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Use of optimization software to determine rugged analysis conditions in high-performance liquid chromatography

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Abstract

The ruggedness evaluation of an analytical method is now generally required for further validation. By considering ruggedness at an early stage of method development, major disappointments and amount of work could be avoided. This work shows that the optimization software OSIRIS can be helpful for the chromatographer during a method development, as it takes into account the method ruggedness. The ruggedness of the analysis conditions is then evaluated all along the selectivity optimization procedure. This optimization software belongs to the interpretive methods that consist of predicting the optimum conditions by modeling first the solute retention over the parameter space using a minimum number of preliminary runs. The choice of a response function is studied. This response function must be able to take into account several individual criteria: analysis time, minimal resolution and ruggedness of each parameter. Some optimum separations, determined using a ruggedness criteria or not, are given and compared in terms of long term repeatability. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

A liquid chromatographic method development includes three stages. The first one concerns the selection of a satisfying parameter space represented by the parameters that have a significant effect on the separation quality and their bounded values. Two or at most three parameters have to be selected: for example, the solvent composition and the column temperature in case of neutral solutes, the solvent composition and the pH of the mobile phase in case of ionogenic solutes.

The second stage consists in optimizing the ana-

lytical conditions. Different optimization procedures have been developed [1,2] and some of them are incorporated into commercially optimization software packages [3–5]. Today, however, these tools in spite of their convenience, are still not great commercial successes.

The third stage concerns the method validation process. It involves different tests to assess the quality of the analytical method. One of these is the ruggedness test. Different procedures may be used to test the ruggedness during the method validation. The analytical method is generally found rugged when small deliberate changes in its critical analytical parameters give consistently the same method performances. However, if the analytical method is

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not found rugged, the method development has to be started again from the second stage. This approach may be very time-consuming. It is possible to avoid a large amount of work by evaluating the ruggedness during the second stage described above.

In our opinion, the interest for optimization softwares may be greatly enhanced by this problem approach especially when two or three parameters are simultaneously optimized. Therefore, a response function based on a multi-criteria decision making (MCDM) process which takes into account the separation quality, the analysis time and the ruggedness as well, has been added to the optimization software OSIRIS developed in our laboratory [5,6].

By way of examples of simultaneous optimization of two parameters, some improvements were generated in method development using such a procedure.

2. Theory

Our optimization procedure, like most of the optimization procedures for liquid chromatography, belongs to interpretive methods and includes two steps, i.e., modeling and optimizing.

2.1. Modeling

The first step consists of modeling the solute chromatographic behavior using a minimum of preliminary experiments. The optimum conditions delivered by the optimum procedure depend strongly on the accuracy of the mathematical models applied to describe the solute behavior. Numerous works deal with the choice of both the models and the experimental strategy to calculate them. These choices are obviously related to the type of parameters [1] to be optimized but they are also generally related to the extent of the parameter space.

For solvent optimization, a linear retention model [7,8] is valid provided that neutral solutes and binary mobile phases are used and that the retention factors are within the interval 1–10. On the contrary, it is no longer valid in case of either ternary or quaternary mobile phases [9], in case of ionogenic solutes [10] or in case of a large interval of retention factors [11]. When the linear retention model can be applied, two preliminary gradient runs are sufficient for the model

estimation [12,13]. Otherwise, it has been shown for binary mobile phases, that accurate predictions of solute retention times are given by a quadratic model provided that it is calculated from a data set of three experiments: two gradient runs and a third experiment under isocratic mode at a strong solvent composition [14].

For pH optimization [15,16], three experiments are necessary to calculate the retention models. OSIRIS operates using a procedure detailed in a previous work [6] that allows one to calculate both the retention models and the variation of peak widths from a set of three experiments. The predicted results have been proved to be accurate within a pH range of two units or less. This procedure has been extended to the simultaneous optimization of both solvent composition and pH [10]. In this case, nine experiments are required: three experiments (two gradient and one isocratic runs) performed at three different pH values.

For neutral solutes and the simultaneous optimization of solvent composition and temperature, two gradient runs performed at two different temperatures values are required [17] for modeling both the retention behavior and the variation of peak widths [18].

2.2. Optimizing

Modeling the solute behavior allows calculation of chromatograms for any values of the parameter space. The aim of this second step (optimizing) is evaluation and comparison of the quality of computerized simulated chromatograms. This is performed by using a good quality response function in order to fulfill the objectives of the chromatographer. Each requirement is described by an elementary criterion and then, the different objectives may be expressed by the threshold values of these different elementary criteria.

2.2.1. Elementary criteria

Two elementary criteria, the separation quality and the analysis time, are usually of prime interest. The first one is related to the analysis performance, the second one deals with the analysis cost.

The minimum resolution, $R_{s,\min}$ is a good criterion to evaluate the separation quality as it allows to take

into account a threshold value for the less separated pair of peaks. The resolution determination is given in Appendix A.

The analysis time may be related to the retention time of the last eluted solute.

In addition to these two elementary criteria, we were interested by the estimation of the method ruggedness. The ruggedness may be characterized by the method's ability to maintain its performances despite some variations that can occur when the method is implemented. In accordance with this definition, the ruggedness cannot be readily quantified. Only the robustness can be quantified during the validation procedure. According to Jenke [19], it is "a measure of the method's ability to remain unaffected by small but deliberate variations in method parameter". Then, a robust method maintains its performance by changing an operational parameter by a known amount, that is not the case for a non robust method. Then, the robustness, evaluated during the validation process, behaves as a Boolean quantity. However, it is obvious that the larger are the possible variations of parameter values, the more rugged the method will be. According to the above definition, the robustness may be easily incorporated into an optimization procedure. However, the corresponding function will be much better if it may vary with the parameters values as the resolution and the analysis time do. In such a case, a compromise between the different criteria is then possible.

In this way, a criterion has been developed by Vanbel et al. [20]. It is based on the derivative of the minimum resolution versus the parameter P_i that is to be optimized.

In our point of view, this kind of criteria has some drawbacks: (1) when more than one parameter is to be optimized, a derivative function is defined for each of the parameters regardless of the others and then, the corresponding robustness criteria are not likely to consider the simultaneous variations of all parameters. (2) Threshold values for these criteria are difficult to estimate on the single basis of the experience. (3) This robustness criterion is defined by considering the quality of the separation alone, without considering the retention time constraints.

Hence, we prefer a global robustness factor, Rob, that is defined as follows:

The method is required to maintain its performances, in terms of separation and retention factors, for any variation higher than or equal to threshold values, $P_{th,i}$, fixed for each parameter P_i . These threshold values are assigned at the outset by the chromatographer according to his own requirements concerning the analysis. For each point of the parameter space, there is a window centered on this point with dimensions corresponding to the threshold values, $P_{th,i}$. Every point inside this window, has to fulfill the required conditions for both separation quality and retention factors. It is the basis window. However, a larger window, called the robustness window, may exist with all basis dimensions increased by the same factor, Rob. Then, $Rob \times P_{th,i}$ represents the extent to which the parameter P_i may vary, without losing the quality of the analysis. The required minimum value for Rob is 1. It corresponds to the minimum window likely to insure the robustness of the method.

An example is given in Fig. 1 for the particular case of two parameters P_1 and P_2 .

2.2.2. Response functions

Numerous response functions [21–23] which allow one to optimize simultaneously two elementary criteria have been used for qualifying the quality of a chromatogram. Most of them combine the resolution and the analysis time. Response functions, based on MCDM have been found to be more reliable [20]. Three different functions have been developed: the pareto optimality concept [24], the Multiple Threshold Approach [25] and the desirability functions [26].

