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An experimental design methodology applied to the enantioseparation of a non-steroidal anti-inflammatory drug candidate☆

R. Ficarra [a], P. Cutroneo [b], Z. Aturki [c], S. Tommasini [b], M.L. Calabrò [a], R. Phan-Tan-Luu [d], S. Fanali [c], P. Ficarra [b,*]

[a] *Dipartimento di Scienze Farmaco-Biologiche, Università di Catanzaro "Magna Græcia", Complesso "Ninì Barbieri", 88021 Roccelletta di Borgia, Catanzaro, Italy*
[b] *Dipartimento Farmaco-Chimico, Università di Messina, Viale Annunziata, 98168 Messina, Italy*
[c] *Istituto di Cromatografia del C.N.R., Area della Ricerca di Roma, P.O. Box 10, 00016 Monterotondo Scalo, Rome, Italy*
[d] *LMRE, Faculté des Sciences de St. Jérôme, Av. Escadrille Normandie-Niemen, Université d'Aix-Marseille III, F-13397 Marseille Cedex 20, France*

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Abstract

An experimental design methodology has been applied to the enantioseparation of a new synthesized aryl propionic acid of pharmaceutical interest, namely 2-[(4′-benzoyloxy-2′-hydroxy)phenyl-propionic acid] (DF-1770y) by chiral capillary zone electrophoresis (CCZE). The chiral separation of the studied compound has been achieved employing vancomycin as the chiral selector. The partial filling-counter current method has been used in order to avoid the presence of the absorbing chiral selector in the path length of the detector and to increase the method sensitivity. A central composite design has been employed to optimize the experimental conditions for a fast separation of the enantiomers of the new synthesized aryl propionic acid. Critical parameters such as chiral selector concentration, pH and temperature have been studied to evaluate how they affected responses such as resolution and migration times. The desirability function approach has been employed in order to find the best compromise between the different experimental responses. The proposed CCZE method provided the baseline enantioseparation of the investigated drug. A Britton-Robinson buffer at pH 6.4 supplemented with 7 mM of vancomycin at 22 °C and −20 kV were the optimum experimental conditions allowing to achieve the highest enantioresolution of DF-1770y in less than 8.5 min.

Keywords: Chiral capillary zone electrophoresis; Arylpropionic derivative; Vancomycin; Experimental design; Central composite design; Desirability function

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* Corresponding author. Fax: +39-90-6766407
E-mail address: pficarra@pharma.unime.it (P. Ficarra).

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1. Introduction

The development of new separation methods for the analysis of drug enantiomers and related compounds is continuously requested by researchers working in the pharmaceutical and/or biomedical fields. It has been widely reported that the two enantiomers of a certain drug can exhibit different biological or pharmacological effect because they do not possess the same physico-chemical properties [1,2]. Thus analytical separation methods offering high efficiency and high resolution are necessary for performing, e.g. drug chiral purity control, pharmacodynamic or pharmacokinetic studies, etc.

Among the analytical techniques employed for the above mentioned purposes, capillary electrophoresis (CE) can offer several advantages over other techniques because allows fast separations with high efficiency employing several selective separation mode and minute amount of buffers [3].

In CE the enantiomer separation is usually achieved in a chiral environment responsible for the diastereoisomer formation during the run changing selectively the mobility of the two isomers.

Among the chiral selectors used in CE, glycopeptide antibiotics (GA) such as vancomycin, teicoplanin were studied for the chiral separation of mainly acidic compounds [4,5]. Besides the above mentioned chiral selectors showed a very high stereoselectivity towards a wide number of analytes including those of pharmaceutical interest, they presented some drawbacks such as strong UV absorption causing low sensitivity. This problem was resolved using the partial filling-counter current method where the GA was occupying only part of the capillary and moving in the opposite direction of analytes keeping the detector free of absorbing material [6,7].

A large number of experimental parameters can be varied to influence the separation selectivity and the performance in a capillary electrophoretic method. Conventionally, in order to assess the impact of each of these variables, each factor may be varied one at a time, whilst the others are kept constant.

