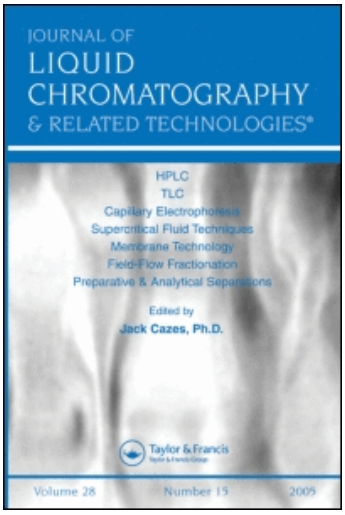

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Optimization Strategies using Response Surface Methodologies in High Performance Liquid Chromatography

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Abstract: Development of effective chromatographic separation involves judicious deciding of the selection of optimal experimental conditions that can provide an adequate resolution at a reasonable run time for the separation of interested components. The isomers of 2-methyl-6-nitroaniline and 2-methyl-4-nitroaniline were selected as the model compounds for the application of optimization strategies by high performance liquid chromatography. Response surface methodologies based on three level, three variable designs such as the Box-Behnken design, central composite face centered, central composite circumscribed design, and full factorial design were comparatively used for the optimization with respect to column temperature, flow rate, and the percentage of eluent. Statistical interpretation of the variables on different responses such as resolution and retention time of the last component was performed. The optimum conditions of these variables were predicted by using a second order polynomial model fitted to the results obtained by applying four designs. The response surface plots using three experimental designs revealed a separation optimum with a 25°C column temperature, a flow rate of 1.0 mL/min, and ACN percentage of 70%. The significance of the statistical designs was confirmed by the generally good agreement obtained between predicted responses and actual experimental data. We have concluded that experimental designs offer a rapid means of optimizing

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several variables and provide an efficient test for the robustness of the analytical method.

Keywords: 2-methyl-4-nitroaniline, 2-methyl-6-nitroaniline, Experimental design, High performance liquid chromatography, Response surface methodology

INTRODUCTION

Many methods have been developed in order to optimize the parameters of interest in high performance liquid chromatography (HPLC) and related techniques.[1,2] In chemometric approaches, experimental measurements are performed in such a way that all variables vary together. An objective function is utilized in which the analyst introduces the desired criteria (selectivity, resolution, retention time). The advantage of chemometrics tools is that no explicit models are required, and when models are available optimization is easier to perform by regression methods. Optimization of a HPLC separation condition is a critical step, since the wide array of variables such as mobile phase composition, pH, column temperature, flow rate, column length, and injection time can influence the separation efficiency, retention time, resolution, and etc. and a complete and quite general physicochemical model in HPLC is not popular.[3–5] One approach to achieve an optimal separation is to vary the experimental parameter steps while keeping other parameters constant. But the search for the optimal separation condition by this approach requires too much experimental work and is tedious and time consuming. Furthermore, when interaction appears, an independent univariate optimization is not appropriate to find the best experimental conditions since the influence of any given variable depends on the magnitude of other variable.

A suitable alternative to overcome the aforementioned shortcomings lies in experimental design techniques. Moreover, the number of experiments to be carried out can be reduced drastically when following these chemometric strategies.[6–9] Chemometrics are involved in the preliminary stages for the establishment of an HPLC method and the analysis of HPLC data to extract the maximum amount of significant information. It allows a large number of parameters to be screened simultaneously, and to achieve this in a small number of mathematical runs, is the most important aspect of mathematical design and will provide a mathematical framework. Experimental designs such as Plackeet-Burman design (PBD), Box-Behnken design (BBD), central composite face-centered design (CCFD), central composite circumscribed design (CCCD), full-factorial design (FFD), etc. have been used for the separation studies of HPLC and related technologies.[10–15] Several studies have been reported on the use of multivariate statistical analysis to optimize HPLC

methods. Depending on the design, the response model can show the relationship between each parameter. BBD, CCFD, CCCD, and FFD are the response surface methods used to optimize the chromatographic and electrophoretic separation.[16,17] As an efficient statistical tool for optimization of multiple variables, it can be performed respectively to predict the best performance conditions by using a minimum number of experiments.

2-Methyl-6-nitroaniline and 2-methyl-4-nitroaniline are aromatic nitro amino compounds, which are generally used as intermediates for dyes and drugs. The aromatic compounds are known to damage the human liver and are registered as toxic substances; quality control concerning the isomers is lacking. In the present work, response surface methodologies including BB, CCF, CCC, and FF designs have been comparably performed to determine the optimal separation conditions by HPLC taking the above isomers as the model compounds. Increased resolution and retention time can be obtained using the experimental design methodology instead of some commercial optimization softwares.[18] Furthermore, in order to find the best compromise between several responses, a multicriteria decision making approach was used, which the resolution response and retention time response can be simultaneously optimized. Baseline separation of the model analytes has been obtained quickly during 5 min with a resolution greater than 1.5.

EXPERIMENTAL

Chemicals

2-Methyl-6-nitroaniline, 2-methyl-4-nitroaniline standard samples were kindly provided by Xinxiang Wanfang Chemical Industry Co. Ltd, thiourea, acetonitrile (ACN), methanol, and other reagents were purchased from Beijing Chemical Reagent Company and Tianjing Kemiou Chemical Reagent Company, China. All solvents used were of HPLC or analytical reagent grade. Distilled water was obtained from a super purification system (Danyangmen Corporation, Jiangshou, China). All solutions were degassed with ultra-sonication and filtered through a membrane (0.45 μm) before use. In a typical chromatographic experiment, mixtures of thiourea and the isomers were dissolved in methanol and injected for peak identification. For the separation the analytes, the compounds were properly mixed and used for the optimization of conditions and effective separations.

Apparatus

Chromatographic measurements were made on a HP1100 Series HPLC system (Agilent Technologies, Inc., Walbronn, Germany) equipped with

a quaternary pump, a vacuum degasser module, a Rheodyne injector with a 20 μL sample loop, a temperature controlled column compartment, and a variable wavelength UV detector set at 254 nm. An HPLC separation was performed on an 15 cm×4.6 mm, i.d., stainless steel column packed with ZORBAX SB-C18 (5 μm particle size, 100 A pore size).

Software

The Microsoft Excel Program (Microsoft Corporation) and Design Expert 7.0 Software (Stat-Ease, Inc. Minneapolis, MN) were used for setting the polynomial equations and the statistical analysis of the response variables. Design Expert 7.0 Software was used for making the Response surface diagrams.

