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A SIMPLE PROCEDURE TO IMPROVE THE ANALYTICAL PERFORMANCE OF FLOW INJECTION SYSTEMS

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ABSTRACT

The optimisation of flow injection analysis (FIA) manifolds is usually performed with the univariate procedure, although in many cases they do not allow to find optimum conditions due to the interaction errors caused by the interdependence between variables. However, many authors prefer the univariate methodology instead of the more correct multivariate methods, probably due to the higher theoretical difficulty of the latter. In this work we demonstrate that by applying a very simple multivariate procedure, the results obtained are better than those obtained by using the univariate optimisation. To illustrate this, the conditions for a FIA manifold used for the

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determination of phosphates (P-PO_4^{3-}) in natural water has been studied by using two different procedures: the classical univariate method and a second one based on experimental design. Experimental design procedures take into account the interactions between the variables optimised, which are ignored by univariate procedures. The classical univariate procedure found experimental conditions that produced less precise and accurate results ($RSD = 13.6\%$; $\varepsilon_r = 11.1\%$) than those obtained when the performance of FIA system was studied with the simpler method based on 2^n experimental design ($RSD = 4.2\%$; $\varepsilon_r = 6.3\%$). Sensitivity was also improved and lower limits of detection were obtained with experimental design (river water: 5.6 $\mu g\ L^{-1}$; seawater: 5.9 $\mu g\ L^{-1}$) than with the univariate methodology (river water: 87.1 $\mu g\ L^{-1}$; seawater: 88.9 $\mu g\ L^{-1}$). Besides, the use of experimental design allowed reducing both waste production and cost of analysis.

Key Words: FIA; Optimisation; Chemometrics; Experimental design; Phosphate; Natural waters; Seawater

INTRODUCTION

The term optimisation has been used in analytical chemistry for a long time. The aim of any optimisation process is to find the conditions that produce the best performance of the system. Although the concept of optimum is clear and unique, the terms optimum and optimisation are often applied to the process of seeking the best operational conditions within a range of several variables that must be previously selected, even when optimum may be not included within this range. When performed, this task has been traditionally carried out by monitoring the influence of one variable at a time on an experimental response. The main disadvantages of one factor optimisation are that it is time consuming and that there is a risk of misinterpreting the results if important interactions between factors are present.[1] The latter reason is especially important if the optimisation of the chemical and/or physical conditions of a flow injection analysis (FIA) manifold is performed, due to the high interdependence presented by variables as sample loop and reaction coil lengths, flow rate, and chemical composition of carrier and reagent solutions. In general, it is thought that the need and advantages of using multivariate optimisation procedures is a well-known issue. However, a simple bibliographic revision demonstrates that it is not true. Searching in some of the journals on Analytical Chemistry with higher impact (in alphabetical order: Analusis, Analyst, Analytica Chimica Acta, Analytical Chemistry, Fresenius Journal of Analytical Chemistry and Química Analítica) we could find up to 57 papers, published during the year 2000, which studied the optimisation of a flow injection manifold. Among them, 47 (82.5%) used the univariate method, while only 10 (17.5%) applied some multivariate strategy. Thus, it follows the necessity of emphasising the convenience of using chemometrics as a tool for the correct optimisation of FIA systems.

Two multivariate strategies for optimising chemical and physical performance of a FIA system can be applied. On the one hand, when the experimenter has a previous knowledge about the optimum conditions, simultaneous designs can be applied, factorial design being the method most frequently used.[2,3] Factorial designs are based on the study of all the combinations between the factors controlling the response of one experimental system, allowing studying, simultaneously, the influence of each variable and their interactions.[4,5] They identify which variables, and corresponding interactions, are responsible for the analytical response of the system. Besides, the ranges where each variable give better response can be established. The simplest factorial design is the factorial design at two levels, where two different values are assigned to each factor: a low level and a high level. In this case, the response is accepted to be almost linear within the range studied. Thus, they are suitable to estimate first order interaction effects, but they fail when additional effects such as second order effects are significant, and then, a star design is usually employed.[1] On the other hand when the experimenter has no or a limited knowledge about how far the starting conditions are from the optimum region, sequential designs can be applied, simplex optimisation (including modified versions) being the most notable design.[6,7]

The significantly low use of multivariate optimisation procedures may be due to the higher conceptual difficulty of these methodologies, if compared with univariate procedures. This difficulty can be overcome if the very simple experimental design 2^n is used. In many cases, the application of this design does not allow finding the optimum conditions of the system but, as we demonstrate, it gives better results than the classic univariate procedure. In the present work the molybdenum blue spectrophotometric method, currently adopted as a standard method,[8] has been applied to the continuous determination of phosphate in natural waters, including seawater, by using flow injection analysis. Influence of chemical and physical factors has been studied using both the univariate method and the proposed 2^n factorial design. The two methods have been compared in terms of sensitivity, precision, accuracy, economical cost and waste production in the determination of phosphate in real samples.

