R_1 as the elementary criterion). In fact, maximizing the minimum resolution is equivalent to minimizing the required number of theoretical plates. Although frequently used [14,15], the minimum resolution presents certain disadvantages. Indeed, this criterion ignores the analysis time, so that the objectives of the chromatographer are often better expressed by a threshold criterion (minimizing the analysis time, while exceeding the required minimum resolution). Moreover, the minimum resolution considers only the separation of the most critical peaks pair. The distribution of the peaks throughout the chromatogram is not taken into account.

One way to achieve a specified minimum resolution for all pairs of peaks is to use the information content of the chromatograms. This criterion was suggested by Mazerolles et al. [16] and is based on the information theory and the early work of Massart et al. [17]. Its maximum value corresponds to chromatograms in which all peaks are separated by at least a postulated minimum resolution. The adaptation of this criterion to limited optimization is not discussed in this paper and will be addressed in future work.

The normalized resolution product (r) and the calibrated normalized resolution product (r^*) were defined to promote an equal spreading of the peaks over the chromatogram. r is the product of all resolution values, each divided by the average resolution. It reaches its maximum value of one if all resolution values are equal. The objective of this criterion is to obtain an equal distribution of the peaks over the chromatogram. Contrary to r,

Table 2
Overall criteria

Symbol	Name and mathematical description
$c_{\min}$	minimum resolution $C = \min \forall\ c_{ji}\ (i = 1^{a} \text{ to } n-1;\ j = i+1)$ (4)
$c \cap k$	threshold resolution IF $c_{\min} \geq \varepsilon$ THEN $C = \frac{1}{1+k_{\omega}}$ ELSE $C = 0$ (5)
r	normalized resolution product = NRP $C = \prod_{i=1}^{n-1} c_{ji} / \bar{c}$ (6)
r^{*}	calibrated normalized resolution product = CNRP $C = \prod_{i=0}^{n-1} c_{ji} / \bar{c}$ (7)
$[t_{\mathrm{ne}}]_{f,d}^{-1}$	minimum required analysis time (constant flow and particle size) $C = \frac{c_{\min}^{2}}{1+k_{\omega}}$ (8)
$[r_{\mathrm{nt}}^{*}]_{f,d}$	time-corrected resolution product (constant flow and particle size) $C = \frac{\sqrt[n]{r^{*}}\, c_{\min}^{2}}{1+k_{\omega}}$ (9)
$[t_{\mathrm{ne}}]_{p}^{-1}$	minimum required analysis time (constant pressure drop) $C = \frac{c_{\min}^{4}}{1+k_{\omega}}$ (10)
$[r_{\mathrm{nt}}^{*}]_{p}$	time-corrected resolution product (constant pressure drop) $C = \frac{\sqrt[n]{r^{*}}\, c_{\min}^{4}}{1+k_{\omega}}$ (11)

c = selected elementary criterion (S, R_S or R_1); $\bar{c}$ = average value of the elementary criterion; k_{ω} = capacity factor of the last peak; $c_{\min}$ = lowest value of c for any pair of peaks in the chromatogram; ε = threshold value for elementary criterion; n = number of solutes.
[a] $i = 0$ to $n-1$ in case a t_0 peak is considered.

r^{*} includes a hypothetical peak at $t = t_0$ (t_0 is the hold-up time of the column). Using r^{*} will help to avoid situations in which all pairs of peaks show equal resolution values, but at high values of the capacity factor (i.e. long retention times). r^{*} will be equal to one if the resolution between all pairs of peaks is the same and equal to the resolution between the first peak and the (hypothetical) peak at $t = t_0$.

If the length of the column may be changed after the selectivity-optimization process, but if the flow-rate and the particle size (or the diameter of open columns) will be kept constant, then Eqs. (8) and (9) correspond most closely to the objective of realizing a good separation in the shortest possible analysis time. The time-corrected resolution product, $[r_{\mathrm{nt}}^{*}]_{f,d}$, pays some attention to an equal spreading of the peaks over the chromatogram. Similarly, under conditions of constant pressure drop, the optimization criteria described by Eqs. (10) and (11) may be used.

A detailed description of the different criteria can be found in Refs. [1,18].

The three different elementary criteria (Table 1) and the eight overall criteria (Table 2) together create 24 different possibilities. Selecting the most suitable criterion requires a great deal of high-level knowledge. Therefore, expert systems have been developed for helping chromatographers to select the most appropriate optimization criterion [7–9].

2.3. Criteria adapted to limited optimization

So far, we have only considered criteria for situations in which all peaks are treated as being of equal importance. Now, we will focus on situations in which only some components of a mixture need to be separated (relevant peaks) from a matrix of other (irrelevant) peaks. In such cases, which we will refer to as limited optimization, the criteria listed in Table 2 have to be adapted in order to express the quality of the separation.

Of the criteria previously described, some can be applied easily in case of limited optimization. We distinguish the appropriate criteria for limited optimization by underlining the corresponding symbols. For $\underline{S}_{\min}$ or $\underline{R}_{S,\min}$, the lowest value must be selected from the relevant pairs of peaks. A pair of peaks is relevant if at least one of the two peaks is relevant. For $\underline{R}_{l,\min}$ the situation is a bit more complicated. As described above, for each pair of peaks two values of the effective resolution exist, ${}^{i}R_{n}$ and ${}^{j}R_{p}$. In the standard case (complete optimization) we generally keep the lowest value characterizing a particular separation (i.e. ${}^{i}R_{n}$ or ${}^{j}R_{p}$). In limited optimization we have to keep only relevant values and these will not necessarily be the lowest ones.

Fig. 1 illustrates two possible situations. In this figure, R refers to a relevant peak and I to an irrelevant one. In Fig. 1a, we have one relevant peak surrounded by two irrelevant ones. Peaks 1 and 3 (both irrelevant) have no relevant value. The relevant peak (peak 2) has two relevant values of the effective resolution: ${}^{2}R_{p}$ referring to the separation between peak 2 and the previous one and ${}^{2}R_{n}$ referring to the separation from the next peak. In Fig. 1b, peak 1 is relevant and has one relevant value, ${}^{1}R_{n}$, referring to the separation between peak 1 and the next one. Peak 2 has no relevant value. For peak 3, ${}^{3}R_{p}$ and ${}^{3}R_{n}$ are both relevant. For peak 4, ${}^{4}R_{p}$ referring to the separation between this peak and the previous one is relevant. As ${}^{3}R_{n}$ and ${}^{4}R_{p}$ refer both to the separation between peaks 3 and 4, only the lowest of these two values will be kept (as in complete optimization). $\underline{R}_{l\min}$ is the lowest relevant value of R_{l}.

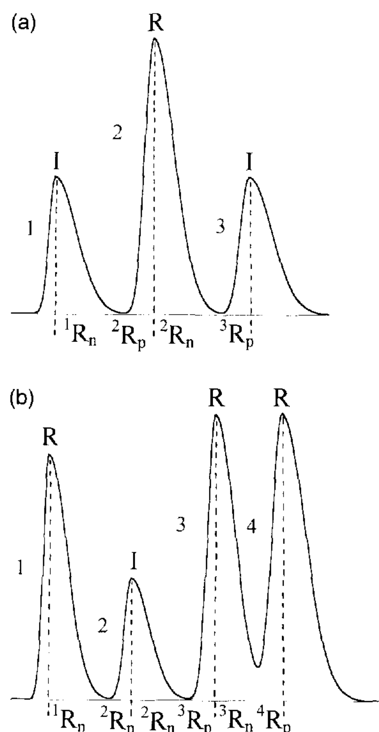

Fig. 1. Two schematic chromatograms illustrating examples of limited optimization.

For the threshold resolution (Eq. (5)) and for the minimum required analysis time (Eq. (8) or (10)), the analysis time is always determined by the capacity factor of the last peak, whether or not this one is relevant. The minimum resolution value is the lowest relevant value of S, R_S or R_1.

