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Review

Experimental design and multiple response optimization. Using the desirability function in analytical methods development

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ABSTRACT

A review about the application of response surface methodology (RSM) when several responses have to be simultaneously optimized in the field of analytical methods development is presented. Several critical issues like response transformation, multiple response optimization and modeling with least squares and artificial neural networks are discussed. Most recent analytical applications are presented in the context of analytLaboratorio de Control de Calidad de Medicamentos (LCCM), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, C.C. 242, S3000ZAA Santa Fe, ArgentinaLaboratorio de Control de Calidad de Medicamentos (LCCM), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, C.C. 242, S3000ZAA Santa Fe, Argentinaical methods development, especially in multiple response optimization procedures using the desirability function.

Contents

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1. Introduction

Due to the increasing quality requirements of regulatory agencies, the high cost of reagents and the large quantity of variables affecting the analytical process, the procedure of development and validation of analytical methods cannot be considered as a simple task. In this context, the term "optimization" seems to refer to improving the performance of the analytical process, i.e. discovering the conditions at which the best response is obtained [1]. In analytical chemistry, optimization is a critical stage to find the value that each factor must have to produce the best possible response. It must be done assuring a good performance in the analytical methods which are being developed in the laboratory, modified from official or standard methods or obtained from the scientific literature.

In this context, the multivariate design of experiments (DOE) is an important issue because it takes less time, effort and resources than the univariate procedures (which are surprisingly still being used in routine method development), and facilitates the gathering of large quantities of information while minimizing the number of experiments [2]. DOE and the response surface methodology (RSM) have been proved to be useful for developing, improving and optimizing processes [3]. The RSM has been extensively used in analytical applications [4–7], industrial world [8–12] and in bioprocesses [13–16].

As it can be appreciated in the flow chart presented in Fig. 1, DOE and RSM are mostly applied to analytical separations and extraction procedures. After a first screening study, a response surface design is built which provides data that must be generally modeled through the least squares fitting or, exceptionally, by artificial neural networks. When a large number of responses should be optimized (following the appropriate criteria), the desirability function is the most popular tool to be applied [17].

In this review, the role of the DOE in the analytical method optimization stage will be analyzed. Several critical issues will be discussed, especially those which have not been addressed extensively in previous reviews, such as response transformation and multiple response optimization. Finally, some analytical applications are presented in the context of analytical methods development, particularly in multiple response optimization procedures using the desirability function.

2. Aim and methodology of experimental design

A major role of experimental design in analytical chemistry concerns method optimizations, where the main purpose is to discover the experimental conditions which produce the best possible analytical performance [1]. Two stages may be considered in method optimization: (a) a screening step, where many factors are studied to identify those with the significant effects on critical variables, and (b) the optimization, where the factors are further examined in order to determine the best analytical conditions. In addition, experimental design is also used in analytical chemistry to evaluate robustness in method validation (to examine the effects that small changes in the analytical method conditions have on the responses) and to build calibration and validation sets to be used for calibration purposes [18].

Two optimization strategies can be distinguished: the univariate and the multivariate approaches. In the first, only one factor is

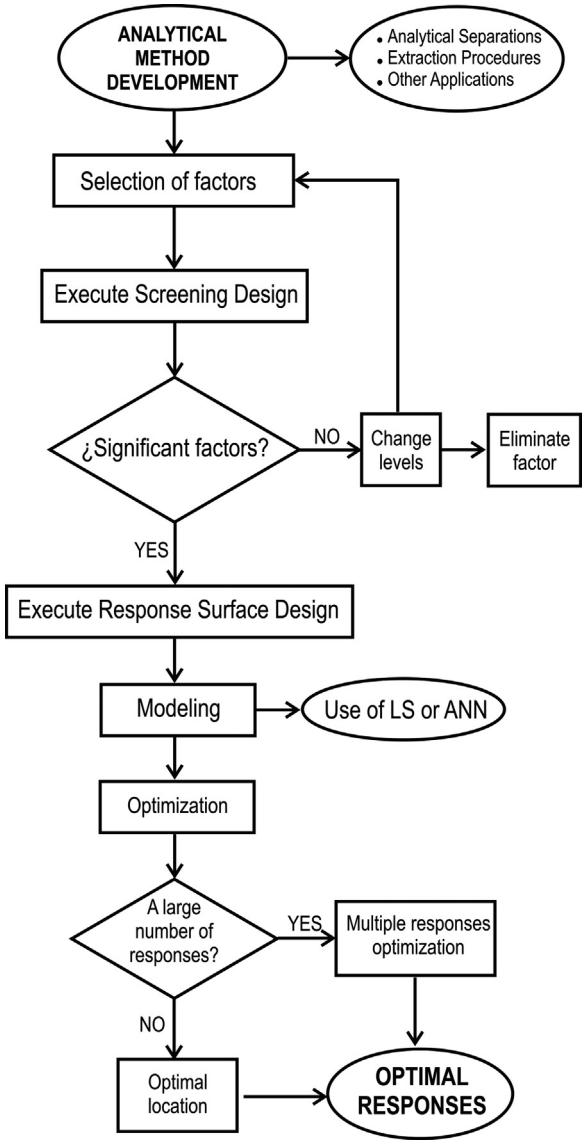

Fig. 1. Flow chart which shows schematically how DOE and RSM are applied in analytical method developments.

varied at a time (OVAT), while the other factors remain constant. This classically applied procedure does not take into account interactions between factors. Moreover, the number of experiments is important when the number of factors increases, and usually the experimental domain explored is smaller compared to that examined with the multivariate approach.

On the other hand, in the multivariate strategy, several factors are simultaneously studied in a predefined number of experiments, varying together the levels of all the factors involved in the process. The multivariate procedure has the following advantages in relation to the univariate strategy, which were discussed in the excellent tutorial written by Leardi [19]:

(a) It is possible to know thoroughly the studied system, having a global knowledge of it in the whole experimental domain. From the results obtained, a mathematical model can be constructed to relate the response to the experimental conditions. The response for any point of the experimental domain can be predicted after an estimation of the coefficients of the model.
(b) The number of experiments is smaller than the number of experiments required in the OVAT approach, reducing cost, effort and time.
(c) It is possible to study the interactions between factors and the non-linear relations with the responses.
(d) Generally, it is possible to find the absolute optimum in the studied domain, while the OVAT approach can find local maximum which depends on the initial conditions of the analysis.
(e) The quality of the information gathered in every experimental point can be known through the leverage.

In order to perform a correct experimental design, most of the authors recommend considering the following steps [3,20]:

2.1. Approach to the problem

It is necessary to have a clear idea about the issue in hand and about the optimization objectives. The experimental design is a tool which allows finding solutions to properly defined analytical problems. The objective of the study should be clearly identified and specified, as well as time and cost of experimentation must be evaluated.

2.2. Selection of the response variables

A variable which can provide the necessary information in the evaluation of the analytical performance of the method must be selected to be subjected to the optimization procedure. This variable is called response and, according to the objective, it may be necessary to observe more than one response. Many variables may be selected as response for example, analyte recovery (accuracy), pre-concentration factor, peak area (sensibility), peak tailing, chromatographic resolution (selectivity), relative standard deviation (precision), migration or retention time (efficiency), etc.

2.3. Selection of factors and their levels

All the factors that may affect the process must be carefully detected and examined. The experimental domain must be defined for each factor and also a way of control and measurement must be established. The factors can be divided into quantitative, qualitative and mixture-related (e.g. volume of solvents).

Since the number of factors to be considered can be important, it is necessary to perform screening experiments to determine the experimental variables and interactions that have a significant influence on one or several responses.

In screening designs, the factors are usually examined at two levels (−1, +1). The range between the levels is the broadest interval in which the factor can be varied for the system under study and is chosen on the basis of the literature information or earlier knowledge.

2.4. Selection of an experimental design

Attention should be paid to the issues to be considered for the selection of the best experimental design for each stage. They are (a) stated objective: type of problem and known information; (b) number of factors and interactions to be studied; (c) statistical validity and effectiveness of each design; (d) operating, cost and time restrictions; and (e) easiness of understanding and implementation complexity of each design.

Table 1
Designs employed for the screening stage.

Design	Factors		Res[a]	Number of experiments	Estimated Effects	O[b]	R[c]
	Type	Number					
Two-level full factorial (2-FFD)	Numerical Categorical	$2 \le k \le 5$	–	2^k 2^{k+1} (considering replicates)	2^k–1 Main effects, interactions (two-way, three-way and k factors)	Yes	Yes
Two-level fractional factorial	Numerical Categorical	>4	III or $>$	2^{k-p}	R=III (main effects confounded with two-factors interactions), R=IV (main effects confounded with three-factors interactions and two-factors interactions confounded with two-factors interactions), R=V (main effects confounded with four-factors interactions and two-factors interactions confounded with three-factors interactions)	Yes	Yes
Plackett–Burman (P–B)	Numerical Categorical	N–1	III	N (multiple of 4)	Main effects confounded with (fractions) of the two and higher order interactions.	Yes	Yes

k=number of factors; p=number of independent generators.

[a] Res: resolution.
[b] O: orthogonality.
[c] R: rotability.

2.5. Execution of the experiments and determination of the responses

In this step, it is recommended to take into account the following considerations: (a) the observations and the errors should be independent random variables; (b) when the number of experiments exceeds the amount that can be done in one day or in a work sequence, the experiments should be performed in blocks (each block corresponds to a different day or work sequence, and it is necessary to take into account the blocking effect); (c) in a first instance it is necessary to apply computations to estimate the factor effects on a response. An estimate is always subject to error, and in order to decide whether or not the effect is significant, the standard deviation of the effect should be known.