The classical 'univariate' approach, widely employed for the development of enantioselective CE separations, may offer many advantages, but it fails to take into account interactions between two or more factors [8].

In chemometric approaches, experimental measurements are performed varying all factors simultaneously. In particular, design of experiments (DOE) is a multivariate technique for systematically applying statistics to experimentation [9]. Generally less time-consuming than univariate procedure, DOE is becoming an essential tool for the development and optimization of analytical methods [10,11]. By performing a planned sequence of experiments, called a design, the effects of factors and interactions between them on response variations can be established. The experimental design strategy can be chosen according to the particular objectives of the process under examination. It has been shown that experimental design can be successfully used in CE in order to find the best experimental conditions for achieving the maximum of analytes resolution [10,12,13].

The aim of this study was to assess adequate experimental conditions in which chiral resolution of the new synthesized arylpropionic acid derivative (DF-1770y) could be obtained in a relatively short analysis time. Generally, optimal enantioresolution exhibits long analysis times. Then, in order to optimize the enantiomeric separation of DF-1770y with the lowest number of experiments, we used experimental design taking into account the physico-chemical parameters (chiral selector concentration, buffer pH, capillary temperature) that more influence an electrophoretic separation. The chemical structure of the investigated drug is reported in Fig. 1. The chiral selector employed for the optical isomer resolution was a GA, namely vancomycin.

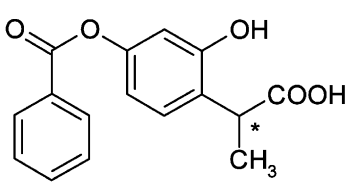
Response surface methodology (RSM) seemed to be the most suitable experimental design strategy. The goal of RSM is to construct mathematical models that predict how changes in input or controlled variables (e.g. temperature, pH, chiral selector concentration, etc.) affect several responses (e.g. resolution, migration time, etc.) in a defined experimental region. In particular, a cen-

tral composite design (CCD) has been used to determine the optimal conditions for chiral separation of the aryl propionic acid. Furthermore, in order to find the best compromise between several responses, a multicriteria decision making approach has to be used. The most popular methodology applied to multiple response optimization is the desirability function approach, as proposed by Derringer and Suich [14]. In our study, this method has been employed to simultaneously optimize the responses resolution and migration times of the two enantiomers.

2. Experimental

2.1. Apparatus

Electrophoretic experiments were carried out on a Biofocus 3000 automated CE system (Bio-Rad Labs, Hercules, CA) equipped with a variable multi-wavelength UV detector, a thermostated carousel and cartridge with air and circulating liquid, respectively. The detector was operated at 195 nm. The used silica capillary was purchased from Composite Metal Services (Hallow, Worcestershire, UK) and was 35 cm (total length), 30.5 cm (effective length), 50 μm I.D. and 375 μm O.D. The capillary was polyacrylamide coated in our laboratory described by Schutzner et al. [15] as a modification of Hjerten's method [16]. The applied voltage was 20 kV and the analytes injected at the cathodic end of the capillary by pressure 5 p.s.i. × 2 s (1 p.s.i. = 6894.76 Pa).



2-[(4'-benzoyloxy-2'-hydroxy)phenyl]propionic acid

DF-1770y

Fig. 1. Chemical structure of the investigated compound.

The background electrolyte (BGE) was 50 mM Britton-Robinson buffer prepared from 150 mM solution containing acetic, boric and phosphoric acids (each 50 mM) after titration with sodium hydroxide to the desired pH and dilution. The BGE was supplemented with vancomycin at different concentrations for the chiral resolution of the aryl propionic acid studied.

The resolution factor (Rs) was calculated by using the following formula:

$$\mathrm{Rs} = \frac{2(t_{m2} - t_{m1})}{w_2 + w_1} \quad (1)$$

where t_m and w are the migration time and the width at the baseline, respectively of the two enantiomers.

Experimental design and statistical analysis were performed by NEMROD software [17].