The calculation of the normalized resolution product is not as obvious in limited optimization as it is in complete optimization. Indeed, we have to promote a situation in which all the relevant peaks are equally distributed throughout the chromatogram, and where, ideally, all irrelevant peaks are confounded into one peak. When using the normalized resolution product ($\underline{r}$), this irrelevant peak may end up anywhere in the chromatogram. In case the calibrated normalized resolution product ($\underline{r}^*$) is used, it is ideally confounded with the t_0 peak. In our opinion, the following expressions are the most appropriate ones for calculating these criteria (see also Ref. [4]). The normalized resolution product ($\underline{r}$) can be defined as

$$\underline{r} = \prod_{k=1}^{d} \underline{c}_k / \bar{c} \tag{12a}$$

where

$$\bar{c} = \frac{1}{p - B} \sum_{i=1}^{n-1} c_{ji} \tag{13a}$$

where c is the elementary criterion (S, R_S or R_1), n the number of solutes, $\underline{c}_k$ the relevant values of c, d the number of relevant values of c, p the number of relevant peaks, and $B = 0$ except when there is no irrelevant peak (then $B = 1$).

When a (hypothetical) peak is assumed at $t = t_0$, the calibrated normalized resolution product can be calculated using a similar expression:

$$\underline{r}^* = \prod_{k=1}^{d} \underline{c}_k / \bar{c} \tag{12b}$$

where

$$\bar{c} = \frac{1}{p} \sum_{i=0}^{n-1} c_{ji} \tag{13b}$$

The products in Eqs. (12a) and (12b) include only the relevant values of the selected elementary criterion, while the sum (Eqs. (13a) and (13b)) is taken over all values of the elementary criterion (relevant or not). The sum is divided by the number of relevant peaks, p (or, $p - B$, for $\underline{r}$). This number corresponds to the minimum number of values of the elementary criterion when all the irrelevant peaks are confounded. A value of $\underline{r} = 1$ can only be obtained if all irrelevant peaks coincide and if all peaks are equally distributed throughout the chromatogram. A value of $\underline{r}^* = 1$ requires in addition that the irrelevant peaks coincide with the t_0 peak.

Finally, the time-corrected resolution products become

$$\left[\underline{r}^*_{\mathrm{nt}}\right]_{f,d} = \frac{\sqrt[d]{\underline{r}^*}\, \underline{c}^2_{\min}}{1 + k_\omega} \tag{14}$$

and

$$\left[\underline{r}^*_{\mathrm{nt}}\right]_{p} = \frac{\sqrt[d]{\underline{r}^*}\, \underline{c}^4_{\min}}{1 + k_\omega} \tag{15}$$

3. Experimental

3.1. Instrumentation

The HPLC system consisted of a Beckman dual pump Model 126 (Beckman Instruments, Fullerton, CA). The system was equipped with an injection valve (Rheodyne model 7725i, Cotati, CA) fitted with a 20 μl injection loop and a diode-array detector from Beckman (Model 168) collecting absorbance data from 200 to 350 nm with a spectral resolution of 4 nm and an acquisition rate of one spectrum per second. System Gold software (Beckman), implemented on an IBM-compatible computer, was used to control the HPLC apparatus and to collect the chromatographic data.

3.2. Chromatographic conditions

A reversed-phase system was chosen for this study. We used a 5 μm 60 RP-Select B LiChrospher column (125×4 mm^2 i.d.) and a 5 μm 60 RP-Select B LiChrospher precolumn (4×4 mm^2 i.d.) from Merck (Darmstadt, Germany). The temperature of the column was maintained at 35°C by a temperature controller (Model 7970, Jones Chromatography, Lakewood, CO). The hold-up time (t_0) was estimated to be 1.22 min. by using replicate injections of 10^{-4} M KI. The flow-rate was 1.0 ml/min. Chromatograms were recorded at 230 nm.

Various combinations of methanol (MeOH), acetonitrile (ACN), tetrahydrofuran (THF) and water constituted the mobile phases. All solvents were HPLC grade (UCB, Leuven, Belgium). Water was obtained from a Milli-Q purification system (Millipore, Milford, MA).

The mixture injected into the HPLC system consisted of nine benzodiazepines: triazolam (10 μg/ml), demoxepam (10 μg/ml), oxazepam (10 μg/ml), flunitrazepam (10 μg/ml), nitrazepam (10 μg/ml), clonazepam (10 μg/ml), diazepam (10 μg/ml), ethyl loflazepate (10 μg/ml), and nordazepam (20 μg/ml). These solutes were kindly provided by the following pharmaceutical companies: Roche, Qualiphar, Upjohn and Sanofi-Winthrop. Stock standard solutions of the investigated compounds were prepared in methanol, acetonitrile or tetrahydrofuran and then diluted as required in the mobile phase. Peak recognition was performed by spectral overlay (the UV spectra of all individual solutes were available).

3.3. Optimization procedure

A conventional procedure was used for optimizing the volume fraction of several organic modifiers in the mobile phase, i.e. ACN, MeOH and THF. The experimental design is shown in Fig. 2. The first binary mobile phase, 57% MeOH/43% H_2O was defined empirically. The two other isoeluotropic mobile phases, ACN/H_2O and THF/H_2O were calculated by using Schoenmakers' empirical transfer rules [19]. After experimental correction, these binary mobile phase mixtures contained 41% ACN (against 43% predicted by the transfer rules) and 34% THF (against 38% predicted), respectively.

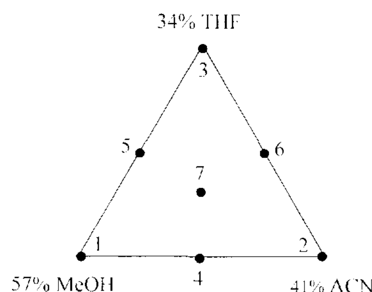

Fig. 2. Experimental design used for the separation of a limited number of solutes from a mixture of nine benzodiazepines.

An exponentially quadratic equation (Eq. (16)) was used to model the capacity factor (k) as a function of the mobile phase composition:

$$k = a_1 \cdot e^{a_2 \cdot \overline{\varphi_1} + a_3 \cdot \overline{\varphi_2} + a_4 \cdot \overline{\varphi_1}^2 + a_5 \cdot \overline{\varphi_2}^2 + a_6 \cdot \overline{\varphi_1} \cdot \overline{\varphi_2}} \tag{16}$$

where $\overline{\varphi_1}$ is the volume fraction of one solvent (i.e. the binary mixture ACN/H_2O) in the mobile phase and $\overline{\varphi_2}$ is the volume fraction of a second solvent (i.e. the binary mixture $MeOH/H_2O$).

3.4. Other optimization examples

In this paper, optimization examples are also described based on experimental data already reported in a previous article [20]. These data concerned a mixture of four acidic solutes (benzoic acid, *m*-nitrobenzoic acid, 3,5-dinitrobenzoic acid and salicylic acid). However, we include here one additional solute (aspirin). A reversed-phase system was chosen for this study. We used a 5 μm C_{18} LiChrospher column (125 × 4 mm^2 i.d.) and a 5 μm C_{18} LiChrospher precolumn (4 × 4 mm^2 i.d.) from Merck (Darmstadt, Germany). The flow-rate was 1.0 ml/min and UV detection was performed at 254 nm.

A 4 × 3 experimental design (three levels of methanol volume fraction and four levels of pH) was used to realize the simultaneous optimization of pH and solvent composition. The volume fraction of MeOH (φ) was varied between 0.30 and 0.40. pH was varied between 2.76 and 6.83 (total ionic strength of 0.05 M).