EXPERIMENTAL

Apparatus

The flow injection manifold is shown schematically in Fig. 1. It consisted on a Minipuls 3 four-path peristaltic pump (Gilson, France) equipped with Tygon tubing, which was used to manipulate the flows of reagent and sample solutions, a Model 5041 six-port sample injection valve (Rheodyne, USA) and a Lambda 11 UV/VIS spectrophotometer (Perkin-Elmer, Germany) equipped with a quartz flow cell with a 50 mm pathlength (Starna, UK). Transport lines and reaction coils were made using 0.8 mm id PTFE tubing. Connections were made of polypropylene (Omnifit, UK).

Reagents and Solutions

Stock solutions of 200 mg L^{-1} P-PO_4^{3-} were prepared by dissolving the necessary amount of potassium dihydrogen phosphate (KH_2PO_4) of analytical grade (Merck, Germany). Further dilutions were made daily as required. For all the studies, a 200 µg L^{-1} P-PO_4^{3-} solution was used. For the preparation of the FIA reagent, three different solutions were previously prepared as follows: 1×10^{-1} mol L^{-1} L(+)-ascorbic acid ($C_6H_8O_6$) of analytical grade (Panreac, Spain); 3.2×10^{-2} mol L^{-1} solution of ammonium heptamolybdate-tetrahydrate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) of analytical grade (Merck, Germany) and 2.5 mol L^{-1} solution of sulphuric acid (H_2SO_4) of analytical grade (Merck, Germany). Then, 30 mL of L(+)-ascorbic acid, 15 mL of ammonium heptamolybdate-tetrahydrate and 50 mL of sulphuric acid were mixed and diluted to 100 mL with bidistilled water to prepare the concentrated FIA reagent, which will be diluted as required.

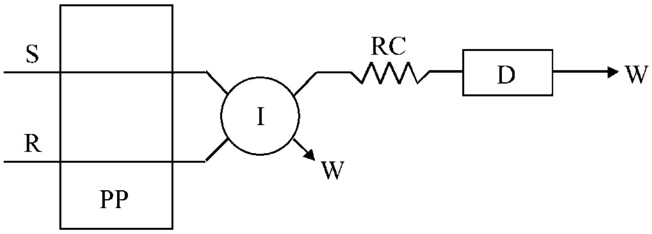

Figure 1. Flow injection manifold. S, sample solution; R, reagent solution; PP, peristaltic pump; I, injection valve; RC, reaction coil; D, detector; W, waste.

Procedure

For the study of the analytical performance of the FIA system, both the absorbance of the obtained phosphomolybdic complex and the precision of its measurement were used as the experimental variables to be maximized. Thus, a response function, *R.F.*, was calculated for each experiment as:

$$R.F. = 0.8 \cdot A + 0.2 \cdot \frac{1}{RSD}$$

where A is the absorbance of the complex (with a weight of 80% in *R.F.*) and RSD is the relative standard deviation of the measurement (with a weight of 20% in *R.F.*).

The study was carried out by varying the reaction coil length (0.5–4 m), sample volume (66–1256 μL), flow rate (0.4–3.4 mL min^{-1}) and concentration of the FIA reagent. The influence of reagent concentration was studied by diluting the concentrated FIA reagent described above, to keep the ratios of the compounds contained in the reagent solution.

Two different optimisation methods were used and compared. On the one hand, the classical univariate method was applied by varying one factor while the others were kept constant. On the other hand a second procedure, based on the use of a factorial design at two levels and four factors, was used. In this method, a set of experiments was carried out in a systematic way in order to predict both the best operational conditions and the interaction effects between factors. High and low levels of each factor are shown in Table 1. For the sake of comparison, the levels were chosen in a wide space to study the same range than that studied with univariate procedure. By using this approach we assume linearity between boundary conditions, even in such a wide range. The runs were carried out in random order. All experimental data were obtained as the average of, at least, three sample injections. Calculations were done using Statgraphics Plus, version 7 (Statistical Graphics Corporation, USA).