The pareto optimality concept usually delivers several pareto-optimal points and requires the chromatographer to decide which of the pareto-optimal point is better. Hence, it is not very convenient for an optimization software. Moreover, it works well provided that there are only two elementary criteria.

The Multiple Threshold Approach is a method which leads to a sufficient resolution (higher or equal to a threshold value set for the minimum resolution) in a minimum analysis time. In previous work [27], we used such a threshold method, combining resolution and analysis time.

This method has been extended for optimizing the

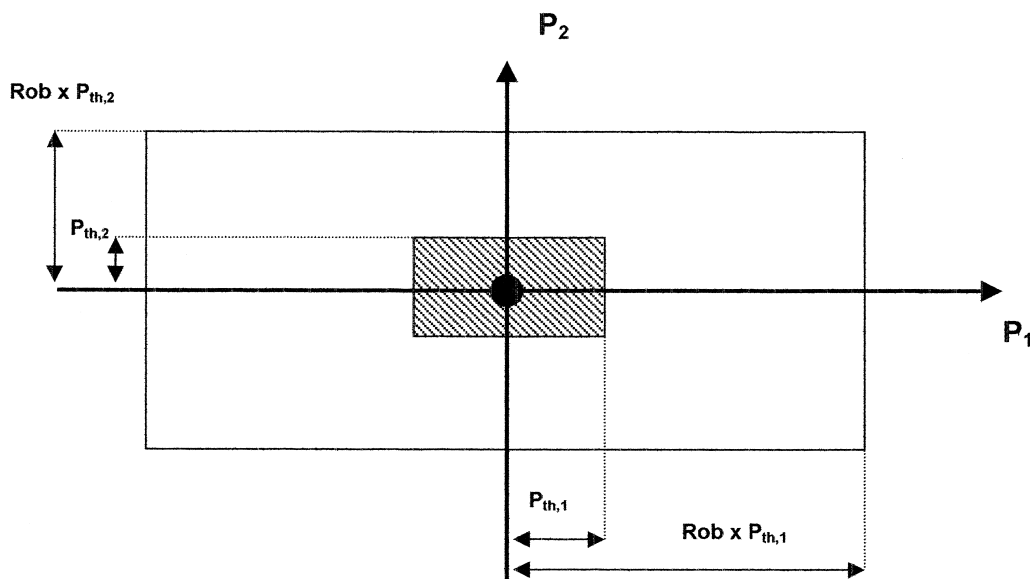


Fig. 1. Schematic example of both obtained and required robustness windows for a given point of the parameter space involving two optimization parameters, P_1 and P_2 (see text for explanations).

robustness factor too [5]. The response function is represented by a set of four requirements as follows:

- $k_{min} \leq k \leq k_{max}$
- $R_{s,min} \geq R_{s,threshold}$
- $Rob \geq 1$
- Minimum analysis time

with k_{min} and k_{max} being, respectively, the minimum and maximum retention factor values.

The advantages and the drawbacks of this method will be discussed later.

The desirability functions are based on a transformation of the individual criteria into a dimensionless value varying from 0 to 1 and then, they may be easily applied to the optimization of more than two criteria. These functions have been applied to the simultaneous optimization of different criteria: the resolution and the analysis time [28], the resolution and the robustness [20,29], or the resolution, the peak asymmetry and the analysis time [30]. In this work, we use desirability functions to simultaneously optimize the minimum resolution, the analysis time and the robustness. The development of this function is given in Appendix B.

In the Discussion, we will first compare the Multiple Threshold Approach to the desirability

functions and then prove the advantage of evaluating the robustness during the optimization procedure.

3. Experimental

3.1. Instrumentation

The liquid chromatograph consisted of two Shimadzu LC10 AD pumps (Touzart et Matignon, France), equipped with a Rheodyne 7520 injection valve (20- μ l sample loop), a 996 photodiode array detector (Waters, Milford, MA, USA) and a Millennium acquisition system (Waters). The polycyclic aromatic hydrocarbons (PAHs, Table 1) were separated on a 250 \times 4.6 mm I.D. Cosmosil column packed with 5 μ m particles. The dead volume of the column was estimated as 2.5 ml (assuming a column porosity of 0.6). The flow-rate was kept constant at 1 ml min^{-1} . The column temperature was varied using a cryogenic apparatus (Julabo F30).

The seven acids and bases (Table 1) were separated on a 150 \times 4.6 mm I.D. Capcell- C_{18} column (Interchim, France), the six substituted anilines

Table 1
Solutives for the different examples of optimization

Temperature and composition optimization		Composition and pH optimization		pK_a
Polyaromatic hydrocarbons		Benzoic acids		
1	Naphtalene	1	3,5-Dihydroxybenzoic acid	4.04
2	Acenaphtylene	2	2,5-Dihydroxybenzoic acid	2.97
3	Acenaphtene	3	Hydroxy-4-benzoic acid	4.48
4	Fluorene	4	Hydroxy-2-benzoic acid	2.97
5	Phenanthrene	5	Benzoic acid	4.19
6	Anthracene	6	Nitro-4-benzoic acid	3.41
7*	Fluoranthene			
8	Pyrene			
9	Benzo(b)fluoranthene	Substituted anilines		
10	Chrysene	1	Aniline	4.63
11*	Benzo(b)fluoranthene	2	<i>p</i> -Toluidine	5.08
12*	Benzo(k)fluoranthene	3	<i>m</i> -Toluidine	4.73
13*	Benzo(a)pyrene	4	<i>o</i> -Anisidine	4.52
14	Dibenzo(a,h)anthracene	5	<i>o</i> -Toluidine	4.44
15*	Benzo(g,h,i)perylene	6	<i>N,N</i> -Dimethyl aniline	5.15
16*	Indeno(1,2,3- <i>cd</i>)pyrene			
		Acids and bases		
		1	Amino-2-phenol	4.66
		2	<i>p</i> -Toluidine	5.08
		3	<i>m</i> -Toluidine	4.73
		4	<i>o</i> -Toluidine	4.52
		5	Hydroxy-2-benzoic acid	2.97
		6	Benzoic acid	4.19
		7	Nitro-4-benzoic acid	3.41

* Solutives of interest.

(Table 1) were separated on a 150×4.6 mm Zorbax-SBC₈ (Touzart et Matignon, France), and the six benzoic acids (Table 1) were separated on a 150×4.6 mm Kromasil-C₁₈ column (Interchim, France). The three columns were packed with 5 μm particles. The column dead volumes were estimated as 1.5 ml (column porosity of 0.6), and the flow-rates were kept constant at 1 ml min⁻¹. For the different experiments, the column temperature was maintained at 30°C using a water-bath.

3.2. Reagents

The solutes and their estimated pK_a values [31] are listed in Table 1. All the experiments were performed using binary mobile phases. The PAHs were separated with water and acetonitrile (ACN). The separation of the substituted anilines was performed with a citrate-phosphate buffer (0.025

M:0.025 *M*) and acetonitrile as organic modifier. The other separations were performed using a citrate buffer (0.05 *M*) and acetonitrile as organic modifier.

Acetonitrile was of HPLC quality. The aqueous buffers were prepared from citric acid and sodium dihydrogenophosphate; the pH was adjusted by adding adequate amount of 6 mol l⁻¹ sodium hydroxide and measured before the addition of the organic modifiers using an Orion 420A pH meter. Buffer solutions were then filtered through 0.65-mm nylon filters.