Table 3
Coefficients describing retention (capacity factor, k), peak height (h), peak area (A) in terms of Eq. (17) and asymmetry factor (A_S) in terms of Eq. (18)

	k°_{HA} [a]	$k^\circ_{A^-}$ [a]	K°_a	S_{HA}	S_{A^-}	Q_1	T_{HA}	T_{A^-}	Q_2
(a) 3,5-dinitrobenzoic acid									
k	$5.50 \cdot 10^7$	12.07	$5.44 \cdot 10^{-2}$	−59.66	−4.00	13.98	49.45	−4.21	−57.86
h	$7.84 \cdot 10^{-4}$	11.98	$3.75 \cdot 10^{19}$	63.60	11.71	$-3.16 \cdot 10^2$	−87.67	−12.92	$4.58 \cdot 10^2$
A	17.26	$1.33 \cdot 10^3$	$3.40 \cdot 10^{13}$	25.45	2.24	$-2.13 \cdot 10^2$	−35.80	−3.34	$3.00 \cdot 10^2$
A_S	$4.79 \cdot 10^5$	$-3.69 \cdot 10^{10}$	$6.48 \cdot 10^5$	−68.89	$-1.38 \cdot 10^2$	−88.88	94.26	$1.81 \cdot 10^2$	$1.14 \cdot 10^2$
(b) benzoic acid									
k	$2.53 \cdot 10^2$	49.19	$2.01 \cdot 10^{-2}$	−12.19	−21.92	−30.01	5.32	21.44	35.92
h	2.10	$2.61 \cdot 10^2$	$2.90 \cdot 10^{-4}$	11.75	−6.20	−17.64	−8.83	9.93	27.74
A	$5.98 \cdot 10^2$	$2.68 \cdot 10^3$	$1.15 \cdot 10^{-1}$	4.26	−5.61	−31.91	−6.58	7.88	32.67
A_S	$2.36 \cdot 10^7$	$-2.41 \cdot 10^{12}$	$2.45 \cdot 10^7$	−89.84	$-1.60 \cdot 10^2$	$-1.08 \cdot 10^2$	$1.22 \cdot 10^2$	$2.15 \cdot 10^2$	$1.45 \cdot 10^2$
(c) salicylic acid									
k	$5.00 \cdot 10^2$	1.50	$2.73 \cdot 10^{-5}$	−6.13	4.54	38.04	−11.80	−18.58	−72.69
h	3.65	51.01	$5.80 \cdot 10^7$	11.46	$2.72 \cdot 10^{-1}$	$-1.25 \cdot 10^2$	−9.69	2.63	$1.24 \cdot 10^2$
A	$2.38 \cdot 10^5$	$1.30 \cdot 10^3$	$1.18 \cdot 10^{-1}$	−24.51	−2.18	−18.76	35.82	2.83	28.89
A_S	$6.96 \cdot 10^2$	$-6.70 \cdot 10^{-3}$	1.42	−30.62	39.34	−15.86	37.21	−88.49	13.85
(d) *m*-nitrobenzoic acid									
k	75.44	1.28	$8.21 \cdot 10^{-7}$	−3.74	4.72	42.01	−7.68	−17.64	−67.56
h	$8.50 \cdot 10^{-4}$	60.72	$3.38 \cdot 10^{11}$	56.60	1.44	$-2.08 \cdot 10^2$	−71.83	$7.81 \cdot 10^{-1}$	$2.94 \cdot 10^2$
A	1.30	$2.18 \cdot 10^3$	$2.14 \cdot 10^{11}$	39.56	−3.91	$-1.74 \cdot 10^2$	−56.04	5.70	$2.29 \cdot 10^2$
A_S	$2.37 \cdot 10^4$	$-2.05 \cdot 10^{10}$	$8.77 \cdot 10^4$	−51.82	$-1.35 \cdot 10^2$	−80.05	69.89	$1.76 \cdot 10^2$	$1.05 \cdot 10^2$
(e) aspirin									
k	$6.72 \cdot 10^2$	7.36	$1.63 \cdot 10^{-3}$	−19.07	−10.35	−10.17	13.50	4.64	9.10
h	$7.48 \cdot 10^{-1}$	17.28	$1.77 \cdot 10^{11}$	20.96	10.95	$-2.08 \cdot 10^2$	−22.21	−13.91	$2.95 \cdot 10^2$
A	$3.36 \cdot 10^2$	$1.78 \cdot 10^3$	$3.60 \cdot 10^{-12}$	8.55	−2.19	98.02	−12.36	3.98	$-1.32 \cdot 10^2$
A_S	$2.75 \cdot 10^7$	$-4.77 \cdot 10^8$	$3.50 \cdot 10^7$	−89.45	−95.22	$-1.06 \cdot 10^2$	$1.18 \cdot 10^2$	91.23	$1.34 \cdot 10^2$

[a] Or equivalent parameters, i.e. h°_{HA} and $h^\circ_{A^-}$, A°_{HA} and $A^\circ_{A^-}$, or the nine parameters in Eq. (18) (b_0, b_1, b_2, S_0, S_1, S_2, T_0, T_1, and T_2).

A sigmoidal model (Eq. (17)) was used for describing capacity factors (k):

$$k = \frac{k^{\circ}_{\mathrm{HA}} \cdot \mathrm{e}^{S_{\mathrm{HA}}\varphi + T_{\mathrm{HA}}\varphi^2} \cdot 10^{-\mathrm{pH}} + k^{\circ}_{\mathrm{A}^-} \cdot K^{\circ}_{\mathrm{a}} \cdot \mathrm{e}^{(S_{\mathrm{A}^-} + Q_1)\varphi + (T_{\mathrm{A}^-} + Q_2)\varphi^2}}{10^{-\mathrm{pH}} + K^{\circ}_{\mathrm{a}} \cdot \mathrm{e}^{(Q_1\varphi + Q_2\varphi^2)}} \quad (17)$$

where k°_{HA} and $k^{\circ}_{\mathrm{A}^-}$ are extrapolated capacity factors of, respectively, the protonated and the dissociated forms of the solute in pure water, K°_{a} is the extrapolated acid-dissociation constant in pure water, S_{HA} and T_{HA} are parameters describing the variation of retention with φ for protonated species, S_{A^-} and T_{A^-} are corresponding parameters for dissociated species, and Q_1 and Q_2 are coefficients describing the variation of the acid-dissociation constant with φ. Eq. (17) was also used to model peak heights and peak areas.

A quadratic expression (Eq. (18)) was used to model the asymmetry factors (A_S):

$$A_S = b_0 \cdot \mathrm{e}^{S_0\varphi + T_0\varphi^2} + b_1 \cdot \mathrm{e}^{(S_1\varphi + T_1\varphi^2)} \cdot \mathrm{pH} + b_2 \cdot \mathrm{e}^{(S_2\varphi + T_2\varphi^2)} \cdot \mathrm{pH}^2 \quad (18)$$

The coefficients describing retention (capacity factor), peak height, peak area and asymmetry factor are listed in Table 3.

A detailed description of the chromatographic conditions and optimization procedure can be found in Ref. [20].

3.5. Software

Different 'in house' programs were developed to model capacity factors and other chromatographic parameters (peak heights, peak areas and asymmetry factors) and to generate response surfaces. Software is also available for generating predicted or simulated chromatograms. All the programs were written in Pascal (Turbo Pascal 7.0, Borland International, Scotts Valley, CA) and implemented on an IBM-compatible computer. Data generated by Pascal programs were imported directly in Excel software (version 5.0) in a Windows environment (Microsoft) for further manipulation and graphical presentation.

4. Results and discussion

4.1. Separation of five acidic solutes

Due to its obvious significance and intrinsic simplicity, the minimum resolution is frequently used in optimization procedures. The minimum *effective* resolution is used to optimize the separation of five acidic solutes (benzoic acid, *m*-nitrobenzoic acid, 3,5-dinitrobenzoic acid, salicylic acid and aspirin). The optimized parameters are the pH of the mobile phase and the percentage of MeOH in the mobile phase. A complete description of the experimental data can be found in Ref. [20]. Fig. 3a shows the optimum chromatogram selected using the minimum effective resolution when all the five solutes are relevant (complete optimization). The minimum effective resolution is equal to 1.2. However, if only two of the five solutes are relevant (i.e. benzoic acid and dinitrobenzoic acid), another chromatogram (Fig. 3b) is selected as the optimum. In Fig. 3b and subsequent figures, black peaks are relevant ones. In comparison with the complete optimization process, the value of the minimum effective resolution is higher ($\underline{R}_{1,\mathrm{min}} = 2.95$) and the analysis time is about 6 min instead of 12 min. The latter is not a direct result of the optimization process. The analysis time is not considered if $\underline{R}_{1,\mathrm{min}}$ is selected as the optimization criterion.