2.2. Chemicals and reagents

Phosphoric acid (85% w/w) and acetic acid, of analytical grade, were from BDH (Poole, UK). Boric and nitric acid were purchased from Carlo Erba (Milan, Italy). Acrylamide 99.9% and ammonium persulphate were from Bio-Rad Labs. 3-(Trimethoxysilyl)propylmethacrylate and sodium hydroxide were purchased from Fluka (Buchs, Switzerland). The racemic new synthesized aryl propionic acid, 2-[(4′-benzoyloxy-2′-hydroxy)phenyl-propionic acid] (DF-1770y) was a kind gift from Dompè S.p.A. (l'Aquila, Italy) [18].

3. Results and discussion

The separation of acidic chiral compounds can be usually performed by using BGEs supplemented with modified cyclodextrins or GA (e.g. trimethylated-β-CD or vancomycin) [5,6,19]. Based on preliminary results and considering the chemical structure of the studied aryl propionic acid (Fig. 1) we decided to investigate the usefulness of vancomycin for the enantiomer separation of 2-[(4′-benzoyloxy-2′-hydroxy)phenyl-propionic acid] (DF-1770y). The GA was added to the BGE (pH range 4–7) and used as run buffer in the partial filling-counter current with the aim to

Table 1
Experimental domain for the three studied factors

Factors	Center	Variation step
U_1: chiral selector concentration (mM)	5.5	2.7
U_2: pH	5.5	0.9
U_3: temperature (°C)	22.5	4.5

achieve the highest resolution factor in the shortest analysis time.

3.1. Response surface methodology

Response surface designs permit to define empirical models (usually quadratic polynomials) that describe accurately how responses behave at all values of the studied variables in the experimental region [20]. The aim of RSM is to determine conditions that provide process improvement.

In order to calculate quadratic regression model coefficients, each design variable has to be studied at three distinct levels at least and, consequently, the CCD is often used to provide estimation of a second-order equation. Among the standard designs applied in RSM, the CCD represents a good choice because of its high efficiency with respect to the number of runs required.

A CCD for k factors consists of 2^k factorial points, $2k$ axial or 'star' points and $n_0 \geqslant 2$ center points. The axial points are located at a distance, α, from the design center with a choice of $\alpha = \sqrt[4]{N_F}$, where N_F represents the number of factorial runs [9].

The key factors, selected during the optimization process, were: chiral selector concentration (U_1), pH (U_2) and temperature (U_3).

The center value and the variation step taken for each variable defined the spherical experimental domain, as reported in Table 1.

The experimental matrix at three factors (CCD) consists of fifteen experiments, expressed in coded

Table 2
CCD: coded and real variables

No.	Coded variables (experimental matrix)			Real variables (experimental plan)		
	X_1	X_2	X_3	U_1	U_2	U_3
1	−1	−1	−1	2.8	4.6	18.0
2	1	−1	−1	8.2	4.6	18.0
3	−1	1	−1	2.8	6.4	18.0
4	1	1	−1	8.2	6.4	18.0
5	−1	−1	1	2.8	4.6	27.0
6	1	−1	1	8.2	4.6	27.0
7	−1	1	1	2.8	6.4	27.0
8	1	1	1	8.2	6.4	27.0
9	−1.6818	0	0	1.0	5.5	22.5
10	1.6818	0	0	10.0	5.5	22.5
11	0	−1.6818	0	5.5	4.0	22.5
12	0	1.6818	0	5.5	7.0	22.5
13	0	0	−1.6818	5.5	5.5	14.9
14	0	0	1.6818	5.5	5.5	30.1
15	0	0	0	5.5	5.5	22.5
16	0	0	0	5.5	5.5	22.5
17	0	0	0	5.5	5.5	22.5
18	0	0	0	5.5	5.5	22.5
19	0	0	0	5.5	5.5	22.5

The relation $U_i = U_i^0 + X_i \Delta U_i$ allows to switch from coded variables to real variables. U_i^0, value of the real variable, i, at the center of the experimental domain; ΔU_i, variation step of the real variable, i, for a unit variation of the coded variable X_i.