RESULTS AND DISCUSSION

Response Surface Methodology

In the present work, column temperature, flow rate, and percentage of the eluent (ACN) in the binary mobile phase (ACN/H_2O, v/v) system were optimized to have effective separation of the analytes by employing the experimental design strategy similar to the methodology adopted elsewhere.[19] Fifteen experimental conditions were chosen from the strategic positions in a cubic diagram (Figure 1). These experimental conditions are described in Table 1. Maximum and minimum column temperature (x_1) was fixed as 15.0°C and 35.0°C, respectively. Likewise, a minimum and maximum for flow rate (x_2) for the experimental designs was selected as 0.5 mL/min and 1.5 mL/min, respectively. The percentage of ACN (x_3) was kept in between 55% and 85%. The resolution among the isomers and the maximum retention time (for the last eluting peak) were noted as responses with these experiments.

Initial method screening to determine the most significant variables for the analytes did not require an experimental design approach due to our knowledge.[20,21] As shown in Table 1, three important variables were chosen for the optimization designs, namely column temperature, flow rate, and percentage of ACN. In order to calculate quadratic regression model coefficients, each design variable has to be studied at three distinct levels or five levels at least.

BB, CCF, CCC, and FF models were comparatively used for the multivariable approaches. For BB design, the experimental plan for a three parameter design is laid out according to the following pattern: two variables have a combination of their extreme levels, while the other

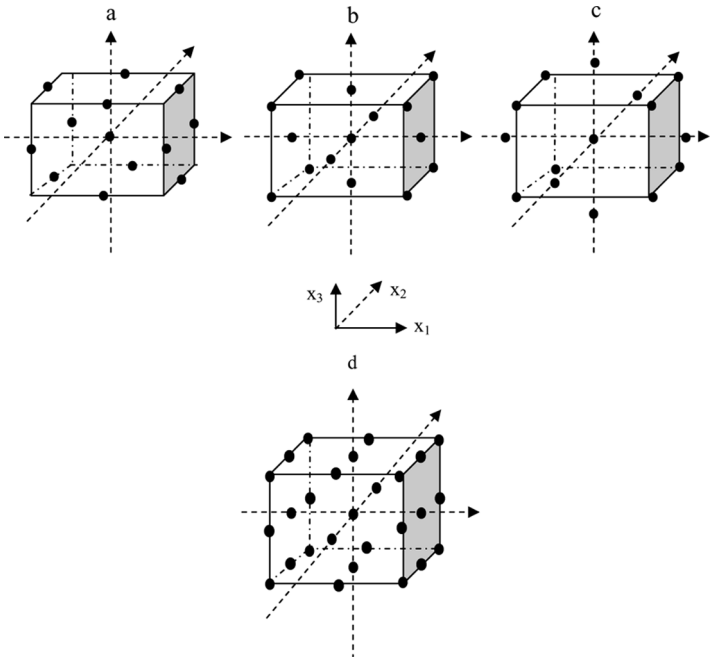


Figure 1. The representation of BB, CCF, CCC, and FF design models.

is set to its mean value. For a three parameter design, all experimental points are located on the edges of a cube around the centre points (Figure 1a). The CCF and CCC designs are based on a full factorial design, which is augmented by centre points and axial or start points (Figures 1b and 1c). There are, therefore, 8 cube points (for a full factorial) with levels of -1 and $+1$, 6 axial or start points with levels of $-\alpha$ and $+\alpha$, and 6 replicates of the centre point. Depending on the α value, three types of models are distinguished: central composite face centre if

Table 1. Coded and true values of variables of the BB, CCF, CCC, and FF design models

	Code		Level				
Variables	Coded value	True value	-1.682	-1	0	1	1.682
Column temperature (°C)	X_1	x_1	8.2	15	25	35	41.8
Flow rate (mL/min)	X_2	x_2	0.159	0.5	1	1.5	1.841
ACN percentage (%)	X_3	x_3	44.8	55	70	85	95.2

$\alpha = 1$, central composite circumscribed if $\alpha > 1$, and central composite inscribed (CCI) if $\alpha < 1$. The first type is spherical designs, while the CCF and CCI is a cubic design. Here, for three variables and three or five levels, a total of 20 experiments were considered. As with the CCD model, the BB design is a response surface method used to examine the relationship between one or more response variables and a set of quantitative experimental parameters. The BBD is not directly based on a full factorial design as it uses middle points instead of corner points. The BB design requires fewer experiments than CCF and CCC but cover a slightly smaller experimental region. It is also a spherical design. A full factorial design of experiment measures the response of every possible combination of factors and factor levels (Figure 1d). These responses are analyzed to provide information about every main effect and every interaction effect.

The levels of three variables for four designs are shown in Table 1. For BB, CCF, CCC, and FF designs, maximum and minimum concentration for column temperature (x_1) were fixed as 15°C and 35°C, respectively. Likewise, flow rate (x_2) and ACN percentage (x_3) were chosen as $0.5 \sim 1.5$ mL/min and $55 \sim 85\%$, respectively. For the CCC design, five levels of three variables should be performed with a α value of 1.682. The BB model with a total of seventeen experiments (the twelve middle points of the edges on a cube and 5 centre points, the CCF and CCC models with a total of twenty experiments, the FF design with a total of twenty seven experiments are depicted in Tables 2–5, respectively. All other experimental plans and the runs were randomized to exclude any bias. The resolution response (R_s) and the retention time (t_{end}) were monitored during processing. The calculated responses for the analytes were also respectively included in Tables 2–5.

In order to define the relationship between the responses and the variables, a quadratic regression model should be applied on the basis of a multiple linear regression (MLR). The selected model included 10 coefficients (the constant term, B_0, three main effects, three quadratic terms, and three interaction terms, as indicated in the equation.[22–24]

$$y = B_0 + \sum_{i=1}^{n} B_i x_i + \sum_{i=j=1}^{n} B_{ij} x_i x_j$$

In our studies, it can be changed into the following equation according to the n value and the coded values of three variables as follows:

$$y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2$$

Table 2. Experimental design and response results using BBD model

Run	x_1	x_2	x_3	R_s Exp.	Pred.	T_{end} Exp.	Pred.
1	−1	−1	0	2.86	2.94	5.90	6.26
2	1	−1	0	2.48	2.49	5.27	5.50
3	−1	1	0	2.62	2.61	1.96	1.73
4	1	1	0	2.22	2.15	1.73	1.38
5	−1	0	−1	5.08	5.07	5.84	6.08
6	1	0	−1	4.19	4.24	4.56	4.93
7	−1	0	1	1.49	1.44	2.04	1.67
8	1	0	1	1.34	1.35	1.95	1.70
9	0	−1	−1	5.01	4.95	10.68	10.07
10	0	1	−1	4.52	4.54	3.52	3.51
11	0	−1	1	1.64	1.62	3.99	4.01
12	0	1	1	1.29	1.35	1.33	1.93
13	0	0	0	2.56	2.53	2.75	2.75
14	0	0	0	2.60	2.53	2.75	2.75
15	0	0	0	2.52	2.53	2.74	2.75
16	0	0	0	2.51	2.53	2.75	2.75
17	0	0	0	2.48	2.53	2.75	2.75