Two other examples of limited optimization are given in Fig. 3c and d. Fig. 3c is the optimum chromatogram selected using the minimum effective resolution when dinitrobenzoic acid is the only relevant peak. In Fig. 3d, nitrobenzoic acid is the only peak of interest. Again, the optimum chromatograms are different if the relevance of the peaks is different. In limited optimization only the relevant peaks need to be separated from all other peaks,

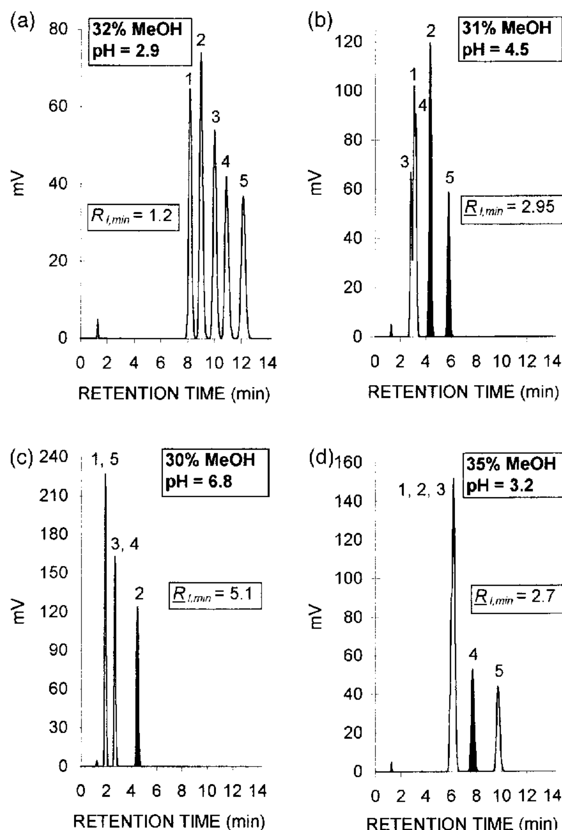

Fig. 3. Optimum chromatograms defined by the minimum effective resolution when (a) all five solutes are relevant, (b) only benzoic acid and dinitrobenzoic acid are relevant, (c) only dinitrobenzoic acid is relevant and (d) only nitrobenzoic acid is relevant. Peaks: 1 = aspirin; 2 = 3,5-dinitrobenzoic acid; 3 = salicylic acid; 4 = *m*-nitrobenzoic acid and 5 = benzoic acid. In chromatograms b, c and d, the black peaks are the relevant peaks.

whereas irrelevant peaks do not need to be separated from each other. The minimum effective resolution is selected from the relevant pairs of peaks so that the response surface can be different, leading to different optimum conditions. Particularly favorable is the example given in Fig. 3c where the minimum effective resolution is equal to 5.1 with an analysis time of less than 5 min. Indeed, in this example, the irrelevant peaks are almost all confounded and appear at the beginning of the chromatogram. These examples demonstrate clearly the importance of considering the relevance of the peaks during an optimization process.

Although it is frequently used, the minimum resolution has a number of disadvantages. For example, it does not consider the analysis time. Consequently it can lead to lengthy separations. A threshold criterion can be used to avoid this problem. If the threshold resolution value (e.g. 1.5) is reached, then the factor $1/(1 + k_{\omega})$, which is inversely proportional to the analysis time, is calculated and subsequently maximized. If the threshold value is not reached, the criterion value is equal to zero.

However, the minimum resolution and the threshold resolution do not reflect the distribution of the peaks over the chromatogram. The normalized resolution product (r) and the calibrated normalized resolution product (r^*) were defined to promote an equal distribution of the peaks throughout the chromatogram. As explained in the theoretical part, in limited optimization $\underline{r}$ and $\underline{r}^*$ promote situations in which all the peaks are equally distributed over the chromatogram (as in complete optimization) and all irrelevant peaks are confounded. When these two conditions are met, $\underline{r}$ is equal to 1. $\underline{r}^*$ will not reach a value of 1, unless all irrelevant peaks coincide

Table 4
Volume fractions of methanol (φ_{MeOH}), acetonitrile (φ_{ACN}) and tetrahydrofuran (φ_{THF}) corresponding to the seven experimental locations. The $\bar{\varphi}$ values correspond to the volume fractions of the three binary mixtures ($MeOH-H_2O$; $ACN-H_2O$; $THF-H_2O$).

No.	φ_{MeOH}	$\bar{\varphi}_{MeOH}$	φ_{ACN}	$\bar{\varphi}_{ACN}$	φ_{THF}	$\bar{\varphi}_{THF}$
1	0.57	1	0	0	0	0
2	0	0	0.41	1	0	0
3	0	0	0	0	0.34	1
4	0.285	0.5	0.205	0.5	0	0
5	0.285	0.5	0	0	0.17	0.5
6	0	0	0.205	0.5	0.17	0.5
7	0.19	0.333	0.14	0.333	0.11	0.333

with the t_0 peak. Obviously, such an ideal situation is not often reached in practice. However, an important practical consequence of using this type of criteria is that they promote situations in which relevant peaks are adjacent. The products in the normalized resolution product (Eq. (12a)) and in the calibrated normalized resolution product (Eq. (12b)) include only the relevant values of the selected elementary criterion, while the sum (for calculating the 'average' value of the elementary criterion) is taken over all values of the elementary criterion and divided by the number of relevant peaks, p (or the minimum number of relevant pairs of peaks, $p - B$, for $\underline{r}$).

4.2. Separation of nine benzodiazepines

In order to illustrate the behavior of normalized-resolution-product-type criteria, we describe the application of $\underline{r}^*$ to the optimization of the mobile-phase composition (volume fractions of MeOH, ACN and THF) for a mixture containing nine benzodiazepines. The volume fractions of MeOH, ACN and THF corresponding to the seven experiments defined by the experimental design in Fig. 2 are listed in Table 4. Table 5 gives the capacity factors of the nine solutes at the seven experimental locations. The model coefficients describing these retention data in terms of Eq. (16) are listed in Table 6. Assume we are interested in the separation of only some of the solutes. Fig. 4 shows four different optimum chromatograms corresponding to four different examples of limited optimization. These chromatograms are selected as optimum using $\underline{r}^*$ as the overall criterion and the separation factor as the elementary criterion. As we can see in Fig. 4, $\underline{r}^*$ yields different optimum mobile-phase compositions depending on the relevance of the peaks. Fig. 4a shows the optimum chromatogram selected by $\underline{r}^*$ when three solutes, i.e. demoxepam, oxazepam and flunitrazepam, are relevant. The three relevant peaks are adjacent while three irrelevant peaks (nitrazepam, clonazepam and diazepam) are confounded. In Fig. 4b, flunitrazepam,

Table 5
Capacity factors of the nine solutes at the seven experimental locations defined by the experimental design (t_0 = 1.22 min)

No.	Capacity factors								
	D	N	O	C	F	T	No	Di	L
1	1.43	1.82	2.51	1.94	2.13	2.88	3.94	4.99	5.39
2	1.15	2.03	1.87	2.35	3.16	2.71	3.11	5.17	5.58
3	1.20	3.63	2.51	3.63	3.13	0.92	3.44	3.63	5.42
4	1.81	2.50	2.86	2.76	3.27	3.97	4.72	6.74	7.72
5	1.24	2.67	2.47	2.79	2.47	1.48	3.56	3.77	4.89
6	1.14	2.25	2.08	2.57	2.57	1.51	3.06	3.76	5.17
7	1.43	2.51	2.59	2.70	2.70	2.14	3.91	4.65	5.97

D = demoxepam, N = nitrazepam, O = oxazepam, C = clonazepam, F = flunitrazepam, T = triazolam, No = nordazepam, Di = diazepam and L = ethyl loflazepate.