Table 3
Experimental responses

No.	First enantiomer migration time (MT1) Y_1	Second enantiomer migration time (MT2) Y_2	Resolution (Rs) Y_3
1	13.67	13.96	0.85
2	14.60	15.24	1.86
3	6.84	7.14	1.55
4	8.55	9.12	2.58
5	12.08	12.35	0.74
6	12.85	13.45	1.72
7	6.04	6.29	1.19
8	7.36	7.86	2.32
9	6.43	6.52	0.49
10	9.00	9.65	2.50
11	13.58	14.03	0.91
12	6.06	6.41	1.70
13	7.94	8.39	2.07
14	6.78	7.14	1.80
15	7.90	8.32	1.92
16	7.58	8.01	1.92
17	7.61	8.02	1.90
18	7.62	8.06	1.92
19	7.62	8.06	1.90

variables X_i, while the corresponding experimental plan, carried out in the laboratory, gives the runs expressed in real variables U_i, as shown in Table 2. The central point was repeated five times to estimate the experimental error variance.

All experiments were performed in randomized order to minimize the effects of uncontrolled factors that may introduce a bias on the measurements.

Three experimental responses were studied: Y_1 = first enantiomer migration time (MT1); Y_2 = second enantiomer migration time (MT2); Y_3 = resolution (Rs). The experimental results are reported in Table 3.

A classical second-degree model was postulated for each experimental response Y_i, as follows:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon_i$$

where X_1, X_2, X_3 are the coded variables, β_i represent the model coefficients and ε_i the experimental error.

All experimental results were computed by NEMROD software [17].

The coefficients of the second-order polynomial model were estimated by the least squares regression [21]. The equation models for Y_1, Y_2, Y_3 were as follows:

$$Y_1 = 7.59 + 0.66X_1 - 2.71X_2 - 0.53X_3 + 0.42X_1^2 + 1.17X_2^2 + 0.30X_3^2 + 0.17X_1X_2 - 0.07X_1X_3 + 0.17X_2X_3$$

$$Y_2 = 8.02 + 0.82X_1 - 2.74X_2 - 0.56X_3 + 0.41X_1^2 + 1.17X_2^2 + 0.30X_3^2 + 0.15X_1X_2 - 0.073X_1X_3 + 0.16X_2X_3$$

$$Y_3 = 1.91 + 0.55X_1 + 0.28X_2 - 0.10X_3 - 0.14X_1^2 - 0.20X_2^2 + 0.02X_3^2 + 0.02X_1X_2 - 0.01X_1X_3 + 0.05X_2X_3$$

Classical statistical tools, as analysis of variance (ANOVA) and residual analysis, were employed to validate each model [9,22]. The statistical analysis showed that the models represented the phenomenon quite well and fitted accurately to the experimental data. Following validation of the model, graphs of surface responses can be drawn,

by plotting the response variation against two of the factors, while the third is held constant at a specified level, usually the center value.

It can be observed that the responses MT1 (Y_1) and MT2 (Y_2) show the same behavior in the studied experimental domain. For this reason, only the response surfaces for MT2 (Y_2) and Rs (Y_3), considering all possible variable interactions, are reported in Fig. 2a–c and Fig. 3a–c, respectively. The areas of interest for the minimization of migration time and maximization of resolution are examined. As reported in Fig. 2, an increase in pH (U_2) results in a decrease of MT2 (Y_2), while the temperature (U_3) has no important effect in the studied domain on the considered response; as regards the chiral selector concentration (U_1), it can be observed a little increase of MT2 (Y_2) at higher values.

As Rs (Y_3) is concerned, Fig. 3 shows as the chiral selector (U_1) has to be considered at the upper levels for its maximization, while it shows a maximum for a value of buffer pH (U_2) corresponding to 6.4. A temperature variation, between 15 °C and 30 °C, has no significant effect on the studied response.

3.2. Derringer's desirability function

The desirability function approach to multiresponse optimization is a useful technique for the analysis of experiments in which several responses have to be optimized simultaneously.