Table 1. Factors and Levels for the 2^4 Factorial Design

Factor	Low Level (−)	High Level (+)
Reagent flow rate, mL min^{-1}	0.6	3.2
Sample volume, μL	66	1256
Reactor length, m	0.5	4
Reagent dilution (n:100)	n = 2	n = 25

RESULTS AND DISCUSSION

Univariate Method

All the factors controlling the behaviour of the FIA system were firstly studied by using the univariate method. The reagent stream flow rate was varied from 0.4 to 3.4 mL min^{-1}. As can be seen in Fig. 2, the response function, *R.F.*, becomes maximum at a flow rate of 2 mL min^{-1}, where optimum value was chosen. Although higher absorbances were obtained at lower flow rates, they produced lower *R.F.* because of the instability of the signal recorded. At optimum flow rate, the influence of the injected sample volume was investigated within the range 66–1256 μL. Results are shown in Fig. 3. As can be observed, *R.F.* increased rapidly with sample volume up to 650 μL, and then it decreased, mainly due to the higher instability of the signal. The highest absorbance was recorded for 1256 μL, but involved a very low reproducibility of the measurement due to the poor mixing between

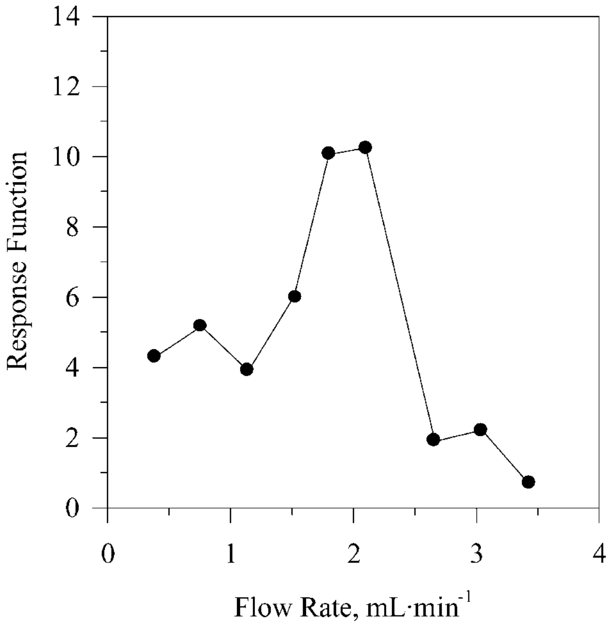

Figure 2. Effect of flow rate on the Response Function. Sample volume: 566 μL; reaction coil: 3 m; reagent dilution: n = 10.

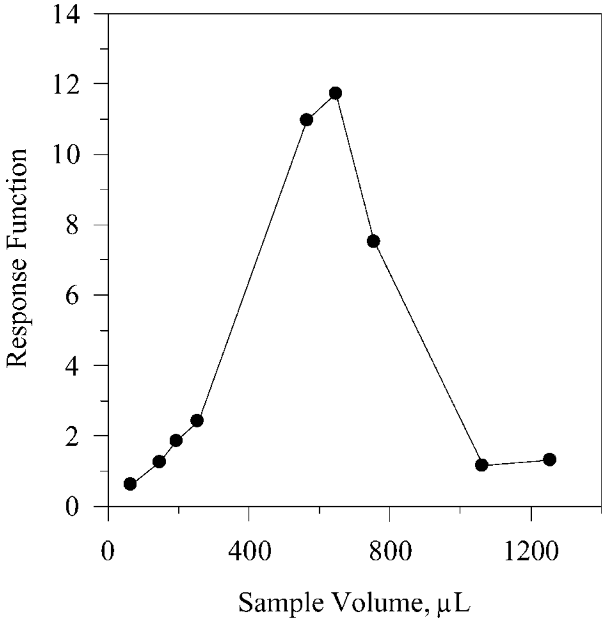

Figure 3. Effect of injected sample volume on the Response Function. Flow rate: 2 mL min^{-1}; reaction coil: 3 m; reagent dilution: n = 10.

sample and reagent at present conditions. As optimum, a 650 µL sample loop was selected. Figure 4 shows the variation of *R.F.* with the length of reaction coil, which was varied within the range 0.5–4.0 meters. An optimum value, when the mixing effect was higher than dispersion effect was reached at 3 m length. Finally, the effect of the concentration of FIA reagent was studied by diluting the stock solution described before. With this aim, a certain volume (n mL) of the concentrated reagent was diluted to 100 mL. Thus the concentration was varied from the lowest value of 1:100 ($n = 1$ in Fig. 5) to the highest value of 25:100 ($n = 25$ in Fig. 5). The spectrophotometric signal increased with reagent concentration, but also imprecision increased. As can be seen in Fig. 5, the best *R.F.* was obtained for a dilution factor of $n = 20$.