Table 6
Model coefficients describing retention (capacity factor, k) in terms of Eq. (16)

Solute	Model coefficients					
	a_1	a_2	a_3	a_4	a_5	a_6
D	1.196	−0.129	−0.0184	0.08675	0.1948	1.689
N	3.625	−1.321	−0.5316	0.7406	−0.1562	1.635
O	2.509	−0.4096	−0.02053	0.1131	0.01723	1.279
C	3.629	−0.9751	−0.4474	0.5427	−0.1764	1.359
F	3.135	−0.8245	−0.5871	0.835	0.2016	1.944
T	0.9191	0.9222	0.7759	0.1594	0.3647	1.936
No	3.429	−0.3124	0.04614	0.2111	0.09076	1.528
Di	3.619	−0.1813	−0.1284	0.5376	0.449	2.126
L	5.416	−0.2012	−0.3861	0.2312	0.38	1.985

D = demoxepam, N = nitrazepam, O = oxazepam, C = clonazepam, F = flunitrazepam, T = triazolam, No = nordazepam, Di = diazepam and L = ethyl loflazepate.

triazolam and nordazepam are relevant. In Fig. 4c, the three peaks of interest are nordazepam, diazepam and ethyl loflazepate. Again, $\underline{r}^*$ selects experimental conditions where the relevant peaks are adjacent while irrelevant peaks tend to be confounded. Notice that in Fig. 4c four irrelevant peaks (triazolam, oxazepam, flunitrazepam and clonazepam) are coeluting. Finally, $\underline{r}^*$ yields the optimum chromatogram shown in Fig. 4d when four peaks are relevant. Three of these relevant solutes are the same as in the chromatogram given in Fig. 4c (nordazepam, diazepam and ethyl loflazepate). The fourth relevant peak is triazolam. The peaks of interest are again adjacent in this optimum chromatogram. Moreover, in comparison with the chromatogram presented in Fig. 4c, where triazolam was irrelevant and confounded with three other irrelevant solutes, this compound is now separated from the irrelevant peaks.

For these four examples of limited optimization, the optimum $\underline{r}^*$ values (mentioned in Fig. 4) are low. This is due to the fact that the irrelevant peaks are not all confounded and none of them is confounded with the t_0 peak. Moreover, in Fig. 4a, flunitrazepam is not well separated from nordazepam. The optimum chromatogram in Fig. 4c, shows a higher $\underline{r}^*$ value than the other ones. Indeed, in this case four irrelevant peaks are confounded and the three relevant ones are well separated.

These four examples of limited optimization again demonstrate the importance of considering the relevance of the peaks during an optimization strategy. The major advantage of normalized-resolution-product-type criteria is that they promote the separation of solutes into groups (ideally all relevant ones separated from all irrelevant ones).

4.3. Discussion of normalized-resolution-product criteria

The use of (calibrated) normalized resolution products can also present some ambiguities. As mentioned by Schoenmakers [1,18], normalized resolution products are not perfect once two chromatograms are compared which show very different capacity factors for the last peak, especially when both criterion values are low. Moreover, in limited optimization, the same $\underline{r}^*$ (or $\underline{r}$) value can theoretically correspond to different chromatograms showing different distributions of the relevant peaks. This is illustrated in Fig. 5 which shows fictive examples of chromatograms. Each chromatogram includes six solutes and three of them are relevant (black peaks). The hold-up time (t_0) is equal to 1.5 min and the theoretical plates number is assumed to be 2000. The resolution between each pair of peaks is equal to 1.5. The chromatogram in Fig. 5a corresponds to the ultimate optimum value of both $\underline{r}^*$ and $\underline{r}$ (equal to 1). The three irrelevant peaks are confounded with the t_0 peak and the relevant ones are equally distributed over the chromatogram. If the irrelevant peaks are confounded but do not coincide with the t_0 peak, the value of the calibrated normalized resolution product ($\underline{r}^*$) is lower than one. A

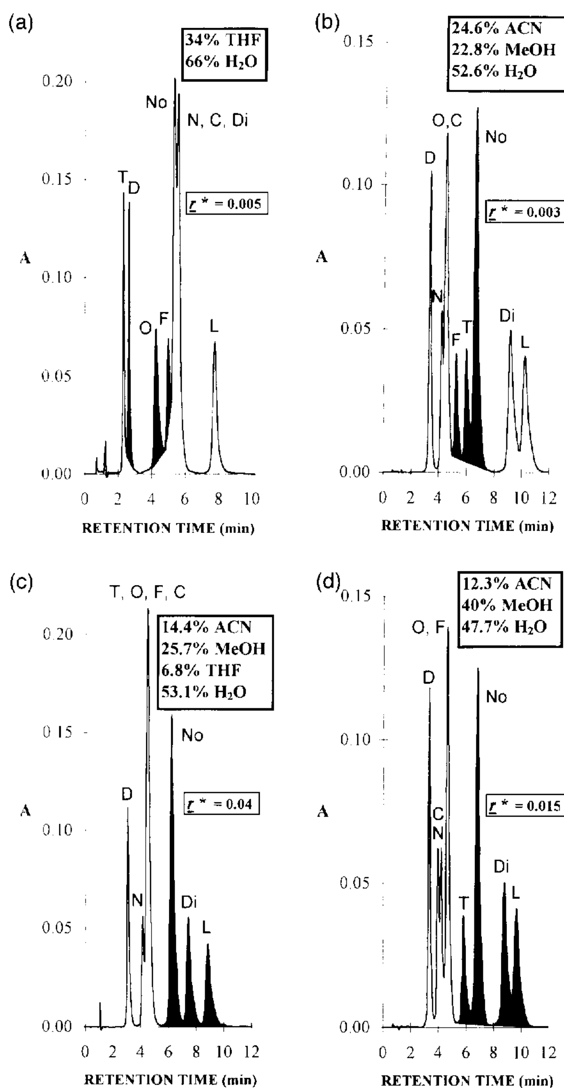

Fig. 4. Optimum chromatograms selected by the calibrated normalized resolution product based on the separation factor when (a) demoxepam, oxazepam and flunitrazepam are the relevant solutes, (b) flunitrazepam, triazolam and nordazepam are relevant, (c) nordazepam, diazepam and ethyl loflazepate are relevant and (d) triazolam, nordazepam, diazepam and ethyl loflazepate are relevant. Peaks: T = triazolam; D = demoxepam; O = oxazepam; F = flunitrazepam; No = nordazepam; N = nitrazepam; C = clonazepam; Di = diazepam and L = ethyl loflazepate. Black peaks are the relevant peaks.

value of $\underline{r} = 1$ requires that the irrelevant peaks coincide but not necessarily at t_0. Indeed, the assumed presence of a hypothetical peak at t_0 affects the calculation of $\underline{r}^*$ but not the calculation of $\underline{r}$ (see theoretical part). In Fig. 5(a–d), $\underline{r}^*$ and $\underline{r}$ yield equal values because at least one (real) peak appears at t_0. For the chromatograms in Fig. 5(b–d), the values of $\underline{r}^*$ and $\underline{r}$ decrease significantly (in comparison with Fig. 5a), because one of the irrelevant peaks is no longer confounded with the two other ones. The chromatograms in Fig. 5(b–d) have the same 'average' resolution value (calculated by using Eq. (13)). The chromatogram in Fig. 5b has three relevant values of the resolution. The chromatograms in Fig. 5c and d have one more relevant value because the irrelevant peaks are not adjacent leading to a lower value of $\underline{r}^*$ and $\underline{r}$. However, the same values are obtained for Fig. 5c and d.