The measured properties of each response Y_i, $i = 1,2,\ldots m$, are transformed to a dimensionless desirability scale (d_i), defined as *partial desirability function*. This makes possible to combine results

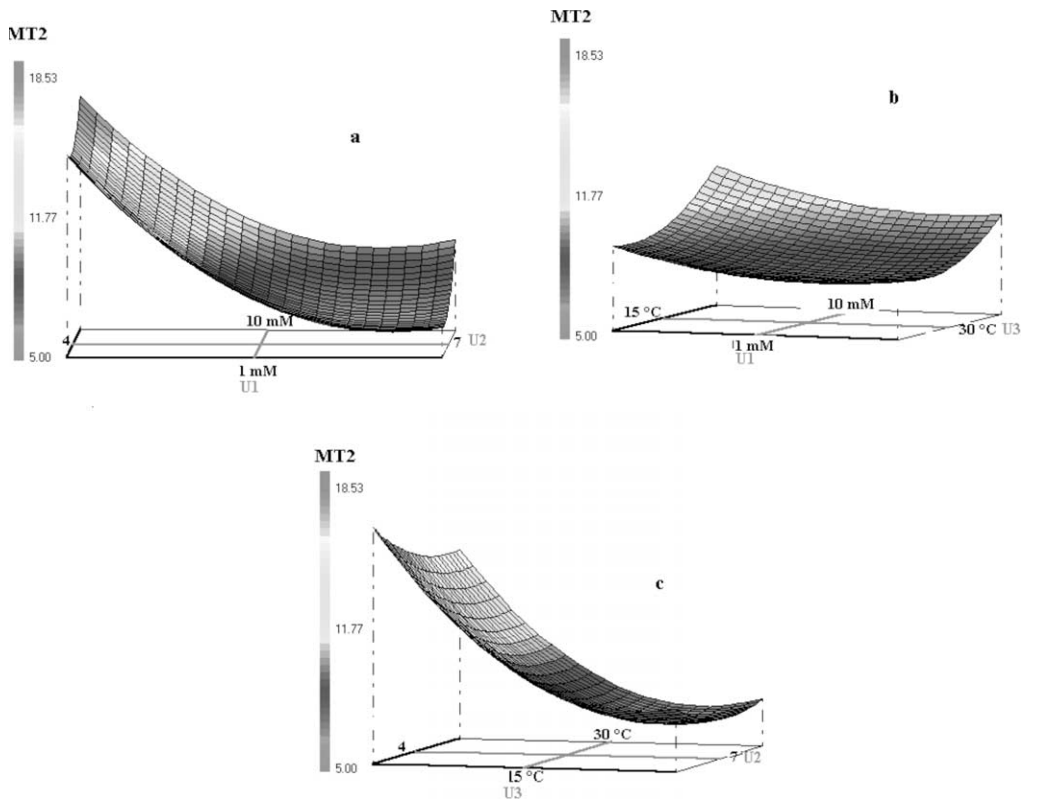


Fig. 2. Three-dimensional plot of second enantiomer migration time (MT2). (a) Response plot of chiral selector concentration (U_1) vs. pH (U_2); (b) response plot of chiral selector concentration (U_1) vs. temperature (U_3); (c) response plot of pH (U_2) vs. temperature (U_3).