This standard deviation is also called standard error on an effect and it can be computed from the standard deviation of an experimental measurement (experimental error, random error, and experimental uncertainty).

Random error is due to common causes of the process and represents the observed variability of the response that cannot be explained through the factors under study. This error may include the effect of the non-studied factors and operator errors made during the execution of the experiments. If the variability due to the two latter cases is important, it is not possible to distinguish what is the true effect that the studied factor exerts on the response. For this reason, it is important that the operator errors remain small or negligible, and to avoid freely variations of any factor with a significant influence on the response.

In some situations the random error is determined as analytical procedure precision. If, on the contrary, it is not previously known, there are different ways to estimate it. A first approach is to replicate the central and/or other points of the design, and estimate the variance. In this case, it is required that replicates be measured under intermediate precision conditions. Measurements under repeatability conditions lead to underestimate the experimental error and then too many effects could be erroneously regarded as significant.

A second approach is that the experimental error can also be estimated from 'a priori' declared negligible effects, e.g. effects of dummies factors and factor-interactions. The dummies factors are imaginary variables for which the change from one level to the other does not represent a physical change. The effects observed for the dummies and for three- or higher-factor interactions are often considered to be due to random errors.

In a third approach, the experimental error is derived from 'a posteriori' declared negligible effect, e.g. using the algorithms of Dong or Lenth, which are based on the distribution of the non-significant effects [21].

3. Designs

3.1. Screening designs

Full factorial, fractional factorial and Plackett–Burman designs, all of them at two levels for each factor (k), are the most widely used in the step of selection of factors because they are economic and efficient. The principal features are presented in Table 1.

As the factorial fractional design is one of the most used for screening purposes, a brief description of this kind of design will be presented next. This design enables the evaluation of a relatively large number of factors in a small number of experiments by fractioning a full factorial 2^k design in a 2^{k-p}design, where p represents the number of independent design generators, chosen to fractionate the design. Different fractional designs can be created for a large number of factors. For example, when examining six factors, a half-fraction factorial design with $2^{6-1}=32$ experiments, a quarter-fraction factorial design with $2^{6-2}=16$ experiments or an eighth-fraction factorial design requiring only $2^{6-3}=8$ experiments, are all possible. However, fractional designs do not enable the estimation of all major and interaction effects separately because some of them are estimated together, i.e. they are confounded (Table 1).

3.2. Optimization designs

They allow modeling a second order response surface (see below). The most widely used designs in this step are full factorial

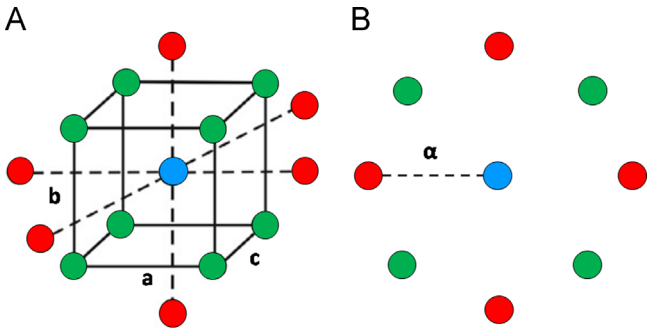

Fig. 2. (A) Three-dimensional representation of a spherical "near- rotatable" central composite design for optimization. Three factors (a, b and c) are assessed each at five levels. The green dots represent the factorial points (Fp), red dots the star points (Sp) and the blue dot the center point (Cp). (B) In a plain projection, the design allocates the points in a circumference of radius α equal to 1.682. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Response-surface designs most commonly used for optimization step.

Design	Type of factors	Factor levels	Number of experiments	Orthogonality	Rotability
Central composite (CCD)	Numerical Categorical	5	$2^k+2k+Cp$	Yes–No[b]	Yes–No[a]
Box–Behnken (BBD)	Numerical Categorical	3	$2k(k-1)+Cp$	Yes	Yes
Full factorial design at three levels (3-FFD)	Numerical Categorical	3	3^k	Completely orthogonal	No
Doehlert Matrix (DMD)	Numerical Categorical	Different for each factor	k^2+k+Cp	No	No
D-Optimal	Numerical Categorical	Different for each model. Irregular experimental domains	Selected subset of all possible combinations	No	Yes

[a] May be rotatable if $\alpha=(f)^{1/4}$, being α the star point distance and f the number of factorial point.
[b] May be orthogonal if $Cp \approx a\sqrt{f+4}-f$, being Cp the number of center points, k=number of factors.

at three levels, central composite, Box–Behnken, D-optimal and Doehlert designs, whose main features are presented in Table 2. All these optimization designs allow to obtain experimental data which are then fitted in a polynomial model of multiple linear regression with the purpose of characterizing a response surface [22,23].

The well-known central composite design will be described as follows: two-level (−1 and +1) factorial design points (*Fp*), axial or "star" points (*Sp*) and center points (*Cp*) with all factors set to 0. Al factors in *Sp* are set to 0, except one factor with the value $\pm\alpha$. The α value determines the location of the *Sp* in the design and usually varies from 1 to $\sqrt{k}$.The former allocates the axial points in the face of the cube or hypercube and is known as "face-centered central composite design". The latter results in experimental points placed in the domain of a (hyper)cube. Rotatable designs will be achieved with $\alpha=(f)^{1/4}$,where *f* is the number of *Fp*. This is an interesting property, since the experimenter does not know at the outset where the desirable response may lie within the design space. Rotatability ensures a reasonably stable distribution of the prediction variance in all the design space. However, for $k>3$ rotatable designs require a large α value, which is not always practical from an operational point of view. In such cases, a spherical design, which results "near-rotatable" is preferable to stabilize the variance in a narrower and acceptable experimental region. In Fig. 2 the experimental points of a spherical central composite design with three factors (*a*, *b* and *c*) are represented. At least fifteen experiments are required in this case in order to construct the mathematical model with an efficient estimation of the quadratic terms.

When the analyzed factors are the components of a mixture, their levels are not independent from one another. In this situation, it is necessary to use a mixture design i.e. a response surface design which allows to study the effect of the variation of the ratios among the variables. In this design, the domain is a regular figure having as many vertices as components, in a space with dimensionality equal to the number of components minus one [19].

The graphical representation corresponding to a mixture design of three components is an equilateral triangle whose vertices correspond to combinations containing 100 percent of a single component. Each of the three sides represents a mixture that does not have one of the three components (binary mixtures). Besides, the internal points correspond to the ternary mixtures. To examine the effects of mixture components on the response variable, simplex designs are used, and simplex lattice or simplex centroid design can be selected among them. A simplex centroid design for the three components is shown in Fig. 3. These designs are usually augmented with additional points in the interior of the experimental region. The models used in mixture designs differ from the polynomials used in response surface for independent variables (see below). They are the well-known Scheffé polynomials, which can be linear, quadratic, full cubic and special cubic [22].

4. Modeling

4.1. Model building for screening

The general approach to the statistical analysis of screening design for every response being analyzed includes (1) estimating factor effects and examining their signs and magnitudes, (2) building an initial model for the response [see Eq. (1)], (3) performing statistical tests, (4) refining the model removing any non-significant variable from the initial model, and (5) analyzing residuals in order to check model adequacy and assumptions [22].

The effect of each factor on each response is estimated as the difference between the average response of the experiments with positive signs and the average response of the experiments with negative signs (in a codified design, see above). An alternative to determine the factor effect is estimating the coefficients of the model. The observations or responses (*y*) in factorial screening experiments can be described by a linear statistical model:

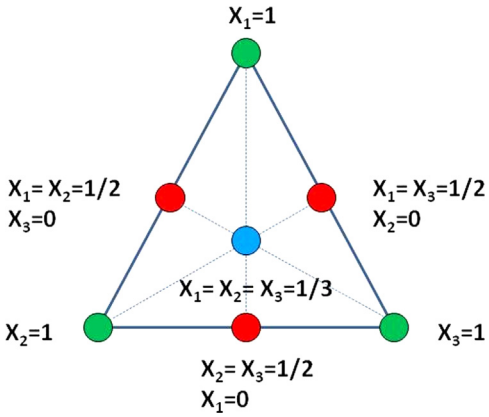

Fig. 3. Schematic representation of a mixture simplex centroid design for three components.

$$y=\beta_0+\sum_{i=1}^{k}\beta_i x_i+\sum_{1\le i\le j}^{k}\beta_{ij}x_i x_j+\varepsilon \quad (1)$$

where β_0 is the overall mean effect, β_I represents the effect of the factor x_i, β_{ij} is the effect of the *ij* interaction between the factors x_i and x_j, and ε is a random error component. This latter term represents other sources of variability not accounted for in the model. Thus, ε includes effects such as measurement error on the response, other source of variation that are inherent in the system like instrumental background noise, the effects of non-studied variables, and so. ε is considered a statistical error, assuming it to have a normal distribution with mean zero and variance σ^2, which can be estimated by performing replicated experiments [3].

One of the most popular ways to assess the significance of the effects and to decide which of them should be considered in the final model and which ones should be included into the error is by using normal and half-normal probability plots. The effects that are negligible are normally distributed, with mean zero and variance σ^2 and will tend to fall along a straight line on these plots. On the other hand, significant effects will have non-zero means and will not lie along the straight line. The apparently negligible effects are combined as an estimate of error and significant effects should be considered in the model. In addition, the selected variables from any graph analysis should be examined by analysis of variance (ANOVA) and, if necessary, the model should be reduced by removing any non-significant variables from the initial model [22].