By using the univariate method, the best performance of the FIA system used for the determination of P-PO_4^{3-} was achieved in the following conditions: flow rate: 2 mL min^{-1}; reaction coil length: 3 m; sample volume: 650 µL and reagent dilution 20:100.

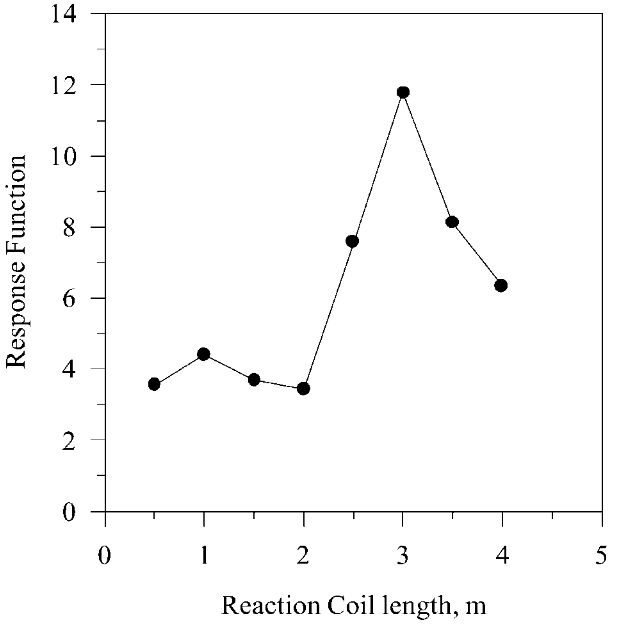

Figure 4. Effect of reaction coil length on the Response Function. Flow rate: $2\,\mathrm{mL\,min^{-1}}$; sample volume: $650\,\mu\mathrm{L}$; reagent dilution: $n = 10$.

FACTORIAL DESIGN

Once the system was studied by using the univariate method, a second procedure based on a 2^4 factorial design was applied. The levels and factors chosen are shown in Table 1. Runs were performed in random order, and the increment of absorbance (sample minus blank) was calculated as an average of, at least, three replicates. The design matrix and results, expressed as the response function, *R.F.*, introduced above, are shown in Table 2. For every factor and interaction, the F-ratio for the null hypothesis (H_0, defined as the no significance of the factor or interaction) and the corresponding P-value were successively calculated by the software used. Interactions of order higher than 3 were not taken into account. The analysis of variance (ANOVA) of the data for the two-level four-factor factorial design is shown in Table 3. The best model of the experimental data set was obtained by calculating it with the principal effects and interactions of two and three factors having values of $P < 0.05$. For this model, the principal and interaction effects were calculated and are shown in Table 4. The model has a

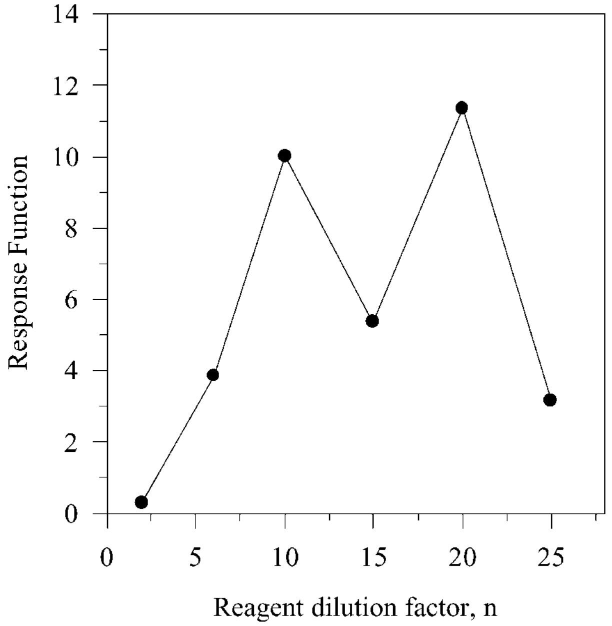

Figure 5. Effect of the dilution of the FIA reagent on the Response Function. Dilution was n:100 with respect to the stock solution described in the text. Flow rate: 2 mL min^{-1}; sample volume: 650 μL; reaction coil: 3 m.

correlation coefficient (adjusted R^2), of 0.9970, explaining 99.70% of the variance in the response values.