3.3. Software

The algorithms required for the different simulations and optimizations reported in this work have been first developed in our laboratory and incorporated then into the commercial version 3.0 of OSIRIS (Datalys, Grenoble, France).

4. Results and discussion

4.1. Comparison of the Multiple Threshold Approach and the desirability function method

The two methods were compared with two examples of simultaneous optimization of pH and organic content of the mobile phase. Nine experiments are required for optimizations: they are described in the Theory section: six gradient runs and three isocratic runs.

The first example concerns the chromatographic separation of six benzoic acids (solutes 1 to 6 in Table 1) by simultaneously optimizing pH and the organic content of the mobile phase (% ACN). The software OSIRIS provides a response surface which shows either $R_{s,\min}$ (Fig. 2a) or the desirability function (Fig. 2b) as functions of both pH and acetonitrile content. The response surface on Fig. 2a is determined from the following threshold values: 1.5 for $R_{s,\min}$, 0.5 for k_{\min} and 15 for k_{\max} . Black areas correspond to retention factor values outside of the interval defined by k_{\min} and k_{\max} . According to these constraints, Fig. 2a reveals two large light areas through which both $R_{s,\min}$ and analysis time requirements are fulfilled. It is obvious that such an information is not sufficient because it cannot be decided which area is the optimum area. The maximum of resolution (point 1), may characterized the best analytical conditions provided that there are no constraints on the robustness.

In order to take into account the robustness too, the global desirability function (Fig. 2b) was calculated with threshold values for the robustness window (Fig. 1): 1% for the acetonitrile content and 0.05 unit for the pH. Point 2 of Fig. 2a indicates the optimum given by the multiple threshold approach, point 3 of Fig. 2b indicates the optimum given by the desirability functions (see Appendix B).

Table 2 summarizes the predicted criteria values for these three points. The corresponding chromatograms are given in Fig. 3.

The analysis conditions corresponding to point 1 lead obviously to the best quality of separation ($R_{s,\min}=4.6$). However, the analysis time is long ($T_a=21$ min) and the method is not robust (Rob=0). It means that a small variation in pH (higher or equal to 0.05 unit) or/and in % ACN (higher than or equal

to 1%) will seriously affect the quality of the analysis.

The analysis conditions corresponding to point 2, i.e., the threshold method, are more attractive as they lead to a fast and complete separation. Furthermore, these conditions fulfill the robustness constraints with a robustness factor value equal to 1. However, at the same time, the resolution has been reduced to 1.6.

The analysis conditions given by point 3, i.e., the desirability function, provide the best conditions with a high resolution value ($R_{s,\min}=4.5$) while the analysis time is only increased from 8 min to 12 min with a robustness factor value equal to 2. It means that any change in % ACN in the range of 2% or/and any change in pH in the range of 0.1 unit will not affect the quality of the analysis. Considering the pK_a (Table 1) of the benzoic acids, it may be noted that the solutes are either partially or fully ionized at this optimum pH (4.5) while it is generally assessed that the robustness increases when the ionization is completely suppressed.

Fig. 3 illustrates the three separations using the experimental conditions previously determined: Fig. 3a, b and c for conditions 1, 2 and 3, respectively.

The second example was the separation of six weak bases (Table 1). The two parameters to be optimized were the pH and the acetonitrile content of the mobile phase. The response surface in Fig. 4a is determined from the following threshold values: 1.0 for $R_{s,\min}$, 0.5 for k_{\min} and 15 for k_{\max} . It reveals the possible area (light area) according to these constraints, black areas corresponding to k values outside the range.

The global desirability function (Fig. 4b) was calculated with the following threshold values for the basis robustness window (Fig. 1): 1% for the acetonitrile content and 0.1 unit for the pH. Point 1 indicates the optimum given by the Multiple Threshold Approach, point 2, the optimum given by the desirability functions (see Appendix B). These two analytical conditions are slightly different and hence lead to different results summarized in Table 3 and shown in the experimental chromatograms (a) to (d) of Fig. 5. It can be seen that the predicted chromatograms are in a very good agreement with the experimental ones.

From conditions 1 to conditions 2 the analysis

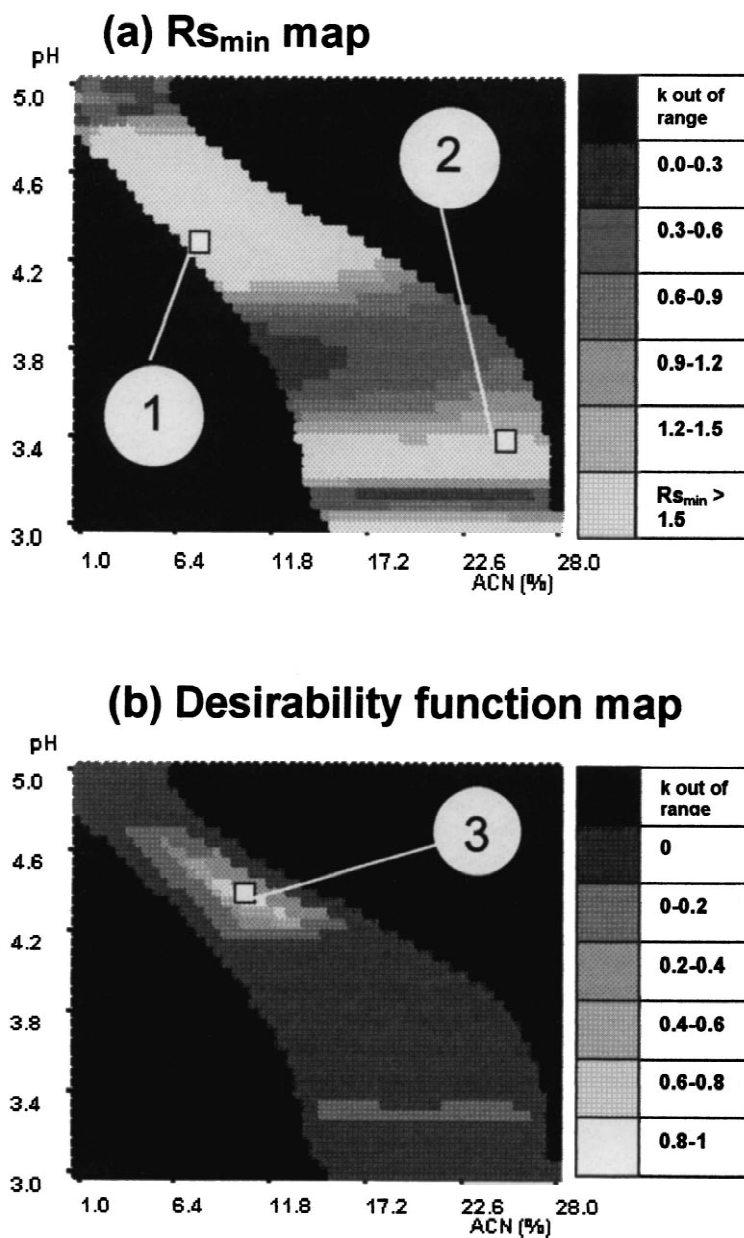


Fig. 2. Response surfaces of the minimum resolution (a) and the desirability function (b) obtained during the optimization of the separation of the six benzoic acids listed in Table 1. Points 1, 2 and 3 represent the optimum given by the maximum of $R_{s\min}$, the Multiple Threshold Approach and the desirability function, respectively.

time is only increased from 5.7 min to 7.1 min, the resolution increased from 1.2 to 1.5 meanwhile, the robustness factor is improved from 1 to 2. It means that a variation in pH, up to 0.2 unit, and/or a

variation in acetonitrile content, up to 2% will not affect the quality of the analysis.