Analysis of variance (ANOVA) is a collection of statistical models used to analyze the differences between group means and their associated procedures, such as "variation" among and between groups. According to Eq. 1, the appropriate hypotheses for model evaluation are

$$H_0:\beta_i=\beta_{ij}=\ldots=\beta_k=0 \quad (2)$$

H_1:$\beta_k\neq 0$ for at least one *k*

Rejection of H_0 in Eq. (2) implies that at least one of the regressor variables x_i, x_j, …,x_k contributes significantly to the model.

ANOVA estimates three sample variances: a total variance based on all the observation deviations from the grand mean, an error variance based on all the observation deviations from their appropriate treatment means and a treatment variance. The test procedure involves partitioning the total sum of squares (SS_T) into

a sum of squares due to the model (SS_R) and a sum of squares due to residual or error (SS_E).

To determinate the statistical significance of the model, the F-test is used. If the statistic F_0 exceeds $F_{\alpha,k,n-k-1}$ the H_0 is rejected and there is at least one variable that contributes significantly to the model.

Finally, the analyst should select all factors that significantly affect each response. When the number of factors is too large, the analyst should resort to experience and knowledge to choose each factor properly and judge its effects. An example of how the analyst must make decisions in such cases can be seen in the development of a method for analyzing sulfur compounds in the aroma of white wines, using ultrasound assisted-emulsification-dispersive liquid-liquid microextraction coupled with gas chromatography-mass spectrometry detection [24]. In this work, six factors were studied and, owing to interactions between them when different responses were analyzed, only one of them could be maintained at a fixed value while the others had to be considered in the next optimization procedure.

4.2. Model building for RSM

Once the data corresponding to the responses evaluated in the optimization stage have been collected, a mathematical model can be built for each response fitting a second order polynomial function. The general equation used for this purpose is the following:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (3)$$

where y, x_i and x_j are the same than for Eq. (1), β_0 is the constant term or intercept, β_i, β_{ii} and β_{ij} represent the coefficients of the first order, quadratic and interaction terms, respectively, and ε is the residual associated with the experiments. Generally, only second-order interactions are taken into account because higher order interactions are not significant and may be confused with the main effects.

The model equation is usually fitted by the least square (LS) methodology, a multiple regression technique that fits a model to set the experimental data finding coefficients values that minimize the residual term ε. Artificial neural networks (ANN) represent another intelligent tool for the non-linear multivariate modeling [25]. In every case, the fitted model must be able to properly describe the data performance in order to make statistical predictions. In the case in which only two factors are optimized, RSM generates a graphical view of the system, since the response can be represented as a solid surface in a three-dimensional space. When more than two factors are being optimized, the graphical representation is made for two of them, maintaining the other ones at constant values; thus, a small fraction of the surface is shown. Besides, contour maps may be plotted as another way of visualization. This contour plot consists of lines of constant response, corresponding to a specific height of the response surface.

4.2.1. Evaluation of the model

When applying LS regression, it is customary to assume that the expected values of the errors are near zero, independent of constant variance and, at least, approximately normally distributed. However, the response is always measured with certain error; therefore, both the parameter estimates $\hat{\beta}$ and the predicted response $\hat{y}$ (x) will have random variations depending on the choice of experiments. That is why these assumptions should be checked [23].

To determine if the multiple regression fit is significant for the second order model, a test of analysis of ANOVA to show that H_1 hypothesis is valid ($H0: \beta_i = \beta_{ii} = \beta_{ij} \ldots\ldots\ldots \beta_k = 0; H_1: \beta_i \neq 0$) should be applied. The model is considered satisfactory when the regression is significant and a non-significant lack of fit is obtained for the selected confidence level.

However, obtaining a significant model does not necessarily mean that it explains correctly the variation in the data. Consequently, it is necessary to evaluate residual plots, the coefficient of determination (R^2) and the adjusted coefficient of determination (R^2adj), representing the percentage of variance explained by the model. Table 3 shows a breakdown of the model diagnostic plots, which are useful to check residual distribution and outlier detection. The normal probability plot indicates whether the residuals follow a normal distribution, one of the basic conditions for the validity of ANOVA. The homogeneity of the variance (other

Table 3
Model diagnostic plots.

Plot	Evaluation	Expected response	Inadequate result	
			Unexpected response	Suggested action
Normal probability	Normal distribution of residual	Straight line	"S-shaped" curve	Response transformation
Residuals vs. Predicted	Constant variance in range	Random scatter	Expanding variance	Response transformation
Residuals vs. Run order	Variables that may have influenced the response during the experiment	Random scatter	Trends	Randomization and blocking of experiments
Residuals vs. Factor	Variance not accounted by the model depending of different levels of a factor	Random scatter	Pronounced curvature	New regression model
Externally studentized residual	Identification of abnormal runs	External residual ≤ 3.5 S[a]	External Residual > 3.5 S[a]	If a special cause is identified for the outlier, it may be rejected.
Leverage	Points with large influences in the model fit	Point leverage < 2 AL[b]	Point leverage $= 1$	Adding points or replicates to model
DFFITS(difference in model fit)	Influence of each point in prediction values	DFFITS $< 2/\sqrt{P/N}$[c]	DFFITS $> 2/\sqrt{P/N}$[c]	Adding points or replicates to model
DFBETAS (difference in betas)	Influence of each point in regression coefficients	DFBETAS $< 2/\sqrt{N}$	DFBETAS $> 2/\sqrt{N}$	Adding points or replicates to model
Cook's distance (CD)	Change in magnitude of regression if the case is omitted	Point CD < 2 ACD[d]	Point CD > 2 ACD[c]	If a special cause is identified for the outlier, it may be rejected.

[a] Standard deviation.
[b] Average leverage.
[c] Where P is the number of model parameters and N is the number of experiments.
[d] Average Cook's distance.

requirement for ANOVA validity) can be evaluated by the plot of residual versus the ascending predicted response values. Two additional plots are also important: (a) the residuals versus experimental run order plot, which allows to detect uncontrolled variables that may have influenced the response during the experiment, and (b) the residuals versus levels of each factor plot, which checks whether the variance not accounted for by the model is different at diverse levels of a factor.

Furthermore, the evaluation of the externally studentized residuals is useful to detect data points that are not well fitted by the selected model (outliers). The leverage is a parameter indicating the potential for a design point to influence the model fit. High leverage points are not desirable because if they carry unexpected errors, these errors would strongly influence the model. Other ways to evaluate abnormal influential points are the DFFITS and DFBETAS plots, which determine the difference in the model fit when a response value is deleted. The DFFITS measures the change in predicted values, while DFBETAS evaluates the difference in each regression coefficient. Finally, Cook's distance, calculated as the square of Euclidean distance between the least squares estimate based on all n points of the vector and the estimate obtained by deleting the ith point, is a measure of how much the regression changes if this point is not taken into account. Outlier points with high leverage and Cook's distance are recommended to be eliminated of the model in order to obtain a better fit.

There are at least three ways to minimize the lack of normality or residuals heterocedasticity [3]: (a) to use non-parametric methods; (b) to use generalized linear model (GLM), a flexible generalization of ordinary linear regression that allows for response variables that have other than a normal distribution; (c) to analyze a transformed response that meets the requirements of normality. In this review the focus was set on option three, owing both to the accessibility and to the practicability of it.

4.2.2. Response transformation

After a general evaluation of the proposed model, it may occur that the implementation of data transformation allows a better fit to the system. This condition is usually found in the following two cases: (a) the range on the response is fairly large, or (b) model assumptions, namely normality and homoscedasticity are not fulfilled (see Table 3). In practice, some response variables follow Poisson, binomial or Gamma distributions, in which the variance of the response (σ_y^2) is not constant, but is related in some way to the mean (μ_y) [23,26]. In such cases, an abnormal distribution of the "horn-type" or "s-shaped" residues graph is produced.

Transformations apply a mathematical function to all the response data, generating a new set of data y' that meets the assumptions that make the ANOVA valid. Then, a new model can be built to better explain the data behavior.

A general and widely used method for transforming data in linear models was developed by Box and Cox [27]. This family of power transformation tools is extensively used to achieve a normalizing transformation on a positive-valued response variable given by

$$y' = \frac{(y^\lambda - 1)}{\lambda}; \text{for} \quad \lambda \neq 0 \tag{4}$$

$$y' = \log_{10} y \quad \text{or} \quad \ln y \; ; \quad \text{for} \quad \lambda = 0 \tag{5}$$

where λ is a scalar parameter defining a particular transformation.

As it was mentioned before, the application of this methodology involves that the data to be transformed are positive values. Otherwise, an option to overcome this limitation is to add a constant value (c) to all the response data, such that $(y+c) > 0$ in every point.

The appropriate choice of a response transformation relies on the subject matter knowledge and/or statistical considerations according to the type of distribution arising from residues. Generally, the best λ value is found at the minimum point of the curve generated by the natural log of the sum of squares of the residuals, i.e. the λ value that generates the set of data with lower residuals dispersion (Fig. 4). If the 95% confidence interval (CI 95%) around this λ includes 1 no specific transformation is necessary. This procedure to choose λ allows for a continuous spectrum in the interval [−3, +3].

In the example given in Fig. 4, the response of a chromatographic resolution was modeled as a function of four variables: column oven temperature, buffer concentration, organic solvent proportion, and pH of mobile phase. A quadratic model was firstly adjusted with the original data set. During normality evaluation, an "*s-shaped*" curve was obtained in the normal distribution plot of the residuals, indicating lack of normality in data behavior. Following Box and Cox methodology, several transformations were evaluated to convert the original data in new data sets using Eqs. (4) and (5) for λ values between −3 and 3. The natural logs of the sum of squares of the residuals obtained with the transformed set of data were plotted vs. the λ values. As it can be seen in Fig. 4, the best λ value was −0.83, with a CI 95% ranging from −0.25 to −1.44. In this particular case, the best choice for λ was a value of −1, i.e., an inverse transformation is recommended to be applied to the analyzed response [28]. It is important to note that, adjusting a model to a transformed response, predicted values for this response should undergo a back transformation (using the mathematical inverse operation of the employed transformation) in order to do a proper interpretation.