The data of Tables 3 and 4 show that all the principal factors are significant in the range studied, their individual effect being in agreement with the trends observed in the results obtained in the univariate optimisation. Besides, up to three interactions of two factors and one interaction of three factors are also significant. The contribution of these interactions to the analytical signal is not considered if univariate optimisation is performed, causing errors in the selection of best experimental conditions.

As mentioned before, we have used a wide factor space with the aim of a correct comparison between the results obtained with univariate and factorial design procedures. To confirm the correct use of the two-level factorial design in such a wide range, we included two central points in the experimental design to check the curvature of the model obtained. The results of the ANOVA showed the no significance of the curvature (P-value = 0.06), indicating the correction of the first order model.

Table 2. Order of Runs and Response Function (R.F.) for the 2^4 Factorial Design

	Factor				
Run	A	B	C	D	R.F.
1	+	+	+	−	0.246
2	+	−	+	−	0.000
3	−	+	+	−	0.284
4	−	−	+	−	2.458
5	−	+	+	+	17.778
6	−	−	+	+	6.124
7	+	−	+	+	1.654
8	+	+	+	+	12.234
9	+	−	−	−	0.000
10	+	+	−	−	0.116
11	+	−	−	+	0.000
12	+	+	−	+	2.060
13	−	−	−	+	0.602
14	−	−	−	−	0.983
15	−	+	−	−	0.000
16	−	+	−	+	3.946

A: Reagent flow rate.
B: Sample volume.
C: Reactor length.
D: Reagent dilution.

By using the factorial design, the best performance of the FIA system used for the determination of P-PO_4^{3-} was achieved in the following conditions: flow rate: 0.6 mL min^{-1}; reaction coil length: 4 m; sample volume: 1256 μL and reagent dilution 25:100.

APPLICATION

Once studied the experimental conditions for the determination of P-PO_4^{3-} by using both methods described, we carried out the determination of P-PO_4^{3-} in three samples of natural water. Two of them were taken in two different rivers of the province of Cádiz (SW of Spain). The first sample was taken in the Guadalete River, and near the city of Jerez (about 200,000 inhabitants). The second sample was taken in the Guadalcacín River, in a less polluted area. The third one was a sample of seawater from the Bay of Cádiz spiked with 80 μg L^{-1} P-PO_4^{3-}. In this case, to correct the effect of the

Table 3. ANOVA for the 2^4 Factorial Design

Effect	F-Ratio	P-Value
A	204.50	0.0438
B	501.64	0.0280
C	888.83	0.0211
D	1320.57	0.0173
AB	1.10	0.4770
AC	68.08	0.0757
AD	67.85	0.0759
BC	202.14	0.0441
BD	752.76	0.0229
CD	697.36	0.0238
ABC	1.91	0.3927
ABD	28.06	0.1172
ACD	28.24	0.1168
BCD	260.20	0.0389

A: Reagent flow rate.
B: Sample volume.
C: Reactor length.
D: Reagent dilution.

Table 4. Principal and Interaction Effects, and Their Standard Errors, for 2^4 Factorial Design

	Estimated Effect	Standard Error
Average	3.0303	0.3671
A	−1.9829	0.7342
B	3.1057	0.7342
C	4.1340	0.7342
D	5.0389	0.7342
BC	1.9715	0.7342
BD	3.8044	0.7342
CD	3.6617	0.7342
BCD	2.2367	0.7342

A: Reagent flow rate.
B: Sample volume.
C: Reactor length.
D: Reagent dilution.

change in refractive index on the measured signal a clean sample of the same seawater was used as a blank.

All the samples were previously analysed by using the molybdenum blue method in batch,[8] and then, the results obtained were compared with those obtained with the two FIA procedures. Results of calibration plots (constructed of five points each) and analysis of the samples (average of five replicates) are shown in Table 5. Results of calibration plots show that the use of factorial design procedure produced a better regression coefficient, an improvement in the slope of the curve and, as a consequence of that, lower limit of detection.