Table 3. Experimental design and response results using CCF model

Run	x_1	x_2	x_3	R_s Exp.	Pred.	T_{end} Exp.	Pred.
1	−1	−1	−1	5.54	5.51	11.76	11.29
2	1	−1	−1	4.55	4.54	9.49	9.44
3	−1	1	−1	5.08	5.07	4.06	4.12
4	1	1	−1	4.10	4.10	3.15	3.01
5	−1	−1	1	1.71	1.71	4.10	4.21
6	1	−1	1	1.58	1.59	3.92	3.82
7	−1	1	1	1.36	1.37	1.36	1.37
8	1	1	1	1.22	1.25	1.30	1.73
9	−1	0	0	2.83	2.87	2.91	3.20
10	1	0	0	2.35	2.32	2.60	2.46
11	0	−1	0	2.74	2.78	5.61	6.12
12	0	1	0	2.42	2.39	1.84	1.49
13	0	0	−1	4.63	4.69	5.11	5.72
14	0	0	1	1.41	1.36	1.99	1.54
15	0	0	0	2.56	2.53	2.75	2.69
16	0	0	0	2.6	2.53	2.75	2.69
17	0	0	0	2.52	2.53	2.74	2.69
18	0	0	0	2.51	2.53	2.75	2.69
19	0	0	0	2.48	2.53	2.75	2.69
20	0	0	0	2.54	2.53	2.75	2.69

Table 4. Experimental design and response results using CCC model

Run	x_1	x_2	x_3	R_s Exp.	Pred.	T_{end} Exp.	Pred.
1	−1	−1	−1	5.54	5.47	11.76	13.25
2	1	−1	−1	4.55	4.56	9.49	11.52
3	−1	1	−1	5.08	5.03	4.06	3.97
4	1	1	−1	4.10	4.11	3.15	2.98
5	−1	−1	1	1.71	1.57	4.10	5.46
6	1	−1	1	1.58	1.51	3.92	5.19
7	−1	1	1	1.36	1.23	1.36	0.52
8	1	1	1	1.22	1.17	1.30	1.00
9	−1.682	0	0	2.77	2.94	2.97	2.41
10	1.682	0	0	2.12	2.12	2.47	1.36
11	0	−1.682	0	2.90	3.00	17.32	14.23
12	0	1.682	0	2.26	2.33	1.48	2.89
13	0	0	−1.682	6.83	6.83	11.04	9.67
14	0	0	1.682	0.90	1.07	1.76	1.45
15	0	0	0	2.56	2.53	2.75	2.79
16	0	0	0	2.60	2.53	2.75	2.79
17	0	0	0	2.52	2.53	2.74	2.79
18	0	0	0	2.51	2.53	2.75	2.79
19	0	0	0	2.48	2.53	2.75	2.79
20	0	0	0	2.54	2.53	2.75	2.79

Where y is the response to be modeled (R_s and t_{end}), B_i are the coefficients of the models by MLR, X_1 is column temperature (in coded variable), X_2 is the flow rate (in coded variable) and X_3 is the ACN percentage (in coded variable).

Coefficients used for four mathematical models of different responses could be calculated with the help of Design Expert 7.0 Software, which have been calculated and plotted from Figures 2a and 2b. The comparative effects for three models have been clearly shown. When the coefficient is not included in the 95% confidence interval, this means that it is statistically different from 0 and, therefore, the variable associated to this efficient has a significant influence on response. If it is positive, it favorably influences response whether it represents a main or quadratic effect or a first order interaction.

As shown in Figure 2a, all terms except x_1, $x_1{}^*x_2$, $x_1{}^*x_3$, and $x_1{}^2$ have high significant influences on retention time for BB design; all terms but $x_1{}^*x_2$, $x_1{}^*x_3$, and $x_1{}^2$ have high significant influences for CCF models; all terms but x_1, $x_1{}^*x_2$, $x_1{}^*x_3$, $x_2{}^*x_3$, and $x_1{}^2$ have high significant influences for CCC model, and all terms but $x_1{}^*x_2$, $x_1{}^*x_3$, and $x_1{}^2$ have high significant influences for FFD model. As for resolution in Figure 2b, all terms except $x_1{}^*x_2$, $x_2{}^*x_3$, $x_1{}^2$, and $x_2{}^2$ have significant influences on resolution

Table 5. Experimental design and response results using FFD model

Run	x_1	x_2	x_3	R_s Exp.	Pred.	T_{end} Exp.	Pred.
1	−1	−1	−1	5.54	5.44	11.76	11.04
2	−1	−1	0	2.86	3.02	5.90	6.53
3	−1	−1	1	1.71	1.68	4.10	4.14
4	−1	0	−1	5.08	5.16	5.84	6.34
5	−1	0	0	2.83	2.77	2.91	2.94
6	−1	0	1	1.49	1.46	2.04	1.64
7	−1	1	−1	5.08	5.02	4.06	4.04
8	−1	1	0	2.62	2.66	1.96	1.72
9	−1	1	1	1.36	1.37	1.36	1.52
10	0	−1	−1	5.01	4.97	10.68	10.19
11	0	−1	0	2.74	2.76	5.61	6.03
12	0	−1	1	1.64	1.62	3.99	3.98
13	0	0	−1	4.63	4.69	5.11	5.66
14	0	0	0	2.56	2.51	2.75	2.59
15	0	0	1	1.41	1.40	1.99	1.64
16	0	1	−1	4.52	4.55	3.52	3.51
17	0	1	0	2.42	2.40	1.84	1.54
18	0	1	1	1.29	1.31	1.33	1.68
19	1	−1	−1	4.55	4.52	9.49	9.38
20	1	−1	0	2.48	2.52	5.27	5.56
21	1	−1	1	1.58	1.58	3.92	3.85
22	1	0	−1	4.19	4.24	4.56	5.00
23	1	0	0	2.32	2.26	2.60	2.27
24	1	0	1	1.34	1.35	1.95	1.66
25	1	1	−1	4.10	4.10	3.15	3.00
26	1	1	0	2.22	2.15	1.73	1.38
27	1	1	1	1.22	1.27	1.30	1.86

for BB models; all terms except $x_1{}^*x_2$, $x_2{}^*x_3$, and $x_2{}^2$ have significant influences on migration time for CCF design; all terms except $x_1{}^*x_2$, $x_2{}^*x_3$, $x_1{}^2$, and $x_2{}^2$ have significant influences on resolution for CCC models; all terms except $x_1{}^*x_2$, $x_2{}^*x_3$, and $x_1{}^2$ have significant influences on migration time for FFD design.