The transformation of the response is an important component of any data analysis. However, the analyst is often reluctant to use them. But if the curvature of RSM can be better accommodated by an alternative approximating function that is simpler and easier to be interpreted and analyzed, then the alternative function should be used [3]. Interestingly, there is a reduced number of papers reporting the use of response transformations when developing analytical methods. An example can be found in Ref. [29].

4.2.3. Evaluation of individual coefficients in models

In each model, the significance of the terms should be evaluated by ANOVA, which performs a comparison of the variation in

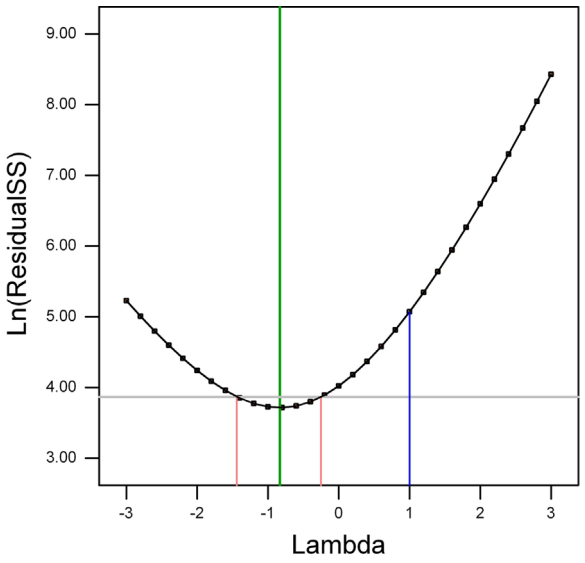

Fig. 4. Box and Cox graphical study which allows to conclude if response transformation is necessary.

the response (due to changes in the combination of variable levels) with the variation due to random errors. Generally, the terms that are not significant in the model are eliminated to obtain the simplest model that describes the system.

More than one possible strategy may be followed in this evaluation: backward elimination, forward selection or step-wise regression [3]. In the backward elimination strategy, a complete model is initially built; then each term is evaluated by ANOVA. The least significant term (the one with the highest partial probability value) is removed from the model. This iteration stops when all the remaining terms satisfy the specified significance (α value) criterion.

When doing forward selection, all blocks and forced terms are first fit to the data. Then, the remaining candidate terms are considered, beginning with a simple regression model and using the single term that has the highest correlation with the response. Terms with the lowest partial probability value (p-value) are added to the model. For designs with categorical factors, terms are added hierarchically. When the p-value of the next term does not meet the specified alpha value, it is not considered. This algorithm may not be as robust as the others because some terms may never get the chance to be included in the model. This will only cause concern if the data exhibits a high degree of collinearity. However, the safest approach may be the backward regression [3].

The step-wise regression is a combination of forward and backward regressions. First, all blocks and forced terms are fitted to the data. Then, a simple regression model, using the single term with the highest correlation with the response is used. After that, terms are added, eliminated or exchanged and the procedure is stopped when there is no further improvement.

An easier way to look at the coefficients and their relative magnitude is the bar plot reported by Leardi [19]. In this tutorial, a graphical representation of the coefficients of the models of two responses evaluated trough a CCD was made with bars with brackets, corresponding to the confidence intervals. A simple visual inspection of the graphic gives a global idea of the relative significance of each model term.

The lack of fit of the resulting model, including significant terms and those left to maintain the hierarchy, are again evaluated by ANOVA. Finally, the adjusted and evaluated model can be linear, linear with interaction, quadratic or cubic. Interestingly, it can be seen that in most of the consulted scientific literature (see Section 6), authors do not inform about the kind of model for each response being optimized.

4.2.4. Artificial neural networks

The ANN methodology is an information-processing chemometric technique especially created to model non-linear information, which simulates some properties of the human brain. The so-called multilayer feed-forward networks [30,31] or multi-layer perceptron (MLP) networks are often used for prediction as well as for classification. The architecture mostly used consists of three layers of neurons or nodes, which are the basic computing units: the input layer, with a number active neurons equal to the number of factors being investigated (predictor variables in regression), one hidden layer with a variable number of active neurons (which should be optimized), and an output layer which has a unit for each response. The neurons are connected in a hierarchical manner, i.e., the outputs of one layer of nodes are used as inputs for the next layer and so on. In the hidden layer, the sigmoid function $f(x) = 1/(1+e^{-x})$ is commonly used, and the output of the hidden neuron j, O_j, is calculated as

$$O_j = f\left[\sum_{i=1}^{m} (s_i w_{ij} + w_{bj})\right] \quad (6)$$

In Eq. (6), s_i is the input from neuron i in the layer above, to neuron j in the hidden layer, w_{ij} are the connection weights between neurons i and j, w_{bj} is the bias to neuron j and m is the total number of neurons in the layer above.

Linear functions are generally used both in the input and output layers. The number of hidden layers and neurons in each hidden layer must be selected to achieve a satisfactory fitting ability of the network, associated to a satisfactory predictive ability [32,33].

It is important to stress that ANN trained with this rule have a remarkable advantage, as there is no need to know the exact form of the analytical function on which the model should be built. Furthermore, neither the functional type nor the number of model parameters need to be given. This is the main difference between modeling by LS regression or ANN.

As an example, Fig. 5 shows the scheme corresponding to a MLP built for modeling data provided by a central composite design for three factors. A vector datum corresponding to each factor is introduced in a neuron of the input layer. In the example, four hidden neurons are necessary to better modeling data. One neuron in the output layer provides the predicted responses corresponding to each experimental combination of factors. After that, the optimal location is searched (see below).

An additional sort of ANN, based on the use of radial basis functions (RBF) has been recently introduced for nonlinear multivariate function estimation and regression tasks [34]. RBF networks have a single hidden layer of neurons incorporating Gaussian transfer functions, and a linearly activated output layer. In comparison with MLP networks, RBF offers some advantages

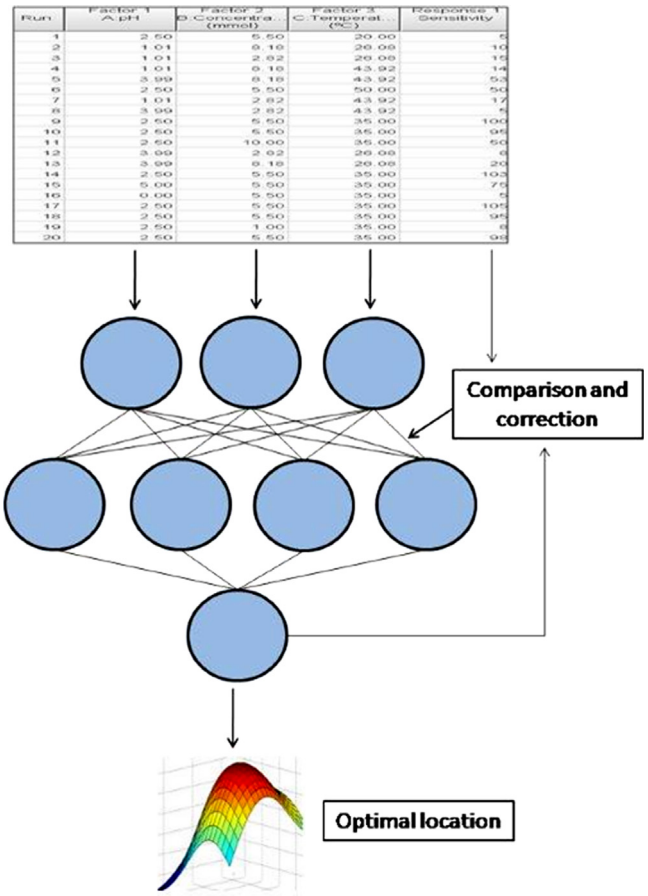

Fig. 5. Scheme corresponding to a MLP built for modeling data provided by a central composite design for three factors.

such as robustness towards noisy data as well as a faster training phase [35].

In the context of regression analysis, recent RBF publications which describe applications to near-infrared analysis of organic matter in soils [36], glucose in blood [37], and water content in fish products [38] can be cited. In the field of optimization, RBF was used for the prediction of optimal culture conditions for maximum hairy root biomass yield [39].