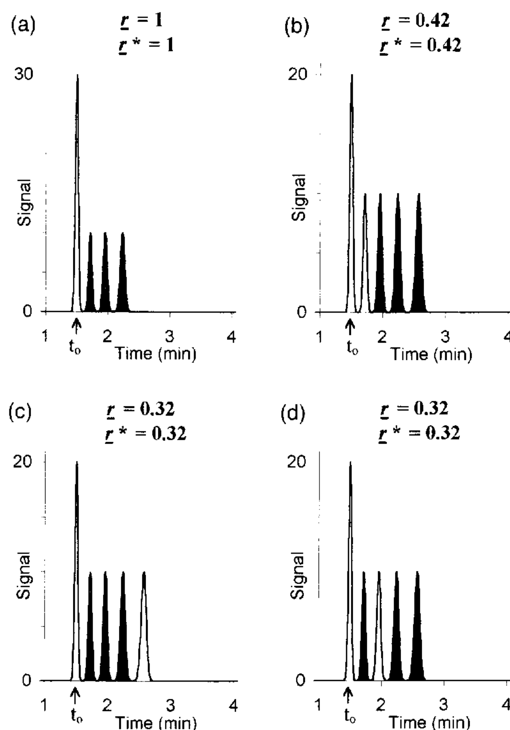

Fig. 5. Examples of fictive chromatograms and the respective values of the normalized resolution product and calibrated normalized resolution product. The resolution between each pair of peaks is equal to 1.5. $t_0 = 1.5$ min and $N = 2000$. Black peaks are the relevant peaks.

Indeed, for an identical 'average' resolution value, these chromatograms present the same number of relevant values of the resolution (i.e. four values), each resolution value being equal to 1.5. This example shows that in some specific situations, the same $\underline{r}^*$ (or $\underline{r}$) value can correspond to different distributions of the relevant peaks over the chromatogram. Although, chromatograms such as these depicted in Fig. 5c and d (showing the same $\underline{r}^*$ or $\underline{r}$ values) will hardly ever appear during the same optimization procedure, they do represent equivalent optima in different regions of the response surface. The calibrated normalized resolution product and the normalized resolution product adapted to limited optimization are obviously imperfect. But, they have the great advantage of strongly promoting the co-elution of irrelevant peaks and of favoring the separation of the solutes into groups (see the chromatograms in Fig. 4). Ideally, the relevant peaks are equally distributed over the chromatogram and the irrelevant peaks are confounded. Moreover, the behavior of the (calibrated) normalized resolution products adapted to limited optimization opens the way towards the optimization of multi-dimensional ('heart-cut') separations (e.g. LC–LC, LC–GC) and, possibly, group-type separations. For example, in two-dimensional separations, relevant peaks do not need to be separated from each other on the first column, but separations in which relevant peaks are not adjacent need to be penalized. In group-type separations, the solutes belonging to a specific group do not need to be separated from each other, but, again, situations where solutes of the same group are not adjacent need to be penalized. A group can consist of one or more solutes.

4.4. *Discussion of all criteria*

Selecting an adequate criterion is a fundamental step for the success of any optimization procedure. As the result of an optimization process depends on the selected criterion, the latter has to be defined in the context of

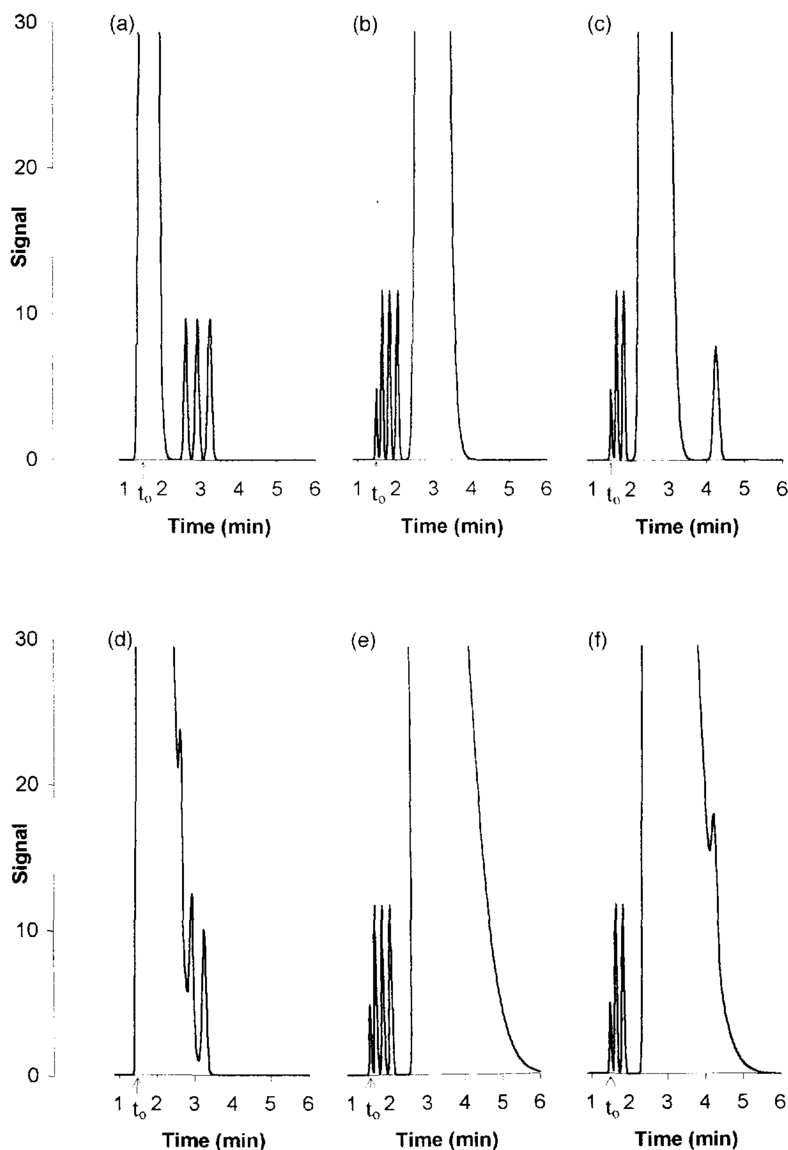

Fig. 6. Examples of computer-simulated chromatograms. The chromatographic parameters are given in Table 7. The huge asymmetrical peak is irrelevant. The three small peaks are relevant. A small hypothetical t_0 peak (irrelevant) is considered at 1.5 min in chromatograms b, c, e and f.

the objectives of the separation. This is illustrated in Fig. 6, which presents six computer-simulated chromatograms featuring one huge asymmetrical irrelevant peak and three small relevant peaks. In Fig. 6b, c, e and f, a small, hypothetical (irrelevant) t_0 peak is considered at $t = 1.5$ min. This peak is considered for calculating minimum-resolution criteria. The chromatographic parameters (retention times, peak heights, number of theoretical plates and asymmetry factors) and the relevant values of separation factor, resolution and effective resolution are given in Table 7. For each chromatogram, Table 8 lists the values of the eight overall criteria adapted to limited optimization which were described in the theoretical part. The calculation of these criteria is achieved by using

Table 7
Chromatographic parameters (retention time, t_R; peak height, h; number of theoretical plates, N; asymmetry factor, A_S) and relevant values of separation factor (S), resolution (R_S) and effective resolution (R_1) corresponding to the computer-simulated chromatograms presented in Fig. 6(a–f)