On the other hand, the results obtained for the analysis of $P\text{-}PO_4^{3-}$ in natural waters turned out to be more precise and accurate when the experimental condition were improved by factorial design. Thus, the average

Table 5. Results of the Calibration Plots and Analysed Samples

	Calibration Plots			
	River Water		Seawater	
	Univariate	Factorial Design	Univariate	Factorial Design
Intercept	0.300	0.299	0.168	0.187
Slope	$1.47 \cdot 10^{-4}$	$6.23 \cdot 10^{-4}$	$1.70 \cdot 10^{-4}$	$4.84 \cdot 10^{-4}$
r^2	0.998	0.999	0.994	0.996
LOD	87.1 $\mu g\,L^{-1}$	5.6 $\mu g\,L^{-1}$	88.9 $\mu g\,L^{-1}$	5.9 $\mu g\,L^{-1}$

	Analysis of Samples[1]				
		Univariate		Factorial Design	
Sample	Known	Measured	ε_r	Measured	ε_r
Guadalete river	1378.0 ± 19.4[2]	1130.6 ± 47.6	−18.0%	1548.6 ± 47.6	12.4%
Guadalcacín river	238.3 ± 5.2[2]	212.2 ± 23.2	−11.0%	245.5 ± 3.7	3.0%
Seawater	80[3]	83.5 ± 12.0	4.4%	77.3 ± 6.1	−3.4%

LOD: Limit of detection = 3·(standard deviation of intercept)/slope.

ε_r: relative error.

[1] Concentration ± standard deviation ($\mu g\,L^{-1}$).

[2] Measured in batch.

[3] Added amount.

relative standard deviations were 13.6% and 4.2% for univariate and multivariate procedures, respectively; while the average relative errors were 11.1% and 6.3% for univariate and experimental design procedures, respectively.

The accuracy of the results was tested by applying the paired *t*-test, which confirmed that the results obtained by the two FIA methods did not differ significantly at the 0.05 level of the results used as reference. The precisions obtained by the two FIA methods were tested by applying the two-tailed *F*-test. The precision obtained with the FIA method studied with experimental design did not differ significantly at the 0.05 level of the precision of the method of reference, but in the case of the FIA method optimised with the univariate method, the precision differed significantly at the 0.05 level (4 degrees of freedom), due to the more inadequate selection of optimum conditions.

Finally, both methods were compared in terms of cost (reagents consumption) and waste production. Thus, the amount of reagent needed in factorial design conditions was 62.5% lower than univariate conditions. Besides, the multivariate approach generated a waste volume 70% lower than univariate procedure.

From the results of this work it follows that, when a FIA system must be used, a very simple procedure, based on the use of a two-level factorial design, provides better results that the more traditional one based on the use of univariate optimisation. The improvements include simplicity, sensitivity, precision and accuracy of the analysis, together, in this case, with lower cost and reduction of waste production.

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REFERENCES

1. Araujo, P.W.; Brereton, R.G. TRAC-Trend. Anal. Chem. **1996**, *15*, 63.
2. Duarte, M.M.M.B.; Neto, G. de O.; Kubota, L.T.; Filho, J.L.L.; Pimentel, M.F.; Lima, F.; Lins, V. Anal. Chim. Acta. **1997**, *350*, 353.
3. Steijger, O.M.; den Nieuwenboer, H.C.M.; Lingeman, H.; Brinkman, U.A.Th.; Smilde, J.J.M. Anal. Chim. Acta. **1996**, *320*, 99.
4. Montgomery, D.C. *Design and Analysis of Experiments*; 4th Edition, John Wiley & Sons: New York, 1997.
5. Bayne, C.K.; Rubin, I.B. *Practical Experimental Designs and Optimization Methods for Chemists*; VCH Publishers, Inc.: Florida, 1986.

6. Gine, M.F.; Tuon, R.L.; Cesta, A.A.; Packer, A.P.; Reis, B.F. Anal. Chim. Acta. **1998,** *366*, 313.
7. Tovar, A.; Moreno, C.; Mánuel-Vez, M.P.; García-Vargas, M. Química Analítica **2001**, *20*, 37.
8. *Standard Methods for the Examination of Water and Wastewater*; 17th edn., American Public Health Association: Washington D.C., 1989.

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