Radial basis function networks also consist of three layers. The input layer serves to distribute the input variables to the hidden layer (as mentioned for MLP). Each of the M neurons of the hidden layer uses a basis Gaussian function as transfer. In order to implement these networks, it is required to find suitable parameters for the Gaussian functions of the hidden layer [40]. They are the centers (contained in the $F \times 1$ vector $\mathbf{c}_m$) and the widths σ, which are usually considered to be equal for all functions. The output from the mth. hidden neuron, for a given input object $\mathbf{s}_i$, is therefore expressed as

$$\text{out}_m = \exp\left(-\frac{1}{2\sigma^2}||\mathbf{s}_i - \mathbf{c}_m||^2\right) \quad (7)$$

where $||\mathbf{s}_i - \mathbf{c}_m||$ is the Euclidean distance between $\mathbf{s}_i$ and $\mathbf{c}_m$. The input value to the output node is the weighted sum of all the outputs of the hidden nodes, with the response of the output node linearly related to its input. Hence, the RBF network output (out_i) for an input object $\mathbf{s}_i$ is given by

$$\text{out}_i = w_0 + \sum_{m=1}^{M} w_m \exp\left(-\frac{1}{2\sigma^2}||\mathbf{s}_i - \mathbf{c}_m||^2\right) \quad (8)$$

where w_0 is the bias and w_m is the weight assigned to the mth. hidden output. These weights are adjusted to minimize the mean square error of the net output. Therefore, two sets of parameters (the centers and widths) in the hidden layer, and a set of weights in the output layer are adjusted. RBF has a guaranteed learning procedure for convergence: given the centers of the M basis functions and a set of I training objects with known factor values ($\mathbf{s}_i$) and target response (r_i), the minimum squared error in the prediction of r is attained when the weights are given by

$$\mathbf{w} = (\mathbf{H}^T\mathbf{H})^{-1}\mathbf{H}^T\mathbf{r} \quad (9)$$

where $\mathbf{w}$ is a vector ($M \times 1$) collecting the weights, $\mathbf{r}$ ($I \times 1$) is the vector of target response values, and $\boldsymbol{H}$ ($I \times M$) is the so-called design matrix, whose elements are calculated as

$$H(i,m) = \exp\left(-\frac{1}{2\sigma^2}||\mathbf{s}_i - \mathbf{c}_m||^2\right) \quad (10)$$

4.2.5. Optimal location

A suitable way to find the optimal location is through the graphical representation of the model. Two types of graphs may result helpful: (a) the response surface in the three dimensional space and (b) the graph of contours that is the projection of the surface in a plane, represented as lines of constant response. Each contour corresponds to a specific height of the surface. In these graphics the response is represented as a function of two factors. According to the established optimization criterion, the optimal value sought may correspond to a maximum, a minimum or a specific value, that can be found by simple visual inspection of the graph. When more than two factors are studied, those who are not plotted must be set at a constant value, thus a limited part of the experimental domain is shown and the optimum is not necessarily seen in the graph. For this reason, the value of the fixed variable must be selected very carefully. The overlaying of contour plots constructed with par combination of three factors allows to search visually for the best compromise region satisfying response requirements. However, if more than three factors are being analyzed, the superposition of contour plots becomes difficult. Therefore, sometimes a more formal analytical approach to the second-order surface is necessary when it is generated in k dimensions. One possible approach is to differentiate the adjusted polynomial with respect to each of the factors, and then allowing the derivative to be set to zero. In this way, the stationary point of the system can be found, which is usually the "optimal candidate" corresponding to a point with a maximum or minimum response in the hyper-surface (k-dimensional), or a saddle point. If the stationary point of the system does not correspond to the wanted optimum, "ridge analysis", a procedure that involves a constrained optimization algorithm is an excellent alternative to find the best operating conditions inside the experimental region [3].Alternatively, if the problem presents low-dimensionality (up to 4 parameters) a grid search would be a feasible manner to find the maxima or minimum.

It should be remarked that a confidence region for the stationary point should be computed. This region is useful because it can provide an idea about the quality of estimation of the stationary point. The size and the direction allows to consider levels of factors which produce a response significantly equal to that produced by the stationary point, and this can provide the advantage to change the level of factors without affecting the quality of the response [41].

5. Multiple response optimization

When the optimization procedure involves more than one response, it is not possible to optimize each one in a separate way, because a number of solutions equal to the variables under study would be gathered. In the optimization of a process or an analytical method, the overall solution must be included in an optimal region, leading to a certain degree of compliance with the proposed criteria for each variable of the system; namely, a compromise solution must be found.

5.1. Graphical optimization

Overlaying the contour plots of each individual response, in the same way as it was discussed for model optimal location, allows to estimate the joint solution provided the number of responses and factors is not too large [3]. As it can be inferred, the graphical representation is only useful when two factors are considered in the optimization of two responses. When the number of responses is equal to or greater than three, the methodology of the contour plot is difficult to interpret and it cannot be usually applied. If the optimal values for each response are localized in different regions, it will be awkward to find graphically the conditions that simultaneously satisfy all responses. The level of difficulty increases as these optimal regions become more distant from each other and do not intersect themselves [3,4]. An alternative is to transform a multiple response problem in a single response one.

5.2. Desirability function

In 1980, Derringer and Suich found one of the solutions to optimize multiple responses by developing the Desirability function, which has been widely used since then in industry [17]. This function is based on the idea that the quality of a product or process that has many features is completely unacceptable if one of them is outside of a "desirable" limit. Its aim is to find operating conditions that ensure compliance with the criteria of all the involved responses and, at the same time, to provide the best value of compromise in the desirable joint response [3]. This is achieved by converting the multiple responses into a single one,

combining the individual responses into a composite function followed by its optimization [17].

Derringer's desirability function allows the analyst to find the experimental conditions (factor levels) to reach, simultaneously, the optimal value for all the evaluated variables, including the researcher's priorities during the optimization procedure. In a first step, an individual desirability function $d_i(\hat{y}_i)$ for each response $\hat{y}_i(k)$ must be created using the fitted models and establishing the optimization criteria. Desirability always takes values between 0 and 1, where $d_i(\hat{y}_i)=0$ for a undesirable response, and $d_i(\hat{y}_i)=1$ represents a completely desirable value, i.e., and ideal response. Intermediate values of $d_i(\hat{y}_i)$ indicate more or less desirable responses.

Different functions may be built, depending on the optimization criteria adopted, within an acceptable range of response values given by $(U_i–L_i)$, where U_i is the upper acceptable value for the response and L_i is the lower. Thus, if the response has to be maximized, $d_i(\hat{y}_i)$ is described by the following equation:

$$d_i(\hat{y}_i(x)) = \begin{bmatrix} 0 & \text{if } \hat{y}_i(x) < L_i \\ \left(\frac{\hat{y}_i(x)-L_i}{U_i-L_i}\right)^s & \text{if } L_i \le \hat{y}_i(x) \le U_i \\ 1 & \text{if } \hat{y}_i(x) > U_i \end{bmatrix} \quad (11)$$

where s is a power value named “weight”, set by the analyst to determine how important it is for $\hat{y}_i$ to be close to the maximum.

The equation for $d_i(\hat{y}_i)$, when it has to be minimized, is

$$d_i(\hat{y}_i(x)) = \begin{bmatrix} 1 & \text{if } \hat{y}_i(x) < L_i \\ \left(\frac{U_i-\hat{y}_i(x)}{U_i-L_i}\right)^t & \text{if } L_i \le \hat{y}_i(x) \le U_i \\ 0 & \text{if } \hat{y}_i(x) > U_i \end{bmatrix} \quad (12)$$

where t is the weight to determine how important is it for $\hat{y}_i$ to be close to the minimum.

Finally, when a target value T_i is the most desirable response, the function is given by:

$$d_i(\hat{y}_i(x)) = \begin{bmatrix} 0 & \text{if } \hat{y}_i(x) < L_i \\ \left(\frac{\hat{y}_i(x)-L_i}{T_i-L_i}\right)^s & \text{if } L_i < \hat{y}_i(x) < Ti \\ 1 & \hat{y}_i(x) = T_i \\ \left(\frac{\hat{y}_i(x)-U_i}{T_i-U_i}\right)^t & \text{if } T_i < \hat{y}_i(x) < U_i \\ 0 & \text{if } \hat{y}_i(x) > Ui \end{bmatrix} \quad (13)$$

Interestingly, factor levels may also be included in the optimization procedure, in order to prioritize the use of certain suitable conditions within the experimental region.

Fig. 6 shows a graphical representation of the desirability functions for the different optimization criteria and how they are modified by s or t. Note that low values for the weight parameters indicate that the response does not require to be strictly near the target value, reaching satisfactory desirability levels for a wide range of responses. In contrast, a choice of large s or t implies that the desirability is very low unless the response gets very close to the target.

Once the n variables (factor and responses levels) are transformed in desirability functions, they are combined in a unique function [17] named Global Desirability (D) to find out the best joint responses using the following equation:

$$D = (d_1^{r_1} x\ d_2^{r_2} x \ldots\ldots x\ d_n^{r_n})^{\frac{1}{\sum r_i}} = \left(\prod_{i=1}^{n} d_i^{r_i}\right)^{\frac{1}{\sum r_i}} \quad (14)$$

where r_i is the importance of each variable relative to the others. In the Design Expert software [42], the importance, established by the analyst, may vary from 1 for the least important variable to 5 for the most important one.

When D reaches a value different from zero, all the variables which are being simultaneously optimized can be considered to have a desirable value. On the other hand, if one of the responses is completely undesirable, $d_i(\hat{y}_i)=0$, D will be zero. The optimization procedure implies to maximize D for which various aggregation schemes may be employed.