Peak	t_R (min)	h	N	A_S	S	R_S	R_1
Chromatogram presented in Fig. 6a = chromatogram a							
0 [a]	1.50	1000	100	3	—	—	—
1	2.62	10	2000	1	0.27	4.4	1.2
2	2.92	10	2000	1	0.054	1.2	1.2
3	3.25	10	2000	1	0.053	1.2	1.2
Chromatogram presented in Fig. 6b = chromatogram b							
0 [a]	1.50	5	2000	1	—	—	—
1	1.65	12	2000	1	0.048	1.1	1.2
2	1.84	12	2000	1	0.054	1.2	1.2
3	2.05	12	2000	1	0.054	1.2	1.2
4	2.73	650	100	3	0.14	2.3	1.2
Chromatogram presented in Fig. 6c = chromatogram c							
0 [a]	1.50	5	2000	1	—	—	—
1	1.65	12	2000	1	0.048	1.1	1.2
2	1.84	12	2000	1	0.054	1.2	1.2
3	2.45	700	100	3	0.14	2.3	1.2
4	4.27	8	2000	1	0.27	4.4	1.2
Chromatogram presented in Fig. 6d = chromatogram d							
0 [a]	1.50	200	17	17	—	—	—
1	2.62	10	2000	1	0.27	4.3	0.5
2	2.92	10	2000	1	0.054	1.2	1.2
3	3.25	10	2000	1	0.053	1.2	1.2
Chromatogram presented in Fig. 6e = chromatogram e							
0 [a]	1.50	5	2000	1	—	—	—
1	1.65	12	2000	1	0.048	1.1	1.2
2	1.84	12	2000	1	0.054	1.2	1.2
3	2.05	12	2000	1	0.054	1.2	1.2
4	2.73	130	17	17	0.14	2.3	2.2
Chromatogram presented in Fig. 6f = chromatogram f							
0 [a]	1.50	5	2000	1	—	—	—
1	1.65	12	2000	1	0.048	1.1	1.2
2	1.84	12	2000	1	0.054	1.2	1.2
3	2.45	140	17	17	0.14	2.3	2.2
4	4.27	8	2000	1	0.27	4.3	0.5

[a] Peak 0 corresponds to the column hold-up time.

three different elementary criteria, i.e. the separation factor, the resolution and the effective resolution. In the following discussion, chromatograms a to f refer to Fig. 6a to f, respectively.

The calculation of some criteria (threshold resolution, minimum required analysis time and time-corrected resolution product) requires the estimation of the analysis time (which is used for calculating the dimensionless column-independent time factor $(1 + k_\omega)$). Generally, the retention time of the last peak is an adequate approximation of the time needed to elute the last peak. However, this approximation is no longer appropriate when a huge asymmetrical peak appears towards the end of the chromatogram (see, for example, Fig. 6e where the retention time of the last peak is equal to 2.73 min and where the analysis time is about 6 min).

Table 8
Values of analysis time ($t_{analysis}$), of ($1 + k_{analysis}$) and of 24 optimization criteria (8 overall criteria combined with 3 elementary criteria) corresponding to the chromatograms presented in Fig. 6a–f

	Chromatogram					
	a	b	c	d	e	f
$t_{analysis}$ (min)	3.40	4.15	4.46	3.40	6.43	5.95
Eq.	(19)	(21)	(19)	(19)	(21)	(21)a
$1 + k_{analysis}$	2.26	2.77	2.97	2.26	4.29	3.97

Elementary criterion	Overall criterion	Chromatogram					
		a	b	c	d	e	f
S	$\underline{S}_{min}$	0.053	0.048	0.048	0.053	0.048	0.048
	$S \cap k$ ($\underline{S}_{min} \geq 0.05$)	0.44	0	0	0.44	0	0
	$\underline{r}$	0.39	0.72	0.55	0.39	0.72	0.55
	$\underline{r}^*$	0.39	0.21	0.12	0.39	0.21	0.12
	$[\underline{t}_{\neq}]^{-1}_{f.d}$	0.00124	0.00083	0.00078	0.00124	0.00054	0.00058
	$[\underline{r}^*_{nt}]_{f.d}$	0.00091	0.00056	0.00046	0.00091	0.00036	0.00034
	$[\underline{t}_{ne}]^{-1}_{p}$	$3.5 \cdot 10^{-6}$	$1.9 \cdot 10^{-6}$	$1.8 \cdot 10^{-6}$	$3.5 \cdot 10^{-6}$	$1.2 \cdot 10^{-6}$	$1.3 \cdot 10^{-6}$
	$[\underline{r}^*_{nt}]_{p}$	$2.6 \cdot 10^{-6}$	$1.3 \cdot 10^{-6}$	$1.1 \cdot 10^{-6}$	$2.6 \cdot 10^{-6}$	$8.4 \cdot 10^{-7}$	$7.9 \cdot 10^{-7}$
R_S	$\underline{R}_{S,min}$	1.2	1.1	1.1	1.2	1.1	1.1
	$R_S \cap k$ ($\underline{R}_{S,min} \geq 1.2$)	0.44	0	0	0.44	0	0
	$\underline{r}$	0.54	0.86	0.67	0.55	0.86	0.68
	$\underline{r}^*$	0.54	0.26	0.16	0.55	0.26	0.17
	$[\underline{t}_{ne}]^{-1}_{f.d}$	0.64	0.44	0.41	0.64	0.28	0.30
	$[\underline{r}^*_{nt}]_{f.d}$	0.52	0.31	0.26	0.52	0.20	0.20
	$[\underline{t}_{ne}]^{-1}_{p}$	0.92	0.53	0.49	0.92	0.34	0.37
	$[\underline{r}^*_{nt}]_{p}$	0.75	0.38	0.31	0.75	0.24	0.24
R_1	$\underline{R}_{1,min}$	1.2	1.2	1.2	0.5	1.2	0.5
	$R_1 \cap k$ ($\underline{R}_{1,min} \geq 1.2$)	0.44	0.36	0.34	0	0.23	0
	$\underline{r}$	1	1	1	0.79	0.88	0.60
	$\underline{r}^*$	1	0.32	0.32	0.79	0.27	0.19
	$[\underline{t}_{ne}]^{-1}_{f.d}$	0.64	0.52	0.48	0.11	0.34	0.06
	$[\underline{r}^*_{nt}]_{f.d}$	0.64	0.39	0.36	0.10	0.24	0.04
	$[\underline{t}_{ne}]^{-1}_{p}$	0.92	0.75	0.70	0.03	0.48	0.02
	$[\underline{r}^*_{nt}]_{p}$	0.92	0.56	0.53	0.03	0.35	0.01

The explanation of symbols can be found in the theoretical part (see also Table 1 and Table 2).
[a] Analysis time determined by one-but-last peak.

The time needed to elute 95% of a Gaussian peak is expressed by

$$t_{elution(95\%)} = t_R + 2\sigma = t_R\left(1 + \frac{2}{\sqrt{N}}\right) \tag{19}$$

where t_R is the retention time, σ is the standard deviation, and N is the number of theoretical plates.

For asymmetrical peaks, a more appropriate expression would read

$$t_{elution(95\%)} = t_R + b = t_R\left(1 + \frac{4A_S}{\sqrt{N}(1 + A_S)}\right) \tag{20}$$

where $A_S = b/a$ is the asymmetry factor measured at 13.5% of the peak height (a and b are the widths of the ascending and descending slopes of the peak, respectively).

Eq. (20) reduces to Eq. (19) if $A_S = 1$. However, if a huge peak is eluted for 95%, it may still be much bigger than the small neighboring peaks (see Fig. 6). For such a huge peak, the elution time should be calculated by using an expression corrected for the difference in peak heights between the huge peak and the adjacent peaks (see also Ref. [11]):

$$t_{\text{elution}(13.5\%\, h_{sp})} = t_R + b^* = t_R\left\{1 + \frac{4A_S}{\sqrt{N}(1+A_S)}\sqrt{1 + 0.5\ln\left(\frac{h_{hp}}{h_{sp}}\right)}\right\} \tag{21}$$

where b^* is the corresponding width of the descending slope of the huge peak at 13.5% of the height of the small adjacent peak ($b^* = b\sqrt{1 + 0.5\ln(h_{hp}/h_{sp})}$), h_{hp} is the height of the huge peak and h_{sp} is the height of the small adjacent peak.