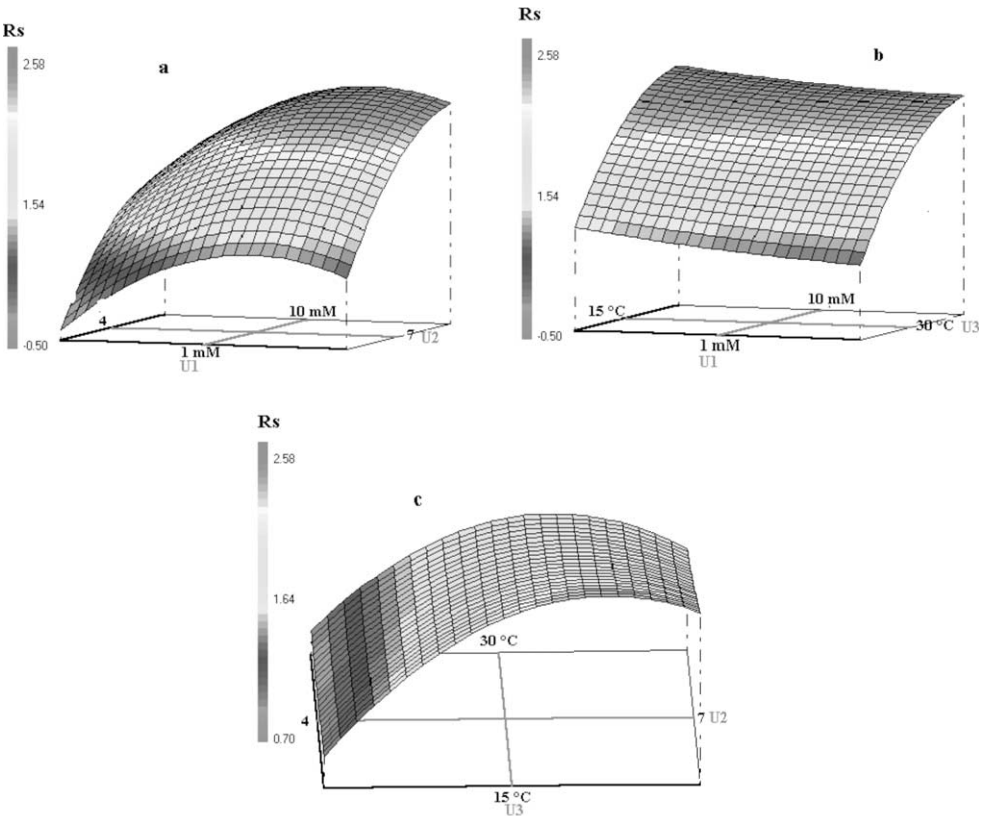


Fig. 3. Three-dimensional plot of resolution (Rs). (a) Response plot of chiral selector concentration (U_1) vs. pH (U_2); (b) response plot of chiral selector concentration (U_1) vs. temperature (U_3); (c) response plot of pH (U_2) vs. temperature (U_3).

obtained for properties measured on different scales.

The scale of the desirability function ranges between $d = 0$, for a completely undesirable response, and $d = 1$, if the response is at the target value.

As shown in Fig. 4a–c, the responses MT1, MT2 and Rs were transformed into an appropriate desirability scale d_1, d_2 and d_3, having regard that migration times of the two enantiomers had to be minimized, while resolution had to be maximized.

Once the function d_i is defined for each of the m responses of interest, an overall objective function (D), representing the *global desirability function*, is calculated by determining the geometric mean of the individual desirabilities.

Therefore, the function D over the experimental domain is calculated, as follows:

$$D = \left(\prod_{i=1}^{m} d_i \right)^{1/m}$$

Taking into account all requirements for m responses, we can choose the conditions on the design variables that maximize D.

A value of D different to zero implies that all responses are in a desirable range simultaneously and consequently, for a value of D close to 1, the combination of the different criteria is globally optimal, so as the response values are near target values.

After calculation by NEMROD software, an optimal separation condition with a CS concentration of 7 mM, temperature of 22 °C, pH 6.4 was predicted using the desirability function. The optimal conditions were obtained with a global degree

of satisfaction of D for the three responses equal to 85.31% and were validated experimentally. The three-dimensional plot of D is presented in Fig. 5. We can note the rather flat area around the optimal conditions which means the values of the three responses near this point are stable. This represents the robustness of the predicted optimal conditions. Fig. 6 shows the electropherogram of the enantiomer separation of the aryl propionic acid studied achieved using the optimized experimental conditions.