Several optimization procedures other than Derringer function and maximization algorithms have been recently analyzed, discussed and evaluated and new alternative methodologies have been proposed [43–46]. Whatever the case, it should be kept in mind that the goal of an optimization procedure is to find a good set of conditions that will meet all the goals, but not to get to a D value equal to 1. This value is completely dependent on how closely the

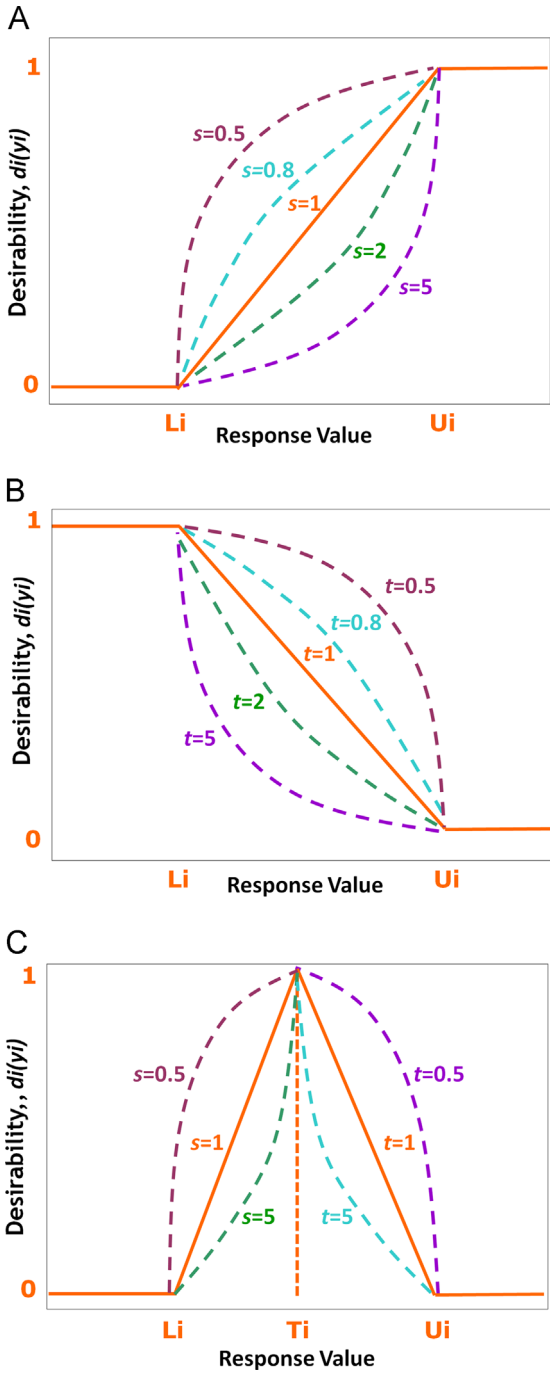

Fig. 6. Graphical representation of the desirability functions for the different optimization criterias.

L_i and U_i limits are set relative to the actual optimum and on the different weights or priorities assigned to each response.

Global desirability can also be graphically represented for the experimental space, calculating several values for D using $d_i(\hat{y}_i)$ for all the responses for a high number of combination of factor levels (a grid). The function usually finds more than one combination of factor levels where the responses are acceptable and represented as a flattened top in the surface of D plot. In these cases, it can be stated that the optimization has generated a robust solution [47].

The final step in multiple response optimization is to predict the values that the different responses can take at the optimal factors combinations using the fitted models. Then, analytical runs should be performed to verify the predictions.

To show the potentiality of the desirability function, a real example carried out in our research group will be considered as follows. It corresponds to the optimization of the pre-concentration and clean-up step by solid-phase micro-extraction (SPME) followed by liquid chromatography with diode array detection analysis in the development of an analytical method for the simultaneous determination of seven non-steroidal anti-inflammatory drugs (piroxicam, sulindac, ketoprofen, naproxen, diflunisal, indomethacin and sodium diclofenac) and the anticonvulsant carbamazepine [48]. In order to find the operational conditions to improve the efficiency of the procedure, a central composite design consisting in 20 experiments was built. Peak areas for all the analytes (as indicators of sensitivity) and analysis time were fitted to polynomial models and validated by the ANOVA. Solving this system of multiple responses by overlaying the contour plots of every combination of factors for each of the nine responses is a very difficult task and, therefore, the use of a simultaneous optimization method was required. For this reason, all the nine responses were simultaneously optimized using the desirability function.

The goal of the optimization task was to minimize the analysis time and to maximize peak areas, between the lower and upper experimental limits, giving more importance to the analytes with smaller areas (carbamazepine and piroxicam), and in decreasing order of importance to the others.

The optimization procedure was carried out and the global desirability function was plotted reaching a level of 0.941for the criteria. (for more details and graphical representation see Ref. [48]). The contour plots were obtained in this work for a given pair of factors, while maintaining the other fixed at its optimal global function (D) value. Besides that these representations were only partial because one of the three factors should remain constant, they allowed us to conclude about the robustness, i.e. the region corresponding to optimal solutions is not very sensitive to small accidental changes in the analyzed factors [41].

It should be remarked that the success of the desirability function approach depends completely on the quality of the models employed in the optimization process. In order to apply this method properly, only statistically validated models should be used to build the partial desirability functions. In this way, the probability of the lack of fit test in these models should be larger than 0.05. Other interesting aspects should be taken into account: (a) the desirability approach consists in a one-dimensional optimization which does not mean that it is always easy to look for the maxima of D, and (b) D is not differentiable, so that alternative algorithms have to be used. In this context, the critical values for acceptability and target, as well as the shape of the function and the relative weight of the different responses, are all (more or less) arbitrarily defined "a priori" by the user. It should be considered that just changing somewhat some of these parameters will lead to totally different landscapes of D.

On the other hand, D is obtained by the product of individual desirabilities, each of which has its own error. Thus, the confidence interval of D can therefore be very high. Consequently, the setting selected as the best (simply because its numerical value of D is the largest) cannot be significantly better than other regions that would be neglected simply because they have lower values of D.

6. Applications

DOE has been widely applied to a variety of analytical techniques. Most of the applications were aimed at solving drawbacks in separation techniques. Recently, in an excellent review presented by Dejaegher and Vander Heyden [7], a complete list of reviews and works, which focuses on the kind of designs used for different applications in a wide variety of analytical techniques, has been presented. Herein, the focus will be set on using multiple response optimization, especially those applications for solving the problem of a large amount of responses being optimized.

6.1. Extraction procedures

The most recent applications on extraction procedures include novel extraction techniques like solid phase micro-extraction (SPME), hollow fiber solid phase micro-extraction (HF-SPME), subcritical fluid extraction (SCFE) and microwave-assisted extraction (MAE). In many cases, such techniques have been combined with experimental design strategies with the aim of increasing the extraction efficiency.

Lu et al. have recently developed a SCFE of carotenoids and chlorophyll from Laminaria japonica Aresch using ethanol-modified subcritical 1,1,1,2-tetrafluoroethane. RSM combined with a Box–Behnken design was applied in this work to evaluate the significance of three independent variables (pressure, temperature and amount of co-solvent) on the yields of carotenoids and chlorophyll by using the desirability function [49].

Another method based on MAE was proposed by Fang et al. for simultaneous extraction of hydrosoluble phenolic acids and liposoluble tanshinones in Salviae Miltiorrhizae radix (*danshen*), which are widely used in traditional Chinese medicine. The key parameters considered for the MAE included solvent type, temperature, microwave power, solvent to material ratio and extraction time. These factors were evaluated by the univariate approach in a first instance. 80% methanol in water was selected as the extraction solvent and proper levels were established for temperature and microwave power. Then, a multivariate optimization procedure was applied in order to find out the optimal experimental conditions giving the maximum extraction yield of nine *danshen* components as a function of extraction time and solvent to material ratio. A central composite design was used, consisting of 13 experiments which corresponded to combinations of the two selected independent variables. Quadratic models were fitted for the nine extraction yields and the responses surfaces were obtained and evaluated. Fig. 7(A–C) shows the interaction of solvent ratio and extraction time in the yield of three of the target tanshinones. Later, the desirability function approach of Derringer was used to optimize all the nine extraction yields simultaneously. A value of five was the importance assigned to the main active components of Danshen, whereas for the least important component the importance of partial desirability functions was equal to 3. Simultaneous extraction of the hydrosoluble and liposoluble components was demonstrated to be feasible by the optimized MAE method [50].

In another application, which deserves to be mentioned, carbon coated Fe_3O_4 magnetic nanoparticles were used as an adsorbent for magnetic solid phase extraction (MSPE) of trace amounts of organophosphorus pesticides from environmental water samples. Their determination was carried out later, using high performance liquid chromatography with ultraviolet detection. Optimization