It can be noted a robust separation was found at a pH value for which the six solutes are ionized while

Table 2

Comparison of the predicted performances for optimum conditions given by maximum resolution (point 1 of Fig. 2a), the Multiple Threshold Approach (MTA) (point 2 of Fig. 2a) and the desirability function (point 3 of Fig. 2b)

	Maximum resolution	MTA	Desirability function
Optimum conditions	% ACN=9%; pH=4.35	% ACN=26.5%; pH=3.3	% ACN=10%; pH=4.5
$R_{s,min}$	4.6	1.6	4.5
Analysis time	21.3 min	8.5 min	12.3 min
Robustness factor	0	1	2
Robustness window		1%×0.05 unit of pH	2%×0.1 unit of pH

no conditions were found as possible at pH values for which the solutes are not ionized.

The Multiple Threshold Approach may be con-

venient for an optimization software as it involves no compromise between the different criteria. It may be advantageous when only two criteria are of impor-

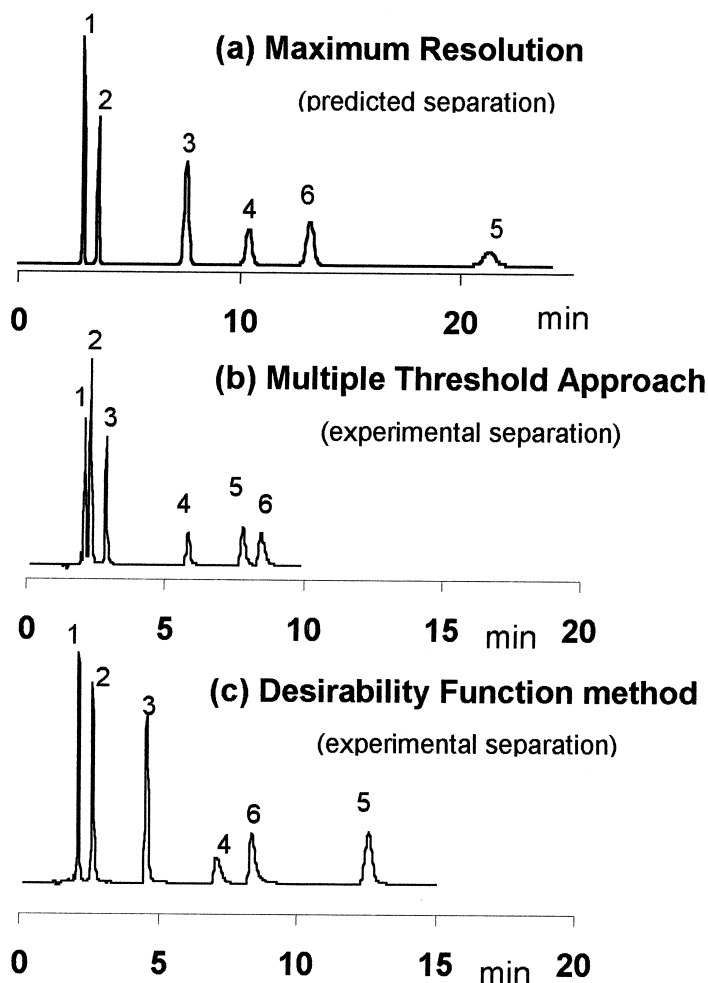


Fig. 3. Chromatograms at the optimum conditions (see Table 2) given by point 1 of Fig. 2a (a), point 2 of Fig. 2a (b) and point 3 of Fig. 2b (c).

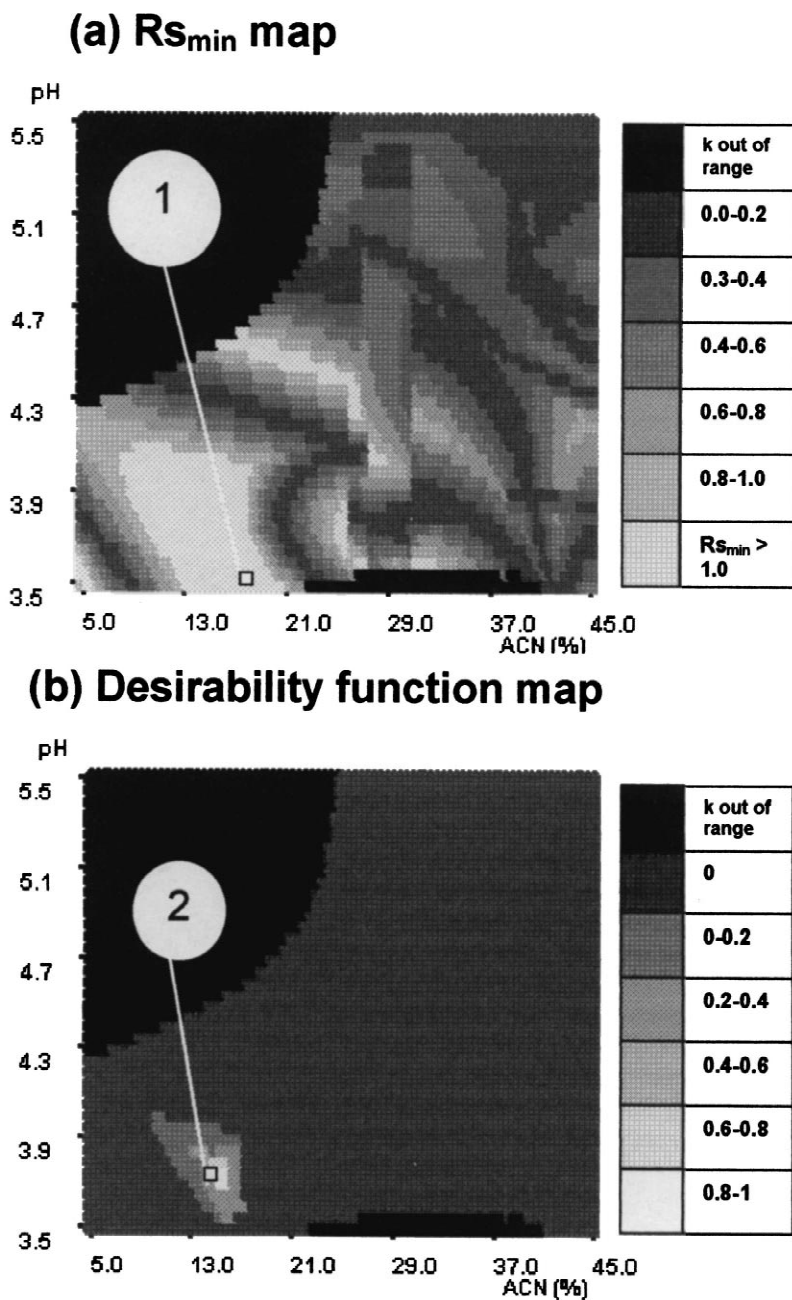


Fig. 4. Response surfaces of the minimum resolution (a) and the desirability function (b) obtained during the optimization of the separation of the six weak bases listed in Table 1. Points 1 and 2 represent the optimum given by the Multiple Threshold Approach and the desirability function, respectively.

tance, for example the resolution and the analysis time. However, in our opinion, the desirability function method is highly recommended when more

than two criteria have to be evaluated, as is the case in this work.