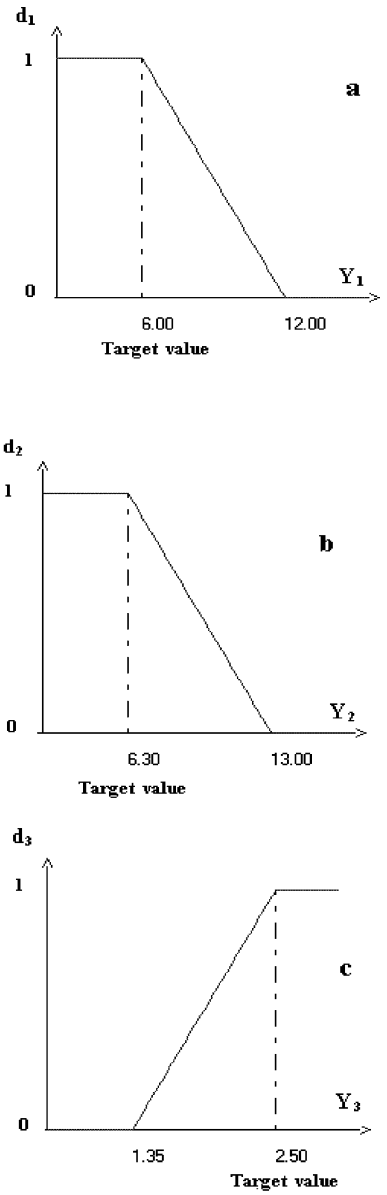


Fig. 4. Shape of the d_i function associated to the responses Y_i. (a) First enantiomer migration time (MT1); (b) second enantiomer migration time (MT2); (c) resolution (Rs).

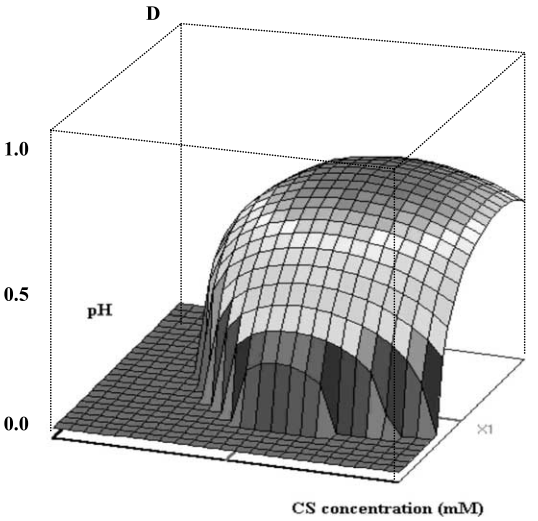


Fig. 5. Graphical representation of the overall desirability function D: degree of satisfaction vs. experimental condition in the plane (CS concentration vs. pH).

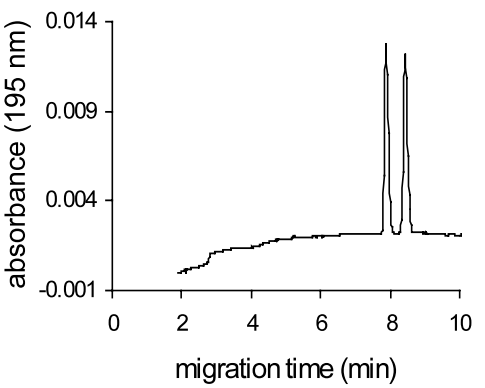


Fig. 6. Separation of enantiomers of 2-[(4′-benzoyloxy-2′-hydroxy)phenyl-propionic acid] (DF-1770y) under the optimized conditions: 50 mM Britton-Robinson pH 6.4 and 7 mM vancomycin; applied voltage, 20 kV; injection 10 psi∗s of 5×10^{-5} M of racemic analyte; capillary temperature 22 °C; capillary, polyacrylamide coated 35 cm (total), 30.5 cm (effective length) × 50 mm I.D.; injection BGE_Vancomycin at 120 psi∗s.

4. Conclusions

This paper shows how an experimental design approach led us to obtain a good CE enantioseparation of the new synthesized aryl propionic acid in a short analysis time with respect of a reduced number of experiments. By means of RSM and multiresponse optimization, the three considered responses were modeled in the experimental domain with a good fitness. In addition, the use of an appropriate chemometric methodology during optimization study has given an indication of method robustness.

Compared to empirical methods, chemometrics can greatly simplify the optimization of chiral CE analysis finding the appropriate experimental conditions.

An appropriate use of DOE ensures that experimental data contain maximum information and provides the availability of answers to real chemical problems, confirming how the application of chemometric techniques in analytical chemistry is needed and can be successfully realized.

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