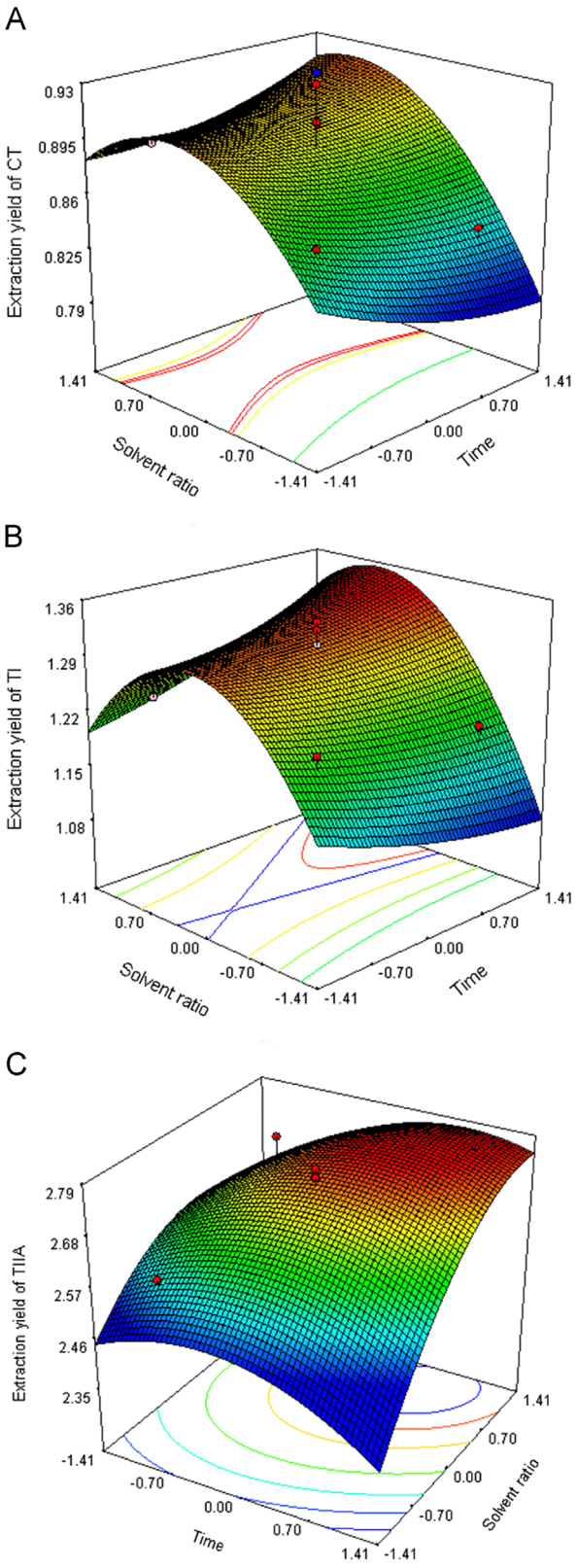

Fig. 7. Response surface plots for extraction yield as a function of solvent ratio and extraction time of: (A) Cryptotanshinone(CT), (B) Tanshinone I(TI) and (C) Tanshinone IIA (TIIA). Reprinted from [50], copyright 2011, with permission of Elsevier.

was conducted using a central composite design. Four factors including the sample solution pH (A), ionic strength (B), amount of the adsorbent (C) and equilibrium time (D) were considered. The experimental range was coded in low (−1), central (0), and high (+1) levels of the factors with the location of the star points in (±2), as shown in Table 4. These designs involved 29 experiments with five replicates at the center point. The criteria selected for the optimization of all the factors and responses studied (peaks areas of the analyte) through the desirability function are presented in Table 5. As it can be seen, the authors looked at this opportunity, not only to maximize the signal of the compounds of interest but also the reduction of the equilibrium time (a factor of the design). A weight of 1 was chosen for all individual desirabilities, while the importance was equal for all responses when constructing the global function. The experimental conditions providing the highest extraction efficiency were found in this way to be in correspondence with a solution pH of 9.16, 97.4 mg of adsorbent, equilibrium time 0 min and 10 mmol $L^{-1}$of NaCl added to the samples. Under optimal conditions, the proposed method was evaluated, and successfully applied to the analysis of organophosphorus pesticides in water samples [51].

Ghafoor et al. developed a method based on SCFE for valuable compounds from grape peel. The process was carried out according to an orthogonal array design consisting of 16 experiments, and the independent variables selected were temperature, pressure and modifier concentration. The experimental responses were extract yields, total phenols, antioxidant activities and total anthocyanins of grape skinextracts, which were fit to a second-order polynomial equation. The prediction of one set of optimal conditions for four response variables was also done by using the desirability function approach [52].

Es'haghi et al. optimized a method for pre-concentration and determination of benzene, toluene, ethylbenzene, and xylene in

Table 4
Factors and value levels used in the central composite design for the optimization made of the MSPE procedure for extracting trace amounts of organophosphourus pesticides from environmental water. Reprinted from [51], copyright 2012, with permission of Elsevier.

Factors	Level				
	α (−2)	Low (−1)	Center (0)	High (+1)	α (+2)
A:pH	4	6	8	10	12
B: Ionic strength (mmol L^{-1}; NaCl)	0	100	200	300	400
C: Amount of the adsorbent (mg Fe_3O_4/C)	5.0	28.8	52.5	76.3	100.0
D: Equilibrium time (min)	0	15	30	45	60

Table 5
Constraints of factors and responses for optimization. Reprinted from [51], copyright 2012, with permission of Elsevier.

Name	Goal	Lower limit	Upper limit	Weight	Importance
pH	Is in range	4	12	1	–
Na CL	Is in range	0	400	1	–
mg Fe_3O_4/C	Is in range	5	100	1	–
Eq. time	Minimize	0	60	1	–
R_{Mala}	Maximize	12,000	133,308	1	5
R_{Diaz}	Maximize	10,004	73,964	1	5
R_{Phos}	Maximize	32,498	470,478	1	5
R_{Chlor}	Maximize	86,136	429,764	1	5

environmental waste water and human hair samples by SPME. In order to obtain high enrichment and extraction efficiency of the four analytes, an orthogonal array experimental design was applied. The matrix was built considering four factors selected according to the researcher's knowledge: stirring speed, volume of adsorption organic solvent, extraction and desorption time of the sample solution, which were evaluated at four levels. The effect of each factor was estimated using individual contributions as response functions in the screening process. Therefore, each response was plotted as a function of the factors and the optimum was selected graphically. Then, ANOVA was employed for estimating the main significant factors and their percentage contributions in the extraction. The final optimum conditions were calculated by OVAT methodology [53].

Finally, Prakash et al. have recently proposed a method for the extraction of betalain pigments and color from prickly pear fruits. The individual and interactive effect of process variables (temperature, time, mass and pH) on the extracted betalain concentration and color of the extract was studied using Box-Behnken response surface design. The experimental data, obtained from 29 experiments, were analyzed by ANOVA and the second-order polynomial models were developed using multiple regression analysis. An optimization study using Derringer's desirability function methodology was performed, the goal being to maximize the extraction of betalain and the color of the extract. Under the optimized conditions, which were obtained with a total desirability value of 0.936, the experimental values of pigment and color agreed closely with the predicted yield [54].

6.2. Analytical separations

In the development of analytical methods based on techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE), the analyst usually has to appeal to multiple response optimization. This is because several responses are frequently of interest, considering the analytical characteristics of the method (separation between analyte peaks, analysis time and peak features such as width, symmetry and theoretical plates, among others). Nevertheless, a special comment should be made for separation problems in which maximizing the minimal resolution can sometimes be inconvenient [55]. This fact may be due to two reasons that will be considered:

(1) Normally when there are two peaks (A and B), eluting once as AB and once as BA, when the separation is the same, resolution (*Rs*) values are the same, e.g. 2. However, for modeling *Rs* it is detrimental because two different situations lead to the same response. In fact, in a proper modeling context, one situation should have been called $Rs=2$ and the other $Rs=-2$. In practice, this has not been done, but researchers prefer computing Rs among the most proximal peaks occurring for each one in each optimization chromatogram (without the identification of the compound) [28].

(2) In optimization, one creates situations and evaluates conditions where selectivity changes occur. The optimum separation is at the conditions where the minimal resolution becomes maximal, i.e. where the worst separated peak pair is separated best. However, at different experimental conditions different peak pairs can be responsible for the critical resolution. Thus, when 5 peaks need to be separated one should model $4+3+2+1=10$ resolutions (between all possible pairs), while at each condition only four are relevant. Therefore alternatively, the retention times (*tr*) of the 5 substances are modeled (in isocratic optimizations also the peak widths (*w*)). In order to find the optimum, a grid is created and at each grid point *tr* and *w* of a substance predicted. Sorting the different *tr* at one set of conditions allows to calculate the Rs between the relevant peak pairs and to select the minimal one. The set of condition where the latter is maximal is then the optimum.

Recently, Bruns et al. developed six procedures to separate different sets of carbohydrates present in food samples by micellar electrokinetic chromatography. The effects of pH, electrolyte and surfactant concentrations on the separation of the compounds were investigated using a central composite design requiring 17 experiments, while several responses were simultaneously studied: resolutions between adjacent peaks, run time and analytical signal to noise ratio. Linear and quadratic models were used to fit the responses. Then, the Derringer and Suich technique was used, stipulating desirability criteria for each resolution. The conditions predicted by the model were confirmed experimentally within the sets of analytical standards. In some cases, more than one satisfactory set of conditions were found. The optimal conditions were applied to real samples in order to evaluate the effect of the matrices. The investigated procedure allowed the separation of the strategic sets of carbohydrates present in each sample [56].

The same authors presented another application of simultaneous optimization of a method to quantify thirteen phenolic compounds in extra-virgin olive oil. Using a central composite design, they investigated changes in boric acid concentration, pH and voltage. Five resolutions between the nearest electrophoretic peaks and analysis time were adjusted to linear and quadratic models and validated by means of ANOVA at the 95% confidence level. The optimum conditions to separate all the 13 peaks were determined by examining response contour graphs (see an example in Fig. 8) and using the Derringer and Suich multi-criteria response technique. Desirability values were established for each individual response and they were combined into their recommended global desirability function. The individual desirabilities were defined to maximize the resolutions while minimizing the runtime. The global desirability function value was around 0.2, which is probably due to the very restrictive criteria employed in this application. However all the peaks were neatly separated in a runtime below fourteen minutes as it was predicted by the model. Fig. 9 contains the obtained electropherogram [57].

Guillén-Casla et al. proposed a method for the determination of serotonin and its precursors in chocolate samples by capillary liquid chromatography–mass spectrometry (MS). The optimization study of the chromatographic conditions was done by using an experimental design involving the following factors: pH and buffer concentration of the mobile phase, injection volume and focusing

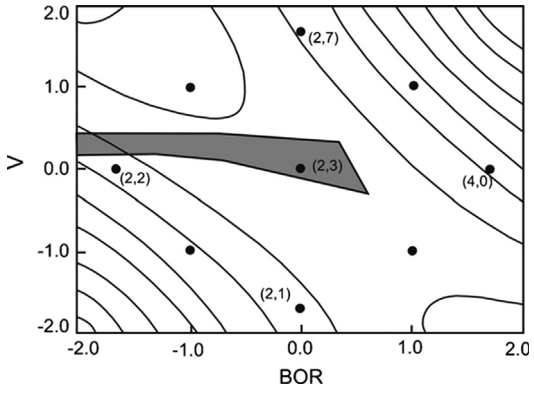

Fig. 8. Contour line graph for the boric acid concentration and voltage showing the experimental region that results in acceptable electropherograms. The pH was held constant at 10.2. The central point shows the optimum conditions. Experimental values for design points are in parenthesis. Reprinted from [57], copyright 2010, with permission of Elsevier.