The significance of the parameters estimated by the least squares can be assessed by using classical statistical tools as ANOVA. When different single response or multi-responses are chosen as the objective function, the most significant variables for different responses will be different. The three models were found to describe the experimental data adequately, with high confidence ($p < 0.05$) and led to different coefficients of determination (R^2), the adjusted coefficients of determination ($R^2_{adj.}$), which is related to the number of parametric

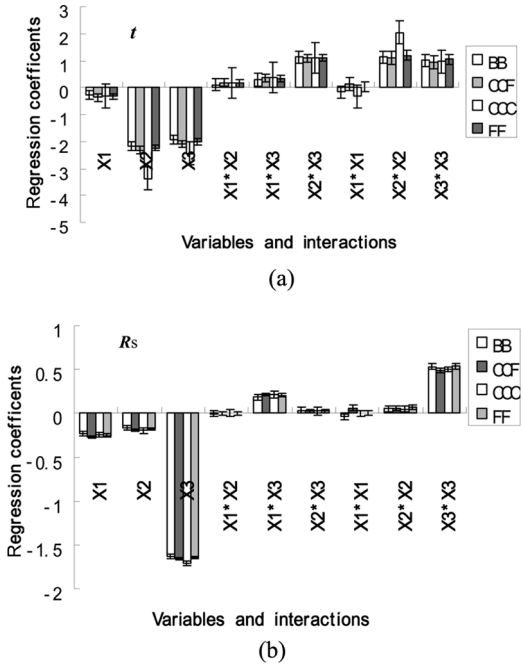


Figure 2. (a) The relationship between the resolution response and the coefficients of three models with corresponding SD; (b) The relationship between migration time of the second peak and the coefficients of three models with corresponding SD. Error bars represent ± SD at level 0.05.

coefficients in the model and the predictive power of the model, which is given by the predicted R^2. In fact, the values of R^2 and $R^2_{\text{adj.}}$ are indicators of the explanatory power of the model and varies from 0, when the model does not explain the response, to 1 for a perfectly explained response. The predicted R^2 is a measure of how well the model will predict the responses for a new experimental condition. The predicted R^2 is the predictive measure corresponding to the measure of fit, while R^2 is the percent variation of the response explained by the model. It gives a lower estimate to how well the model predicts the outcome of new experiments, while R^2 gives an upper estimate. The statistical evaluation of these models is shown in Table 6. This table revealed that R^2, $R^2_{\text{adj.}}$, and the predicted R^2 for the responses, except the predicted R^2 of CCC model for the retention time were higher than 0.859, indicating the good fitting of these models, and allowing to establish response surfaces and contour plots and predict any responses within the experimental domain.

Table 6. Statistical evaluation of each model (date from Design-Expert, Version 7.0.1 Stat-Ease, Inc. Minneapolis, MN 55413)

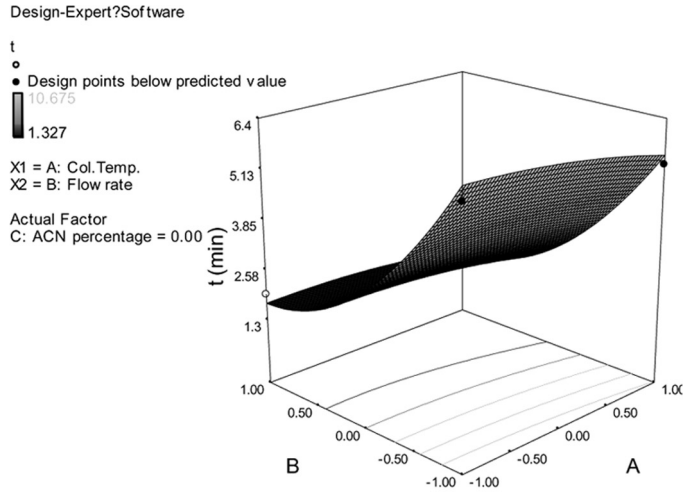
Response	Experimental average value n = 6	BB Pred. value	CCF Pred. value	CCC Pred. value	FF Pred. value
R_s	2.54	2.53	2.53	2.53	2.51
T_{end}	2.75	2.75	2.69	2.79	2.59

On knowing the coefficients,the function of experimental responses related to three variables could be obtained. Based on the mathematical model, the response surface can be explored graphically. In this case, one can plot the response surfaces and their two dimensional contour plots against two of the variables, while the third is held constant at a specified level, usually the center value. Figures 3–10 shows the response surfaces and contour plots for the isomer separation using four designs, obtained plotting column temperature (X_1) versus flow rate (X_2), and ACN percentage (X_3). It can be observed that the response (R_s) show the same behavior among four studied models. An increase in ACN percentage will apparently decrease the resolution, meanwhile column temperature and flow rate has a little effect on it. For the retention time (t_{end}), flow rate and ACN percentage have apparent negative effects, while column temperature has a little effect on it.