In fact, using the desirability function method, the

Table 3

Comparison of the predicted performances for optimum conditions given by the Multiple Threshold Approach (MTA) (point 1 of Fig. 4) and the desirability function (point 2 of Fig. 4)

	MTA	Desirability function
Optimum conditions	% ACN=19%; pH=3.6	% ACN=16%; pH=3.7
$R_{s,\min}$	1.2	1.5
Analysis time	5.7 min	7.1 min
Robustness factor	1	2
Robustness window	1%×0.1 unit of pH	2%×0.2 unit of pH

choice of the threshold values has a minor importance provided that they are reasonable from a chromatographic point of view. Adequate values may be $R_{s,\min} > 1$, $0.5 < k < 15$, and a basis robustness window defined by 1% in organic content, 0.05 unit in pH, 1°C depending on the optimization parameters. In contrast, it is obvious that this choice is crucial with Multiple Threshold Approach. As a priority is given to the analysis time, when threshold values are too low, the area corresponding to suffi-

cient resolution and sufficient robustness is large and it may exist a better optimum than the optimum given by the minimum analysis time. On the other hand, when the threshold values are too high, there is no solution.

Moreover, the desirability function method delivers a response surface and therefore, a response level for the different points of the surface. In contrast, with the threshold method, there is a lack of information as an unique optimum result, is provided.

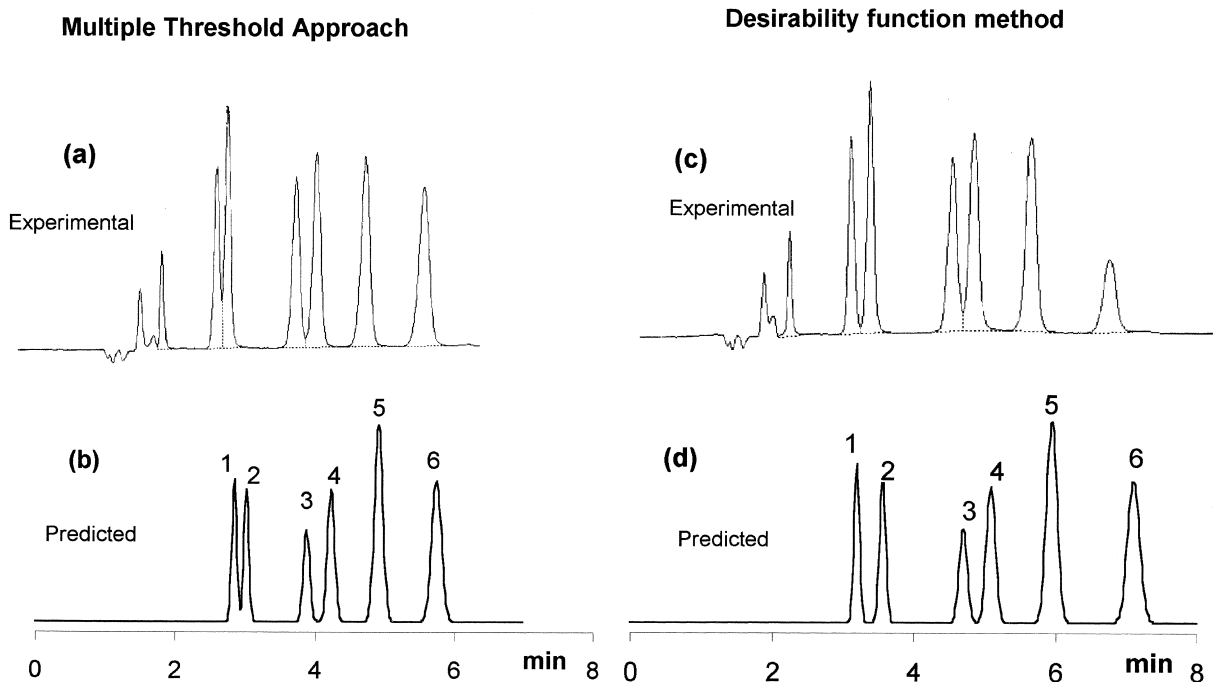


Fig. 5. Experimental (a) and predicted (b) separations for the optimum conditions given by the Multiple Threshold Approach. Experimental (c) and predicted (d) separations for the optimum conditions given by the desirability function. Analysis conditions given in Table 3.

4.2. Advantage for evaluating the three criteria during the optimization procedure

Two examples of rugged analysis conditions research were discussed in the following section.

The first one deals with a mixture of the 16 PAHs listed in Table 1. These 16 compounds are listed by the US Environmental Protection Agency (EPA) as dangerous water pollutants. According to the French standard (AFNOR NF T 90-115), six PAHs, indicated by a star in Table 1, are considered the most dangerous. The problem was then to obtain a separation of the PAHs, but more especially, a convenient separation of the specific PAHs mentioned.

The requirements for this separation were: $R_{s,min} > 1$; $0.5 < k < 20$ and robustness window: $2^{\circ}\text{C} \times 1\%$ ACN.

The purpose of this study was to search for a composition at ambient temperature ($20 \pm 2^{\circ}\text{C}$) that leads to a rugged analysis.

Four gradient runs were performed (two at 0°C and two at 40°C) to model the solute behavior as a

function of both temperature and acetonitrile content of the mobile phase.

The resulting desirability function map is shown in Fig. 6. It was determined by taking into account the three criteria: resolution, analysis time and robustness. Highest response function values (response = 0.8) occurs within a region indicated in white color and corresponding to high acetonitrile content. It appears that for each temperature, bounded by $15^{\circ}\text{C} < T < 35^{\circ}\text{C}$, there is an acetonitrile content leading to a maximum value of the response function. At 20°C , the optimum is located at a value of 93% for the acetonitrile content. Predicted values for $R_{s,min}$, analysis time and robustness factor are 1.4, 13 min and 3, respectively. Another solution is given in Fig. 6. It corresponds to a response value equal to 0.55. In this case predicted values for $R_{s,min}$, analysis time and robustness factor are 1.2, 45.5 min and 3, respectively. Although the robustness factor is the same, the former conditions are much more attractive as regards to the analysis time. The actual separation ($T = 20^{\circ}\text{C}$ and % ACN = 73%) is shown in Fig. 7a

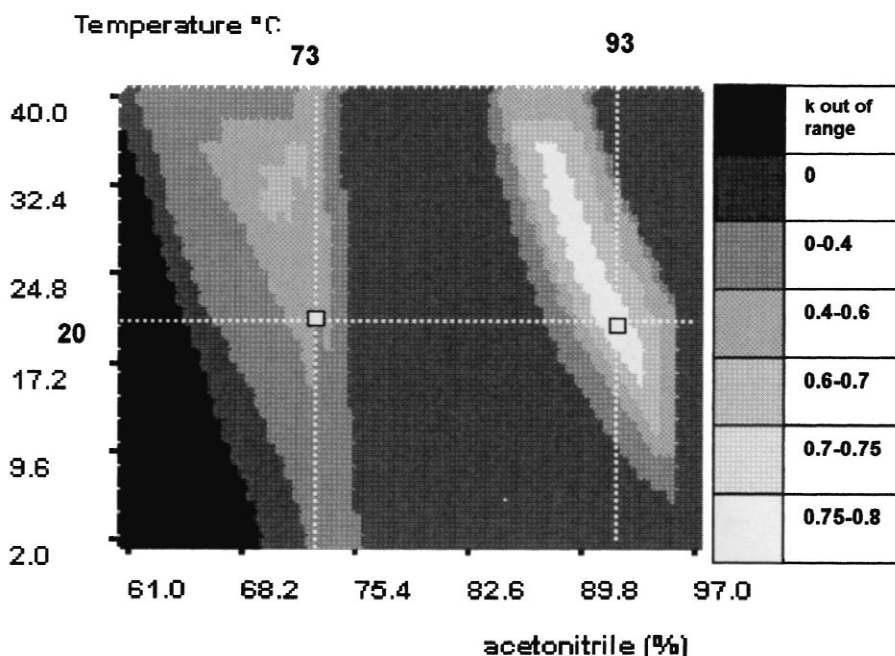


Fig. 6. Response surface of the desirability function obtained during the optimization of the separation of the 16 polycyclic aromatic hydrocarbons listed in Table 1.