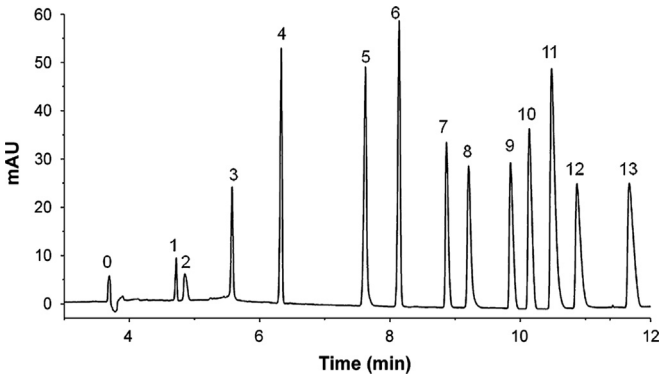

Fig. 9. Eletropherogram for the 13 phenolic compounds after overall optimization. Standard mixture containing 12.9 mg L^1 of each compound.For peak identification refer to original article. Reprinted from [57], copyright 2010, with permission of Elsevier.

conditions, while both peak area and peak width were selected as experimental responses to evaluate sensitivity in MS detection. A first-order polynomial equation was fit, obtaining R^2 higher than 0.781 and good standard estimation errors. The optimization criterion involved maximizing peak area while minimizing peak width. The experimental responses obtained under the optimum conditions ($D=0.7$) were statistically compared to those predicted by the model [58].

De Zan et.al optimized an ion-pairing HPLC method for the simultaneous determination of the two nicarbazin components in bulk materials and feed additives. Four variables, including mobile phase composition and oven temperature were analyzed through a central composite design exploring their contribution to the analytes separation. The experiments were performed in three blocks, and six replicates were made to provide a measure of pure error and to stabilize the variance of the predicted response in the design region. Then, five responses, peak resolutions, 2-hydroxy-4,6-dimethylpyrimidine (HDP) capacity factor, HDP tailing and analysis time, were modeled using second-order polynomial functions. In each model, the terms were evaluated by ANOVA and the backward regression procedure was applied to eliminate the insignificant ones ($\alpha=0.10$). Thus, simplified models, including only significant terms and those necessary to maintain the hierarchy were obtained and evaluated by ANOVA for model significance and lack of fit. Finally, to find out the experimental conditions to reach simultaneously the optimal value for all the evaluated variables, the Derringer desirability function was used. Three responses were maximized (HDP capacity factor and two resolutions while analysis time was minimized, and HDP peak tailing was adjusted to a target value). Interestingly, apart from optimizing the responses, the authors also attempted to minimize the concentration of salts in the mobile phase to reduce costs and extend the column lifetime. In addition, a weight or emphasis was given to each goal: the highest importance was assigned to the HDP capacity factor and peak tailing ($r=5$), whereas a lower importance was given to retention time ($r=1$); the importance of the other variables was kept in an intermediate value. The suggested optimal conditions were then experimentally corroborated [29].

In a very recently reported work, Ferey et al. developed a CE method with laser-induced fluorescence detection for the fast simultaneous separation of 8 heavy polycyclic aromatic hydrocarbons (PAHs) among food and environmental priority pollutants. The first step involved the selection of the most important factors influencing PAHs electrophoretic behavior. Then, a response surface strategy using a central composite design was carried out to model the effects of the selected factors on the normalized migration times. Twenty experimental conditions were finally evaluated. An optimization study using Derringer's desirability function methodology was performed to optimize four responses: the analysis time and the resolutions between 3 critical pairs of PAHs. The goal was to minimize the analysis time, maintaining the resolution higher than 2.5. A weight of 1 was given to all individual desirabilities, except for the analysis time for which a weight of 20 was attributed. From the model, predicted optimum conditions were experimentally validated and full resolution of all 8 PAHs was achieved in less than 7 min [59].

The desirability function was also applied to a microemulsion liquid chromatographic method development for the chromatographic separation of perindopril tert-butylamine and its four impurities. A central composite fractional factorial design was applied for a response surface study of five factors on three responses [60].

In another interesting application, a multiple response optimization strategy for the separation of acetylsalicylic acid, its major impurity salicylic acid and ascorbic acid in pharmaceutical formulations by hydrophilic interaction chromatography was developed using a Box–Behnken design. The effects of four independent variables was studied simultaneously on two responses (resolution and analysis time). The methodology also captured the interaction between variables which enabled the exploration of the retention mechanism involved. From this study, it was inferred that the retention is governed by a compromise between hydrophilic partitioning and ionic interaction [61].

Multiple response optimization using the Derringer's desirability function was also used for the development of a reversed phase HPLC method for the simultaneous determination of lamivudine, tenofovir and efavirenz in commercial pharmaceutical preparations. Twenty experiments, taking the capacity factor of the first peak, resolution between the second and third peaks and the retention time of the third peak as the responses with three important variables as organic phase composition, buffer molarity, and flow rate, were used to design mathematical models. The experimental responses were fitted into a second order polynomial and the three responses were simultaneously optimized to predict the optimum conditions for the effective separation of the studied compounds [62].

Hadjmohammadi and Nazarihave recently reported the separation of five flavonoids with micellar liquid chromatography using experimental design and Derringer's desirability function. They study the effect of four factors in two responses: mobile phase and flow rate. The experiments were performed according to a face-centered cube half fractional central composite design. The optimum mobile phase composition for separation of the flavonoids using a C18 column was [SDS]$=0.040$ mol L^{-1}; 11.2% v/v butanol, 1.4% v/v acetonitrile with flow rate of 1.1 mL min^{-1}. The efficiency of prediction of the polynomial model was confirmed by performing the experiment under the predicted optimal conditions [63].

The development of a reversed-phase HPLC method for the simultaneous determination of pantoprazole, rabeprazole and lansoprazole with domperidone in human plasma samples was carried out by Sree Janardhanan et al. by multiple response optimization employing the Derringer's desirability function. The influence of the independent variables (% acetonitrile, buffer concentration and flow rate) on the output responses: capacity factor of the first peak, resolutions between critical peaks, retention time and a variable defined by the authors as "the chromatography optimization function" (COF) were evaluated. The coefficients of determination R^2 were more than 0.92 for all the models. Optimum conditions chosen for assay were acetonitrile, methanol and 18.65 mM K_2HPO_4 (pH 7.0 ± 0.5) solution (31.41:20:48.59 v/v/v) in a flow rate of 1.10 mL min^{-1}. Total chromatographic analysis time per sample was approximately

Table 6
Recent applications of multiple response optimization using the desirability function, design built, analyzed factors and number of simultaneously optimized responses and references.

Application	Screening of factors	RSM design	Number of factors	Number of responses	Models	Reference
Production of bio-hydrogen	Selected by previous work	Box–Behnken	3	2	Second-order polynomial	[68]
Production of recombinant proteins	Plackett-Burman design	Central composite	4	2	Polynomial and ANN	[69]
Production of bio-hydrogen	Selected by previous work	Box–Behnken	4	2	Second-order polynomial	[70]
Tuber melanosporum fermentation medium	Plackett–Burman design	Draper–Lin small composite design	4	3	Second-order polynomial	[71]
Production of avocado oil	Selected by previous work	Two-level full factorial design	4	5	Second-order polynomial	[72]

9 min. The authors found the method to be simple, sensitive and applicable in bioavailability studies [64].

Different interpretations of the desirability function were made for simultaneous optimization of resolution and analysis time of seven flavonoids in reverse phase liquid chromatography [65]. To judge the extremely different quality aspects of a chromatogram and to find a compromise between conflicting goals such as maximizing the separation while minimizing the analysis time, the authors developed a combined criterion called a chromatographic response function (CRF) which consists of factors related to the time and describing the separation quality. For more details see Ref. [65].

Other applications can be cited as the optimization of a strategy for preconcentration of antibiotic residues in milk and their quantitation by capillary electrophoresis [66] and the development of an HPLC-electrochemical detection method for the determination of captopril in which the optimal conditions were found by superimposition of contour plots [67].

6.3. Other applications

In addition, and even though the field of production processes does not fit within the aim of this review, several recent applications of multiple response optimization deserve to be cited. They are summarized in Table 6, Refs. [68–72].

7. Conclusions

Experimental design and optimization play an important role in the procedure carried out when a new analytical method is developed and validated. This is the reason why analytical methods must meet complex quality requirements, the process generally being affected by a large number of variables (or factors).

The application of RSM is a relevant issue in the field of analytical methods development, and regrettably, nowadays few reports presenting new methods show the application of a multivariate strategy. When RSM is performed, important issues to be considered are selection of the most convenient experimental design, modeling of the experimental data by using preferentially least squares fitting, and location of the optima.

As a result of the increasing complexity on both modern instrumentation and analytical problems, it is common that several responses should be simultaneously optimized, especially in the field of analytical separations and sample pre-treatments. In this scenario, the desirability function of Derringer and Suich has been the option of many researchers, playing an important role in the world of experimental design and optimization, as it can be ascribed from the high number of papers published concerning this topic in recent years.

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