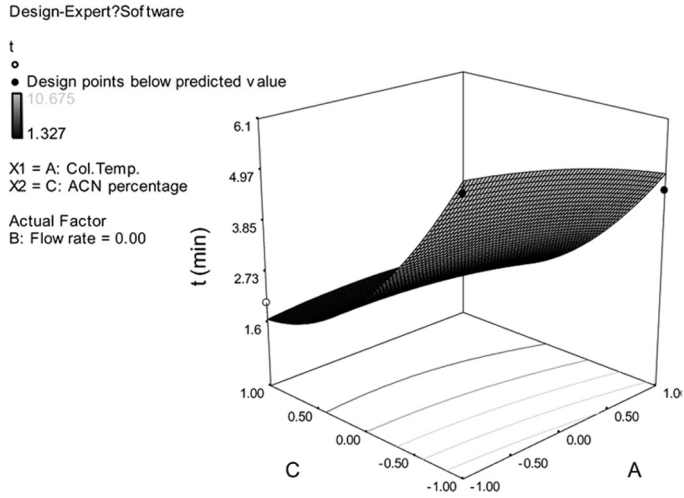
Derringer's Desirability Function

Generally, responses were usually transformed into an appropriate desirability scale to balance between different responses. Frequently, different weight factors should be assigned for each response, with larger weights corresponding to more important responses and smaller weights to less important responses.[25] After the individual desirabilities were calculated for each response, they were combined to provide a measure of the composite desirability of the multi-response system. This measure is the weighted geometric average of the individual desirability or the responses. Sometimes, it is very difficult to choose different weights according to the importance of different variables. The most popular methodology applied to multiple response optimization is the desirability function approach.[11,26]

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 make it possible to combine results obtained



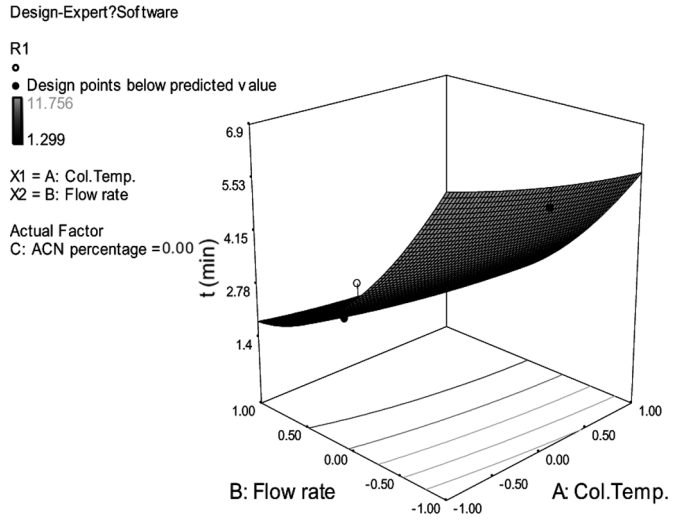
(a)



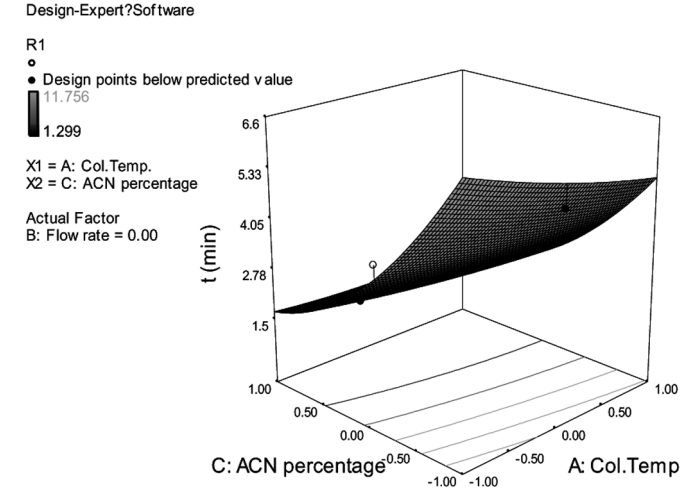
(b)

Figure 3. Response surface plot and its contour curve of R_s using BB design model.

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. Once the function d_i is defined for each of the m responses of interest, an overall objective



(a)



(b)

Figure 4. Response surface plot and its contour curve of t_{end} using BB design model.

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: $D = (\Pi d_i)^{1/m}$. Taking into account all requirements for m

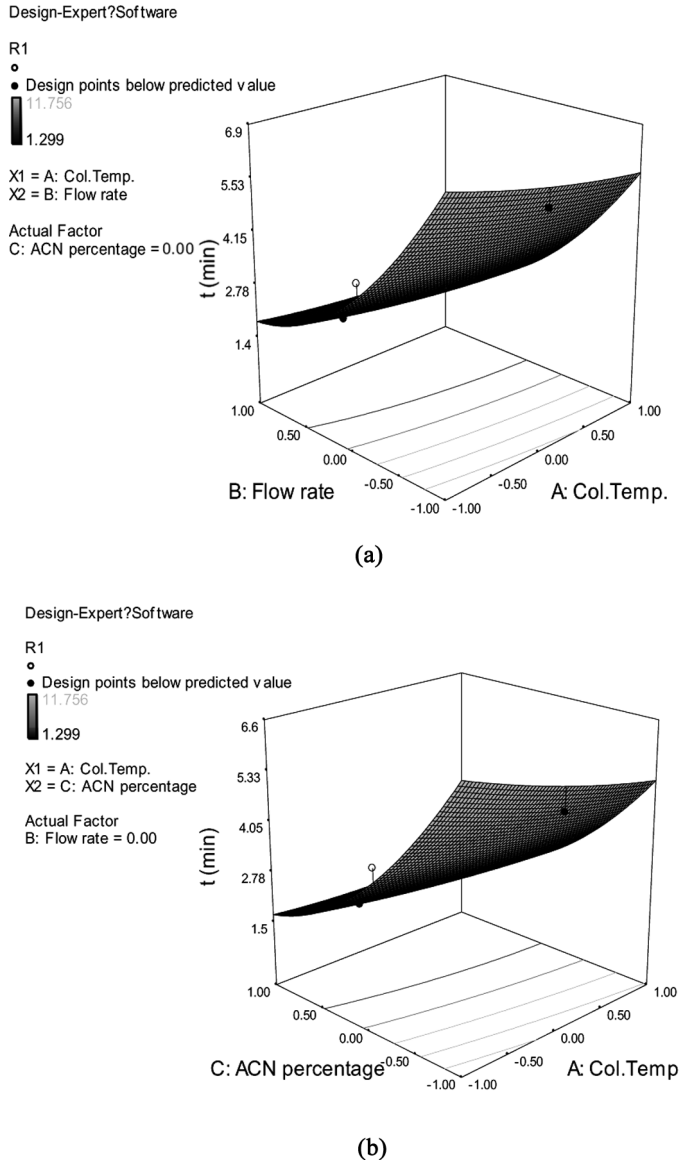
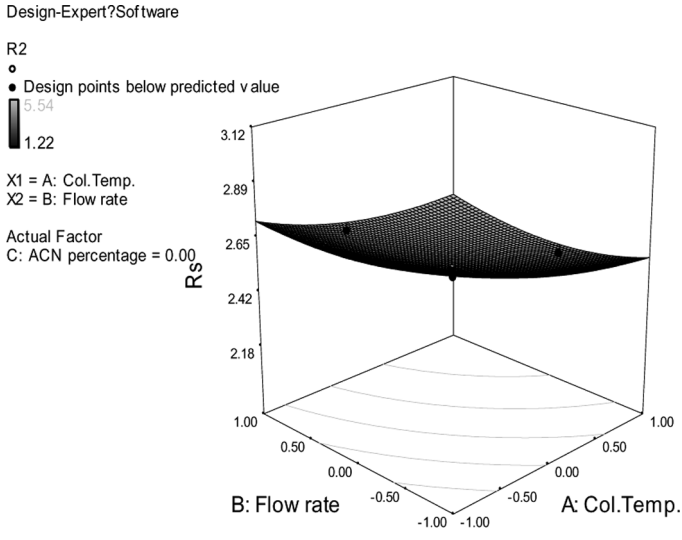
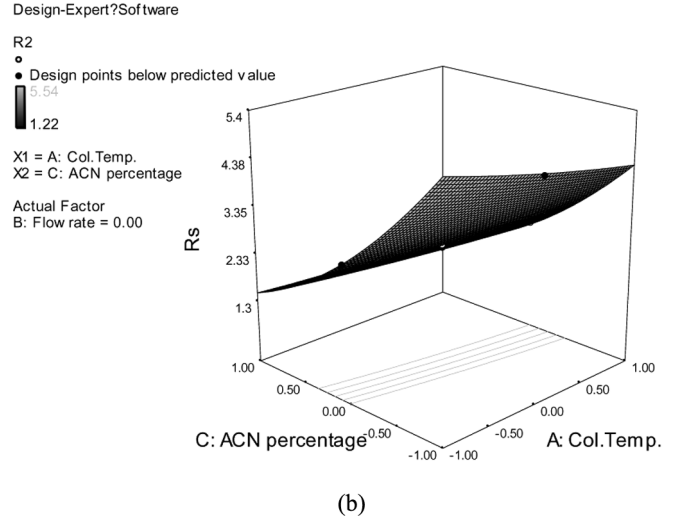