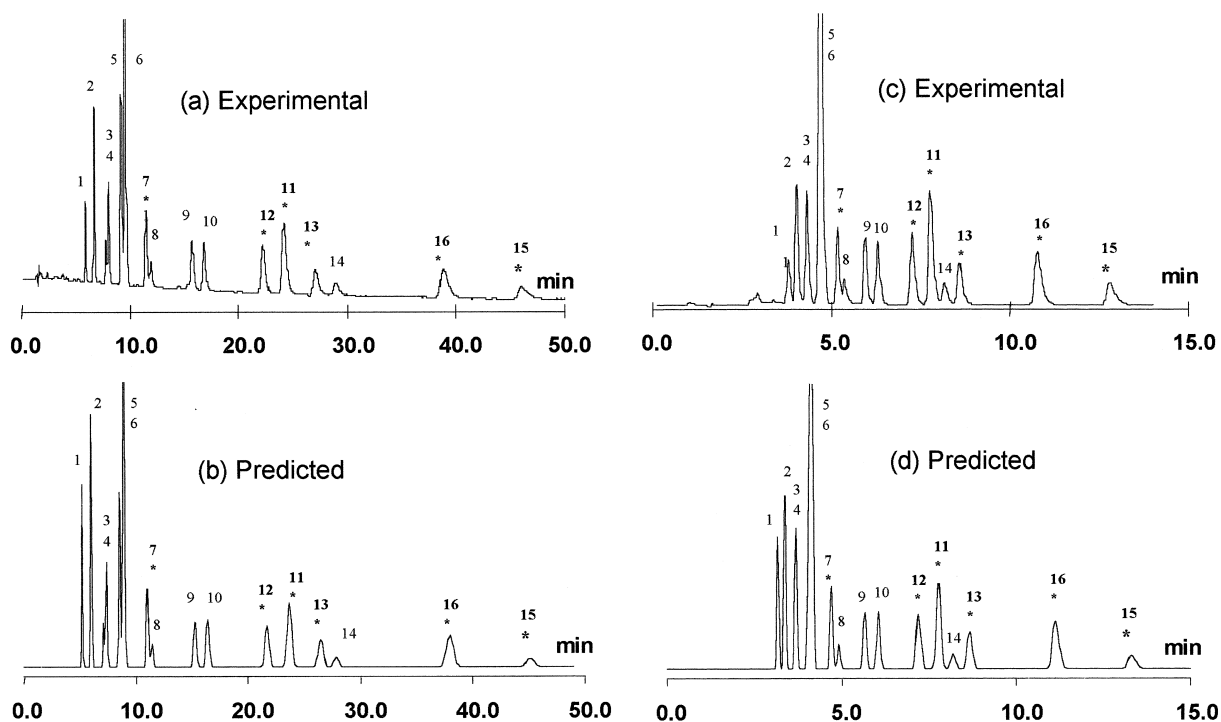


Fig. 7. Experimental (a) and predicted (b) separations for 20°C and 73% acetonitrile. Experimental (c) and predicted (d) separations for optimum conditions (20°C and 93% acetonitrile) given by Fig. 6.

and compared to the predicted separation of Fig. 7b. The optimized actual separation ($T=20^{\circ}\text{C}$ and % ACN=93%) is shown in Fig. 7c and compared to the predicted separation of Fig. 7d. As expected, experimental versus predicted results are in very good agreement..

In order to assess the robustness of the method, a set of experiments has been performed in our laboratory within 3 months, using the optimum acetonitrile content (93%) and a temperature within 18°C and 22°C. It was found that $R_{s,\min}$ values vary within the interval bounded by 1.2 and 1.4, which was satisfactory with respect to our requirements.

The second sample was a mixture of the seven ionogenic solutes, weak acids and weak bases, listed in Table 1. The requirements for the separation were: $R_{s,\min} > 1.5$; $0.5 < k < 20$; robustness window: $1\% \times 0.05$ unit of pH.

Nine experiments were required for the simultaneous optimization of both pH and acetonitrile content of the mobile phase. The obtained response

surfaces are given in Fig. 8. The first one (Fig. 8a) represents the desirability function according to the two studied criteria, the resolution and the analysis time. Several light areas reveal regions where the resolution and the analysis time fulfil the requirements. The complexity of the response surface attests of the many changes in solute elution order.

The second response surface (Fig. 8b) represents the desirability function according to the three criteria, the resolution, the analysis time and the robustness. When the robustness is taken into account, the region where the response function is strictly positive is restrained.

The comparison of the predicted performances given by the two optimum is shown in Table 4. The first optimum (given in column 1) is more attractive as regards to the analysis time but it is not robust as it is confirmed by the experimental chromatogram (Fig. 9a) carried out with optimum conditions given by Table 4 column 1. Variations of experimental conditions within the required robustness window

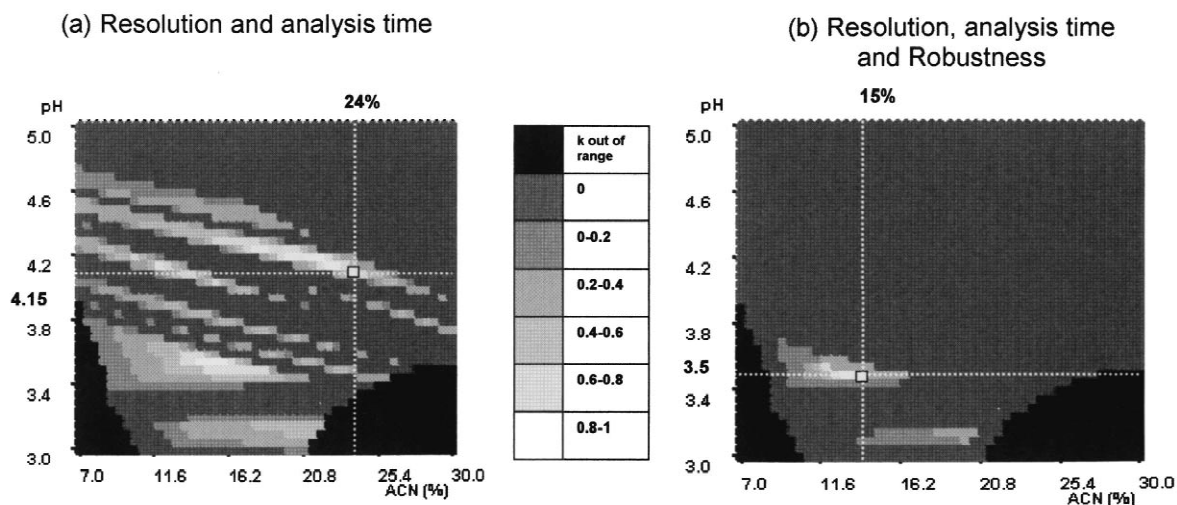


Fig. 8. Response surfaces of the desirability function obtained during the optimization of the separation of seven ionogenic solutes listed in Table 1. Calculated from the minimum resolution and the analysis time (a), from the minimum resolution, the analysis time and the robustness (b).