The values of the analysis time ($t_{analysis}$) and of the factor ($1 + k_{analysis}$) for the six chromatograms of Fig. 6 are given in Table 8. From the analysis time value, the dimensionless column-independent time factor ($1 + k_{analysis}$) is calculated. This last factor is then used (instead of ($1 + k_\omega$)) for the calculation of the threshold resolution, the minimum required analysis time, and the time-corrected resolution product.

If we select the separation factor or the resolution as the elementary criterion, the minimum resolution, the normalized resolution product ($\underline{r}$), and the calibrated normalized resolution product ($\underline{r}^*$) do not differentiate between chromatograms a and d, b and e and, c and f. Notice that the peaks in chromatograms a and d, b and e and, c and f show the same retention times, but that the huge irrelevant peak in chromatograms d, e and f is wider and more asymmetrical than in chromatograms a, b and c. Having the same analysis time, chromatograms a and d are not differentiated by the threshold resolution, by the minimum required analysis time (($[\underline{t}_{ne}]_{f,d}^{-1}$ or $[\underline{t}_{ne}]_p^{-1}$), nor by the time-corrected resolution product (($[\underline{r}_{nt}^*]_{f,d}$ or $[\underline{r}_{nt}^*]_p$) when these criteria are based on the separation factor or the resolution. This demonstrates the fundamental importance of selecting an appropriate elementary criterion. The separation factor and the resolution do not adequately describe the extent of the separation between the huge, irrelevant, asymmetrical peak and the small adjacent peak(s) and they overestimate the resolution values. For example, in chromatogram d, the resolution between the huge peak and the next small one is equal to 4.3 (see Table 7) but the peaks are not well separated. In non-ideal situations, the effective resolution is the recommended elementary criterion. In our example, the effective resolution correctly describes the separation between the huge asymmetrical peak and its neighbors (see Table 7).

In chromatograms a, b and c, the effective resolution is the same between each pair of peaks ($R_l = 1.2$) but only chromatogram a reaches the ultimate optimum value of the calibrated normalized resolution product ($\underline{r}^* = 1$). Indeed, in chromatogram a, the huge irrelevant peak is confounded with the t_0 peak. This is not the case in chromatograms b and c and the corresponding $\underline{r}^*$ values are lower (see the explanations previously given). While chromatogram a is doubtlessly superior to chromatograms b and c, the situation is totally different if we compare chromatograms d, e and f. Chromatogram d has the best value of the calibrated normalized resolution product (based on the effective resolution). Indeed, the huge irrelevant peak is confounded with the t_0 peak and the relevant peaks are more or less evenly distributed over the chromatogram. Chromatograms e and f show lower $\underline{r}^*$ values than chromatogram d, principally because the huge irrelevant peak is not confounded with the t_0 peak. However, in terms of separation, chromatogram e is much better than chromatogram d ($\underline{R}_{l,min} = 1.2$ instead of $\underline{R}_{l,min} = 0.5$). $\underline{r}^*$ promotes situations where relevant peaks are equally distributed over the chromatogram but this objective can be reached without a complete resolution of the peaks. Notice that chromatogram d is a special case where none of the relevant peaks is completely separated from the huge irrelevant peak. The resolution between the small adjacent peaks is not as good as expected because of the interfering big peak (see Table 7).

Contrary to $\underline{r}^*$, $\underline{r}$ (based on the effective resolution) reaches three times the target value of 1. Indeed, in chromatograms a, b and c, the values of the effective resolution are all equal and, at this condition, the unique irrelevant peak can appear everywhere in the chromatogram (and not necessarily at t_0 as for $\underline{r}^*$). As explained previously, the hypothetical t_0 peak (Fig. 6b, c, e and f) is not considered in the calculation of $\underline{r}$. However, as for $\underline{r}^*$,

a 'good' value of $\underline{r}$ can be reached without a complete resolution of all relevant peaks (see chromatogram d for example).

If the principal goal of a chromatographic separation is to obtain a good resolution, the minimum (effective) resolution is the obvious choice. However, the threshold resolution should be preferably used to avoid long analysis times. For example, the target value of the minimum effective resolution can be set at 1.2. From the chromatograms reaching this threshold resolution (chromatograms a, b, c and e in Table 8), chromatogram a, which has the shortest analysis time, is selected as optimum. Similarly, the advantages of the minimum resolution and the calibrated normalized resolution product adapted to limited optimization can be elegantly combined by using a threshold approach. When the threshold resolution is reached, the calibrated normalized resolution product can be maximized.

If the dimensions of the column may be changed after the selectivity-optimization process (system optimization), the minimum required analysis time, $[\underline{t}_{ne}]_{f,d}^{-1}$ (under conditions of constant flow-rate and particle size) or $[\underline{t}_{ne}]_p^{-1}$ (under conditions of constant pressure drop), can be recommended in order to minimize the required analysis time. In Fig. 6, the highest values of both $[\underline{t}_{ne}]_p^{-1}$ and $[\underline{t}_{ne}]_{f,d}^{-1}$ (based on the effective resolution) are obtained for chromatogram a. This chromatogram obtains also the best values of the time-corrected resolution products based on the effective resolution, $[\underline{r}_{nt}^*]_{f,d}$ or $[\underline{r}_{nt}^*]_p$. These last criteria have the same objective as $[\underline{t}_{ne}]_{f,d}^{-1}$ and $[\underline{t}_{ne}]_p^{-1}$, but in addition some attention is paid to an equal spreading of the relevant peaks over the chromatogram.

The precision of criterion values is an important issue. Fortunately, the precision is greatest at the highest values of the resolution, i.e. around the optimum. The precision of elementary criteria will decrease with increasing complexity, i.e. S can be determined more precisely than R_S and R_S will be more precise than R_l. In turn, R_l describes the separation most accurately. The precision of overall criteria is largely determined by that of the minimum resolution (based on S, R_S or R_l as the elementary criterion). The precision of the minimum resolution is a critical factor in almost every optimization process.

5. Conclusions

Limited optimization is required in many situations and in many application areas, because analysts are often interested in the separation of a limited number of solutes in a complex mixture (biological samples, environmental samples, etc). Good criteria are a vital factor. Different criteria adapted to limited optimization have been described in this paper. Among the most useful ones are the minimum resolution and the calibrated normalized resolution product. The minimum resolution is the obvious choice when the ultimate objective of the separation is to obtain a good resolution between the relevant peaks and all the other peaks. As the minimum resolution does not consider the analysis time, it can lead to lengthy separations. The threshold resolution can be used to avoid this problem. However, the minimum resolution and the threshold resolution do not reflect the distribution of the peaks over the chromatogram. The calibrated normalized resolution product promotes an equal spreading of the relevant peaks throughout the chromatogram. The great advantage of this criterion is that it strongly promotes the co-elution of irrelevant peaks and favors the separation of the solutes into groups. Ideally, the relevant peaks are equally distributed over the chromatogram and all the irrelevant peaks are confounded with the t_0 peak. The main disadvantage of normalized resolution products is that high criterion values may be obtained when the actual resolution is low. Therefore, we believe that these criteria, like analysis time, can best be used in a hierarchical, 'threshold' format.

In summary, recommended criteria for the limited optimization of separations on a given column are

1. minimum effective resolution to achieve the best possible separation;
2. threshold resolution to achieve an acceptable separation in the fastest possible time;
3. threshold distribution (i.e. $C = \underline{r}^*$ while $\underline{R}_{l,\min} \geq \varepsilon$, else $C = 0$) to achieve the best possible distribution of the peaks in combination with an adequate separation.

Moreover, the behavior of normalized resolution products adapted to limited optimization opens the way towards the optimization of multi-dimensional separations and, possibly, group-type separations.

When the column dimensions may be varied after the selectivity optimization procedure, the minimum required analysis time (or the time-corrected resolution product) can be recommended.

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