Figure 5. Response surface plot and its contour curve of R_s using CCF design model.

responses, we can choose the conditions on the design variables that maximize D. In our study, only the resolution response (R_s), and the retention time of the second component (t_{end}) have been considered. In order to define D quality response, t_{end}, and R_s were normalized. The shortest t_{end}



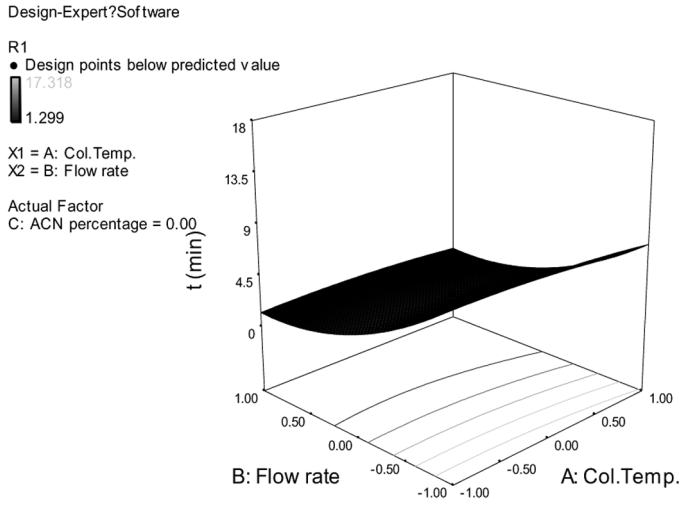
(a)



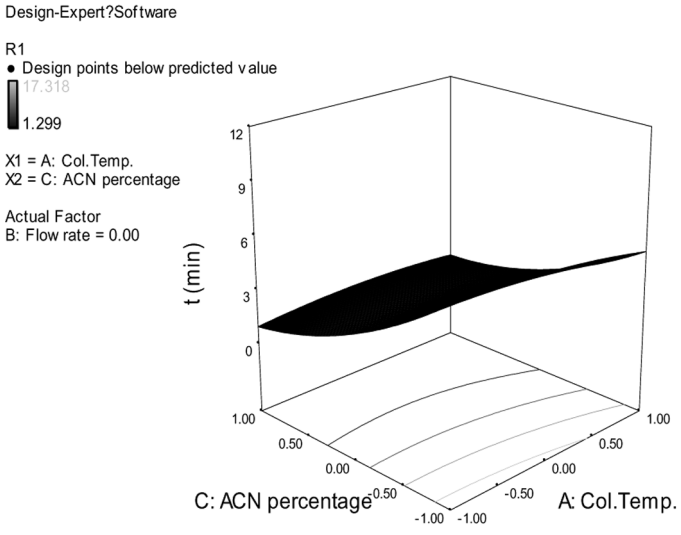
(b)

Figure 6. Response surface plot and its contour curve of t_{end} using CCF design model.

(5.0 min) and the highest R value (3.0) in all experiments were given the value 1 (maximum), while the longest t_{end} (10.0 min) and the lowest R_2 values (the unwanted one, 1.5) were given the value 0 (minimum). This is



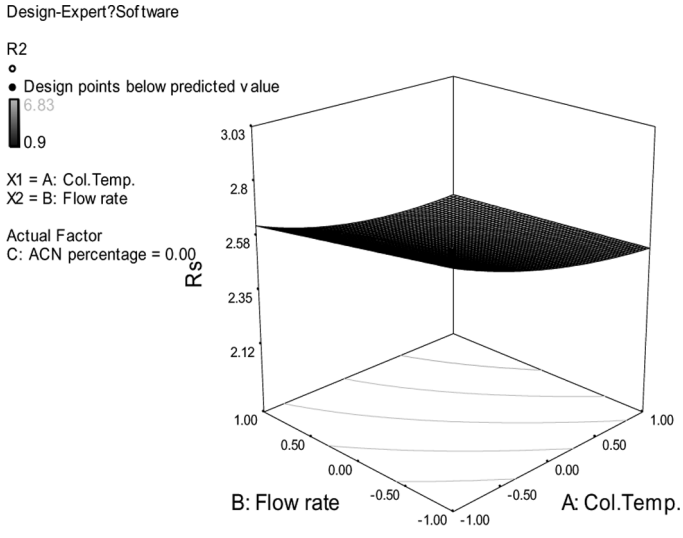
(a)