(Fig. 9b–e) led to the main variation of the separation quality: solute pairs 6/3 (Fig. 9b) and 1/5 (Fig. 9e) are no longer separated.

The second optimum (Table 4 column 2) gave rise to a robust and good separation as confirmed by the chromatograms given in Fig. 10. Hence, the five chromatograms (Fig. 10a–e) fulfill the requirements of resolution and time showing that these conditions are actually robust within the entire robustness window.

In addition, the long term repeatability of these optimum conditions has been tested successfully by a set of 15 experiments performed within 4 months, the $R_{s,\min}$ values varying within the interval bounded

by 2.0 and 2.8 (Table 5). By contrast, the optimum conditions based on the only resolution and analysis time without the robustness, seem to be unsuitable on a reproducibility point of view.

While the first example reveals the great advantage of taking into account the analysis time in addition to resolution and robustness, the second one shows how it is absolutely necessary to take into account the robustness during the optimization procedure in order to avoid a failing of the validation procedure.

Such an approach is also applied in our laboratory to elution gradient optimization to evaluate how the method robustness is affected by changes either in

Table 4

Comparison of the predicted performances for optimum conditions given by the two response surfaces of Fig. 8

	Desirability function based on resolution and analysis time (1)	Desirability function based on resolution, analysis time and robustness (2)
Optimum conditions	% ACN = 24%; pH = 4.15	% ACN = 15%; pH = 3.5
$R_{s,\min}$	1.7	2.5
Analysis time	6.5 min	14.5 min
Robustness factor	0	2
Robustness window		2% \times 0.1 unit of pH

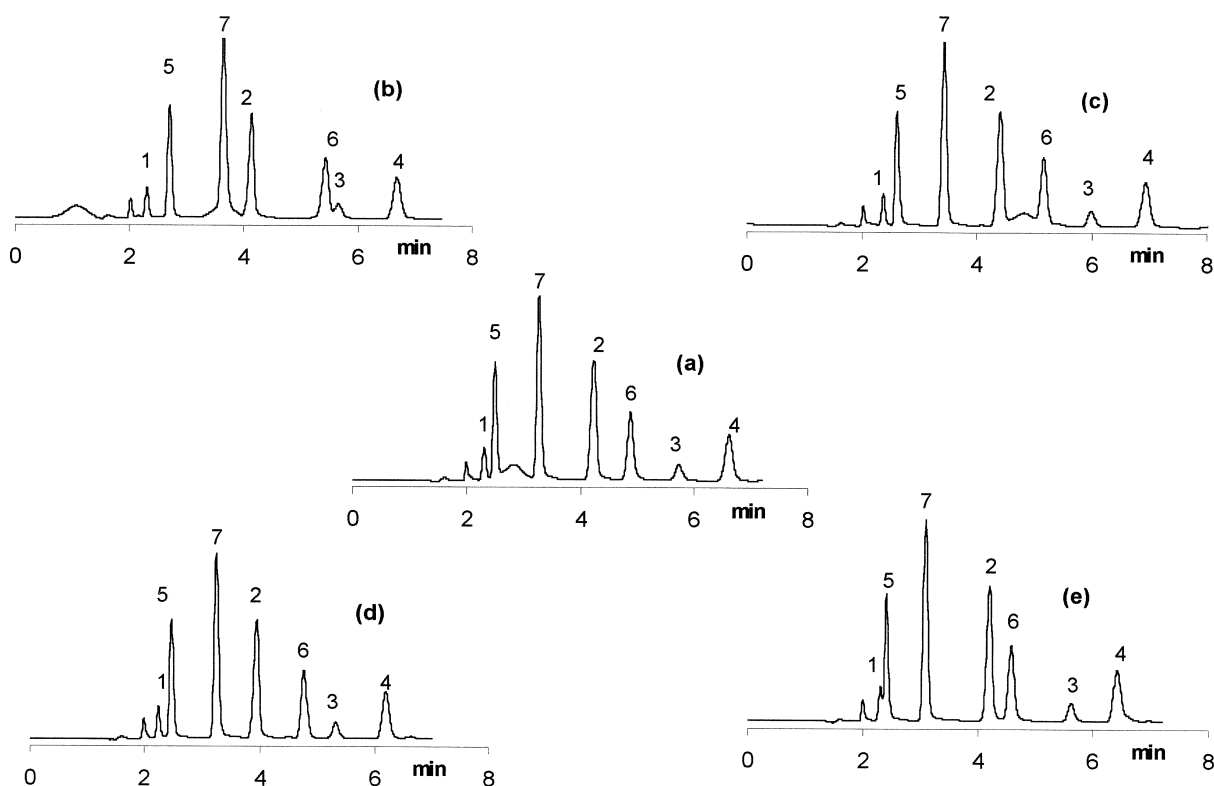


Fig. 9. Experimental separations for the optimum conditions of Table 4 column 1 (a) and their related conditions corresponding to the four corners of the required robustness window: 23% acetonitrile and pH 4.10 (b), 23% acetonitrile and pH 4.20 (c), 25% acetonitrile and pH 4.10 (d), 25% acetonitrile and pH 4.20 (e).

the dwell time or in other parameters. Hence, it is well known that methods based on gradient elution mode are less transferable because of differences in gradient equipment.

5. Conclusions

It has been shown that it is highly recommended to take into account the robustness during the optimization procedure. It allows one to define, without any doubt, the regions of the parameter space that are not robust according to the required performances. Likewise, it allows to define the optimum conditions, if they exist, according to the three relevant criteria, the quality of the separation, the analysis time and the robustness. In all cases, it leads to an advantageous gain of time for method

development. Such an approach is useful provided that two relevant parameters at least have to be optimized simultaneously and consequently, it requires the help of optimization software.

It has been shown that the multiple threshold approach may lead to unexpected results. A multicriteria response function such as the desirability function of Derringer is more convenient as it allows an attractive compromise between the criteria provided that the target values are well defined.