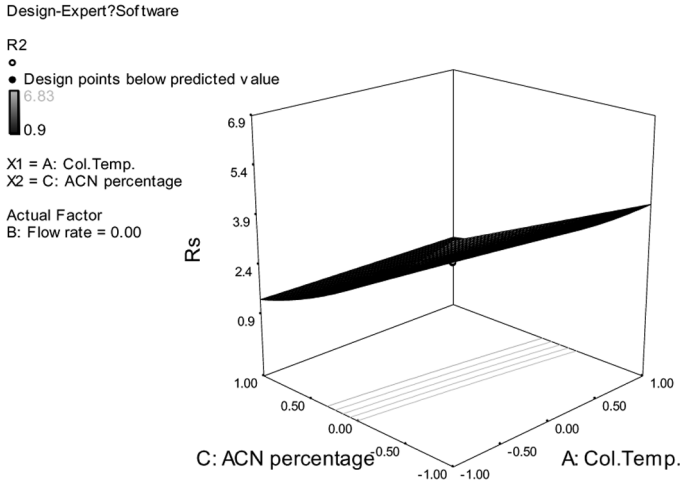
(b)

Figure 7. Response surface plot and its contour curve of R_s using CCC design model.

shown in Figure 11. Linear interpolation allowed us to calculate the normalized values for the remaining t_2 and R, normalized values were called d_1, and d_2, which could be calculated according to the following equations:[25,26]



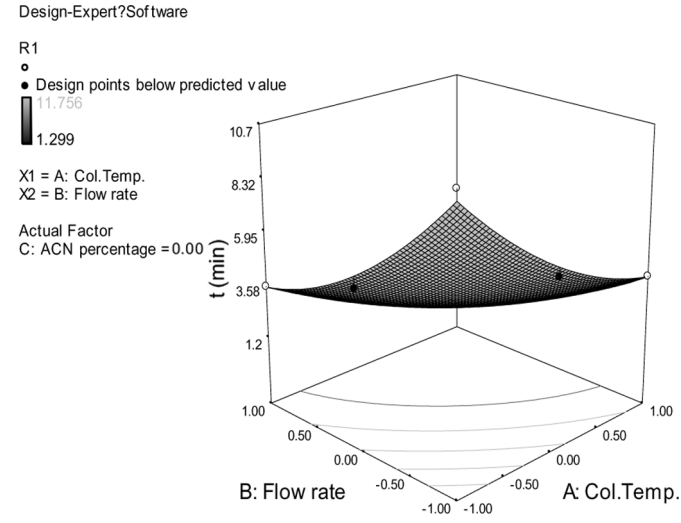
(a)



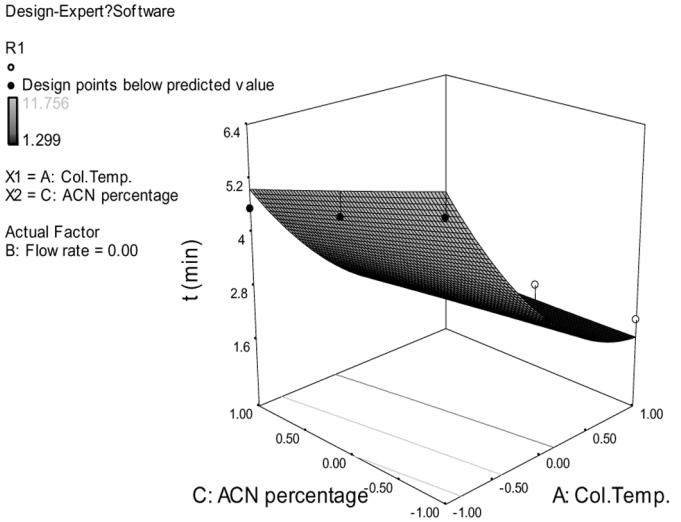
(b)

Figure 8. Response surface plot and its contour curve of t_{end} using CCC design model.

$$d_1 = (R_i - R_{min})/(R_{max} - R_{min}),\ \text{if}\ 1.5 < R_i < 3.0;$$
$$d_i = 0,\ \text{if}\ R_i \leq 1.5; d_i = 1,\ \text{if}\ R_i \geq 3.0. \quad (1)$$



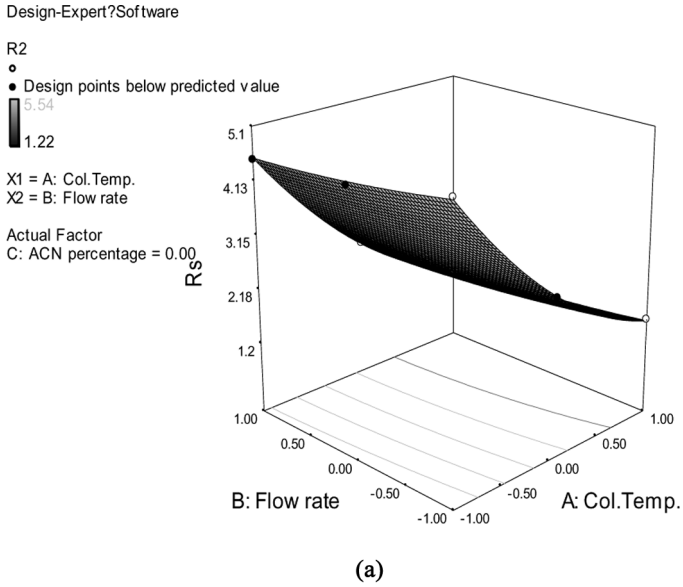
(a)



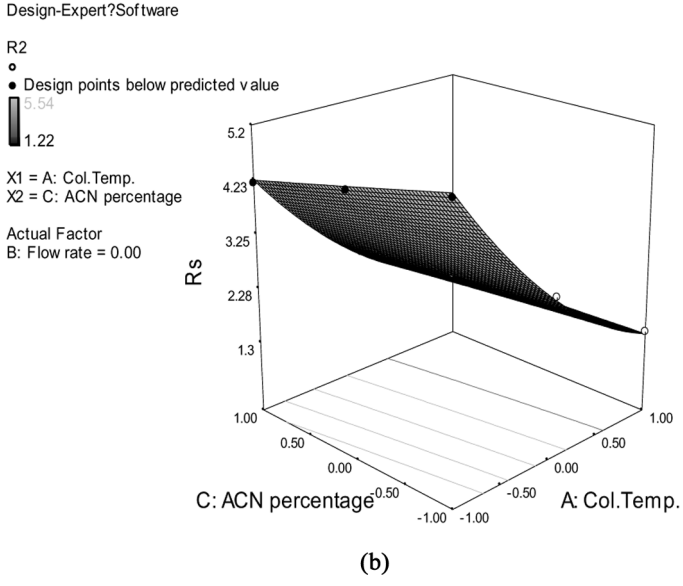
(b)

Figure 9. Response surface plot and its contour curve of R_s using FF design model.

$$d_2 = (t_{max} - t_i)/(t_{max} - t_{min}),\ \text{if}\ 5.0 < t_i < 10.0;\ d_i = 1,\ \text{if}\ t_i \le 5.0;$$
$$d_i = 0,\ \text{if}\ t_i \ge 10.0. \quad (2)$$



(a)



(b)

Figure 10. Response surface plot and its contour curve of t_{end} using FF design model.