Appendix A

In order to determine the quality of the separation between two non ideal peaks (i and j), the resolution is calculated by the expression of the modified

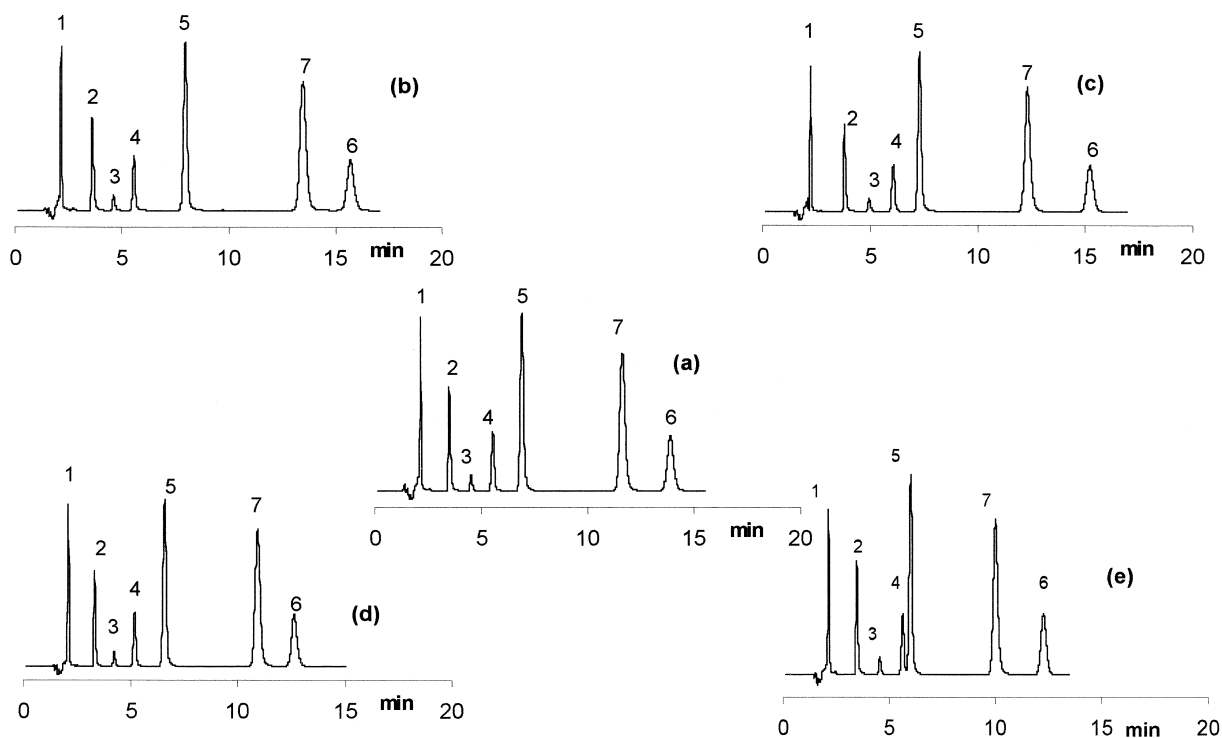


Fig. 10. Experimental separations for the optimum conditions of Table 4 column 2 (a) and their related conditions corresponding to the four corners of the required robustness window: 14% acetonitrile and pH 3.45 (b), 14% acetonitrile and pH 3.55 (c), 16% acetonitrile and pH 3.45 (d), 16% acetonitrile and pH 3.55 (e).

resolution function developed by Schoenmakers et al. [32]:

$$R_s = \frac{(t_j - t_i)(1 + A_{s,i})(1 + A_{s,j})}{A_{s,i}t_i f_i (1 + A_{s,j})\sqrt{N_j} + t_j f_j (1 + A_{s,i})\sqrt{N_i}} \times \frac{\sqrt{N_i N_j}}{4} \quad (\text{A.1})$$

where t is the retention time, A_s is the asymmetry

factor, N is the number of theoretical plates and f is a factor which depends on both the peak height, h , and the solute interest, w (equal to 1 or 0 if the solute is, respectively, of interest or not), according to Ref. [4]:

$$f_i = \sqrt{1 + 0.5 \ln(h_i/h_j)} \text{ and } f_j = 1 \text{ if } h_i > h_j \text{ or } w_i = 0$$

$$f_i = 1 \text{ and } f_j = \sqrt{1 + 0.5 \ln(h_j/h_i)} \text{ if } h_i \leq h_j \text{ or } w_i = 0$$

(A.2)

Table 5

Experimental study of the long term repeatability at the optimum conditions listed in Table 4 (experiments performed within 4 months)

	Optimum conditions based on resolution and analysis time	Optimum condition based on resolution, analysis time and robustness
Minimum $R_{s,\min}$ value	1.4	2.0
Maximum $R_{s,\min}$ value	2.0	2.8
$R_{s,\min}$ standard deviation	0.2	0.2
Number of experiments	11	15

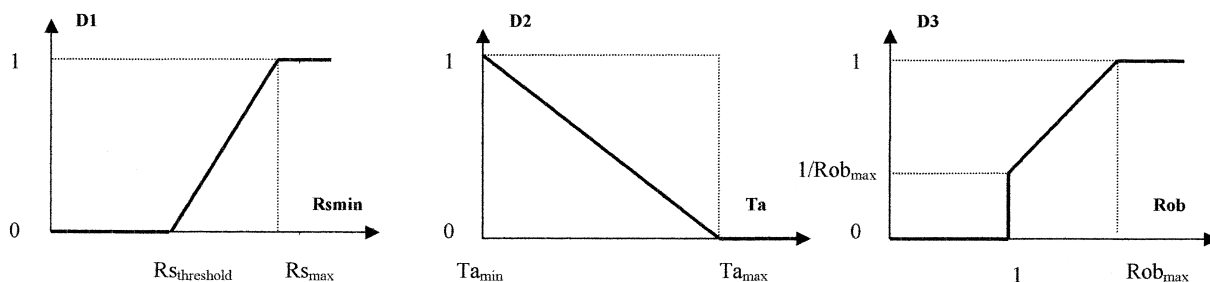


Fig. 11. Individual desirability functions, D_1 , D_2 and D_3 as functions of the three criteria, $R_{s,\min}$, analysis time and robustness factor, respectively.

Appendix B

The response function is a combination of three individual criteria: the minimum resolution, the analysis time and the robustness factor.

The three corresponding desirability functions, D_1 , D_2 and D_3 are the results of the following transformations on these criteria (see Fig. 11):

$$D_1 = 0 \quad \text{for } R_{s,\min} < R_{s,\text{threshold}}$$

$$D_1 = 1 \quad \text{for } R_{s,\min} > R_{s,\text{max}}$$

$$D_1 = \frac{R_{s,\min} - R_{s,\text{threshold}}}{R_{s,\text{max}} - R_{s,\text{threshold}}} \quad \text{for } R_{s,\text{threshold}} \leq R_{s,\min} \leq R_{s,\text{max}}$$

$$D_2 = 0 \quad \text{for } k > k_{\text{max}} \text{ or } k < k_{\text{min}}$$

$$D_2 = \frac{T_{a,\text{max}} - T_a}{T_{a,\text{max}} - T_{a,\text{min}}} \quad \text{for } T_a \leq T_{a,\text{max}}$$

$$D_3 = 0 \quad \text{for } \text{Rob} < 1$$

$$D_3 = 1 \quad \text{for } \text{Rob} > \text{Rob}_{\text{max}}$$

$$D_3 = \frac{\text{Rob}}{\text{Rob}_{\text{max}}} \quad \text{for } 1 \leq \text{Rob} \leq \text{Rob}_{\text{max}} \quad (\text{B.1})$$

where $T_{a,\text{max}}$ is the maximum analysis time given by

$$T_{a,\text{max}} = t_0(1 + k_{\text{max}}) \quad (\text{B.2})$$

$T_{a,\text{min}}$, $R_{s,\text{max}}$ and Rob_{max} , are the minimum analysis time, the maximum $R_{s,\min}$ value and the maximum robustness factor value, respectively that can be obtained within the parameter space. $R_{s,\text{max}}$ and Rob_{max} may be higher than a reasonable limit value and in such a case, they are adjusted to this value. For example, it may be considered that a resolution

higher than 3 and a robustness factor higher than 5 are not relevant from a chromatographic point of view.

The overall desirability function is the geometric mean of the three desirability functions according to:

$$D = (D_1 \times D_2 \times D_3)^{1/3} \quad (\text{B.3})$$

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