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 or equal to 1, the combination of the different criteria is globally optimal, so the response values are near target values.

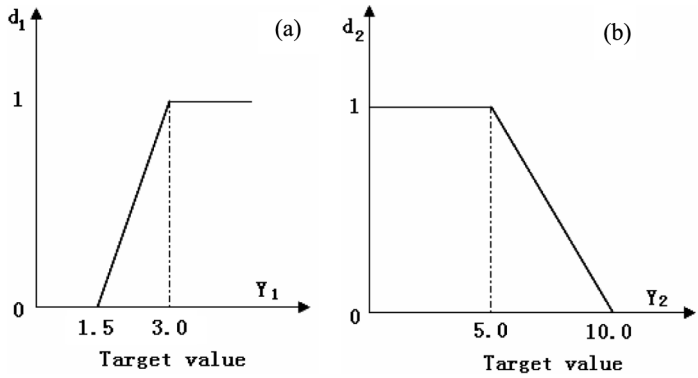


Figure 11. Shape of the d_i function associated to the response Y_i, resolution (R_s); (b) retention time of the last peak (t_{end}).

After calculation by the Design Expert program, the final equations in terms of coded variables using three experimental designs were the following:

$$D_{BBD} = 1.00 - 0.0057X_1 + 0.10X_2 - 0.097X_3 - 0.040X_{12} - 0.046X_{13} - 0.30X_{23} + 0.081X_1^2 - 0.14X_2^2 - 0.34X_3^2 \quad (3)$$

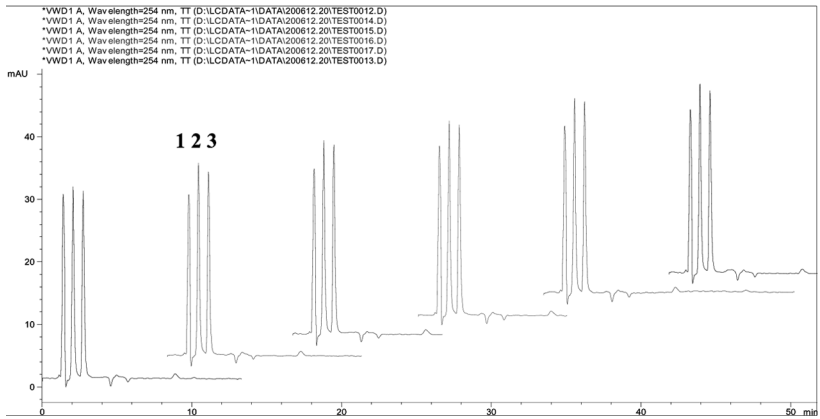


Figure 12. Chromatograms of the isomers using the optimal conditions. Stationary phase: ZORBAX SB-C_{18}, wavelength 254 nm, column temperature 25°C, flow rate of 1.0 mL/min, ACN percentage of 70%. Peak identification: 1: uracil; 2: 2-methyl-4-nitroaniline; 3: 2-methyl-6-nitroaniline.

Table 7. Observed and predicted response for testing of the predictability of the models

		R^2	$R^2_{adj.}$	Predicted R^2
BB	R_s	0.9985	0.9966	0.9815
	T_2	0.9824	0.9598	0.7188
			0.7188	
CCF_s	R_s	0.9993	0.9987	0.9965
T_2		0.9882	0.9775	0.8863
CCC_s	R_s	0.9970	0.9944	0.9788
	T_2	0.9259	0.8592	0.4374
FFD_s	R_s	0.9985	0.9977	0.9960
	T_2	0.9828	0.9738	0.9541

$$D_{CCF} = 1.02 + 0.0096X_1 + 0.13X_2 - 0.060X_3 - 0.045X_{12} - 0.062 X_{13} - 0.26X_{23} - 0.070X_1^2 - 0.089X_2^2 - 0.29X_3^2 \quad (4)$$

$$D_{CCC} = 0.99 - 0.0059X_1 + 0.20X_2 - 0.010X_3 - 0.045X_{12} - 0.062 X_{13} - 0.26X_{23} + 0.0042X_1^2 - 0.16X_2^2 - 0.33X_3^2 \quad (5)$$

$$D_{FF} = 0.98 - 0.20X_1 - 0.04X_2 - 0.23X_3 - 0.015X_{12} - 0.029 X_{13} - 0.058X_{23} - 0.0098X_1^2 + 0.0036X_2^2 - 0.21X_3^2 \quad (6)$$

The optimal conditions for four experimental designs were obtained with a global degree of satisfaction of D for the combination responses. There are several coded variable values (X_1, X_2, X_3) for every model, respectively, corresponding to their maximum D_{BBD} (1), D_{CCF} (1), D_{CCC} (1), and D_{FFD} (1), respectively. Generally, their response surfaces and contour plots of D functions could be represented and visualized for the choice of optimization conditions within the selected ranges (not shown).

The optimal chromatograms of four models using the desirability functions were also represented by another six experiments in Figure 12. Baseline separation of the analytes could be obtained during 5 min with a resolution more than 1.5, using the optimal condition with a coded values of (0, 0, 0). The results from the experiments using the optimal condition and testing conditions were compared to the predicted values from four models Table 7). Close agreement using BB, CCF, CCC, and FF design models could be found in most cases between observed and predicted responses.

CONCLUSIONS

The simultaneous evaluation of the experimental variables was carried out for the separation of typical isomers by means of BB, CCF, CCC, and FF design models with efficient estimation of the first and second order coefficients. For four designs, all design points can be sure to fall within a safe operating zone, which have similar results for optimization and prediction in our studies. An appropriate use of experimental designs 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.[27,28] Compared to empirical methods, chemometrics can greatly simplify the optimization procedure finding the appropriate experimental conditions.

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