Application of Experimental Design to the Development of a Multicomponent Derivative Spectrophotometric Method: Simultaneous Determination of Sulfamethoxazole and Trimethoprim

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A central composite design is used for the development of a spectrophotometric method for the simultaneous determination of sulfamethoxazole and trimethoprim. The method involves multicomponent second-derivative spectrophotometry in the ultraviolet range with a photodiode-array spectrophotometer. Accuracy, precision, linearity and other figures of merit are verified in connection with the application of the proposed method to pharmaceutical quality control. The influence of such variables as wavelength range, derivative order and solvent are discussed.

Keywords: *Multicomponent derivative spectrophotometry; diode array; experimental design; sulfamethoxazole; trimethoprim*

Spectrophotometric methods of analysis have experienced a considerable evolution in the last 20 years. From an early straightforward approach that involved just the measurement of absorbances and their correlation with the concentration of the analyte sought, many instrumental and computer-assisted techniques have evolved, such as numerical data handling, digital filtering, derivative spectrophotometry, matrix multicomponent methods, Fourier-transform spectrophotometry and statistical handling of the results. This has been possible owing to the widespread use of microprocessors and computers in connection with the instruments, as the theoretical basis of these methods was already known.

Along with this trend, more emphasis is being put on the use of rational strategies for the optimization and validation of analytical methods. When dealing with real-world samples, there exists the possibility of multiple interferences and matrix effects. In order to detect these effects and to assess their significance, a considerable number of experiments would be necessary. Extensive experimentation, however, is expensive, hence it is necessary to minimize the number of experiments and to maximize the information extracted from them. This has led to an increasing use of statistically-based techniques, usually called experimental designs.[1,2]

In this paper, experimental designs are applied to the development and validation of a spectrophotometric multicomponent method for the simultaneous determination of two pharmaceutical substances, sulfamethoxazole and trimethoprim, in tablets by second-derivative multicomponent spectrophotometry in the ultraviolet (UV) region.

Sulfamethoxazole [N^1-(5-methylisoxazol-3-yl)sulfanilamide (SMX)] and trimethoprim [5-(3,4,5-trimethoxybenzyl)pyrimidine-2,4-diamine (TMP)] are substances often found associated in pharmaceutical dosage forms used as antibacterials. Therefore, the determination of the two drugs is a frequent analytical problem in quality control of the pharmaceutical industry.

The simultaneous determination by UV spectrophotometry with direct measurements or by a zero-order multicomponent method cannot be performed satisfactorily because of the extensive overlap presented by the spectra. Both drugs can be determined simultaneously by high-performance liquid chromatography (HPLC),[3] which is the technique specified by the United States Pharmacopeia (USP),[4] colorimetry[5] or nuclear magnetic resonance spectrometry.[6]

Derivative spectrophotometry[7,8] is a useful technique for the suppression of additive interferences, and has been used extensively for simultaneous determinations of substances in mixtures.[9–11] One of the extensions of derivative spectrophotometry is the application of multicomponent methods based on the resolution of simultaneous equations, involving measurements at as many wavelengths as components exist.

Korany *et al.*[12] presented a two-wavelength first-derivative technique for the simultaneous determination of the two drugs. In our experience, however, derivative techniques such as this tend to yield results that are strongly dependent on the noise level of the instrument. One of the problems arises from the use of the minimum number of wavelengths necessary for the resolution of the equations system, as compared with overdetermined systems where all available information is used. Besides, interference from excipients, when applying the technique to real-world samples, may not be reduced enough by a first derivative. On the other hand, multicomponent derivative methods based on overdetermined systems allow the use of low-order derivatives, thereby attaining higher signal-to-noise (S/N) ratios, especially if the measurement is carried out by using a photodiode-array spectrophotometer.[13] The very low noise level attained when using this kind of detection, as compared with conventional wavelength scanning, results in a more accurate solution of the system of equations.

The principle on which these methods rely is the solution of the set of simultaneous equations derived from Beer's law:

$$A = \Sigma\, k_i\, c_i$$

or, in matrix notation:

$$\boldsymbol{A} = \boldsymbol{kC}$$

The solution is greatly simplified by the use of matrix calculations involving the generalized inverse matrix. In the instrument used in this work, weighted multiple linear regression is employed for this purpose. The computer algorithm used resides in read-only memory and is not accessible to the user.

Experimental

Instruments

The instrument used was a Hewlett-Packard (Palo Alto, CA, USA) 8450A double-beam photodiode-array spectrophotometer with stoppered 1 cm silica cells. This instrument has built-in software capabilities that permit various spectral

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processings as well as derivative and multicomponent calculations. Graphic plots were recorded with a Hewlett-Packard 7225B digital plotter.

The HPLC determinations were performed with a Shimadzu (Kyoto, Japan) chromatograph composed of an LC-9A pump system and an SPD-M6A photodiode-array absorptiometric detector. Injections were made by means of a Rheodyne (Cotati, CA, USA) 7125 injector with a 20 μl loop in a Hewlett-Packard 20 cm × 4.6 mm stainless-steel column packed with 10 μm LiChrosorb RP-18. The other conditions were set according to the technique described in the USP.[4]

Instrumental Settings

Absorbance measurements were made at 10 s intervals. This allowed 100 spectra to be recorded and averaged each time, thereby ensuring a high S/N ratio. The measurement wavelength range was always 200–800 nm in the instrument used, with a nominal bandwith of 1 nm in the 200–400 nm region and 2 nm in the 400–800 nm region. However, the wavelength range taken into account for the multicomponent calculations was restricted according to the results of previous experiments (described under Results and Discussion). Derivative and multicomponent calculations were always performed in 'post-run' fashion in this instrument.

The following settings were used: zero order, 240–340 nm; first derivative, 250–320 nm; and second derivative, 250–320 nm.

Materials

The SMX and TMP were of pharmaceutical grade and were used as received. All other materials were of analytical-reagent grade.

The SMX–TMP tablets (400 mg of SMX and 80 mg of TMP per tablet, or twice those amounts for 'strong' formulations) used in the suitability tests were of several brand names.

Procedure

Standard solutions containing the amounts of SMX and TMP indicated in each instance were prepared in 0.1 mol l^{-1} methanolic hydrochloric acid.

Second-derivative spectra of the standard solutions were measured and stored, and the respective exact concentrations assigned.

For analytical applications, 20 tablets were reduced to fine powder after determination of the average mass. An amount of powder equivalent to 400 mg of SMX was weighed into a 100 ml stoppered calibrated flask, and 50 ml of 0.1 mol l^{-1} methanolic hydrochloric acid added. The flask was shaken for 15 min, and the solution was made up to volume with the same solvent. After filtration through Whatman No. 41 filter-paper, the first 15 ml were discarded, and 1 ml of the filtrate was diluted to 50 ml with the same solvent.

Standard solutions containing 80 mg l^{-1} of SMX and 16 mg l^{-1} of TMP, respectively, in 0.1 mol l^{-1} methanolic hydrochloric acid were also prepared and analysed.

The second-derivative spectra for the unknowns were recorded and the concentrations of SMX and TMP were determined in the multicomponent mode with the instrumental settings indicated above.

The HPLC determinations were carried out as directed in the USP XXII.[4]

Results and Discussion

Spectra and Order of Derivation

Zero-order spectra for SMX and TMP, as well as their first and second derivatives, are shown in Figs. 1–3. The extreme spectral overlap in the absorption spectrum, which hinders direct multicomponent determinations, and how this overlap is considerably diminished in second-derivative spectra, can be seen. The enhancement of the characteristic spectral features attained in the second-derivative spectra is reflected in the two figures of merit provided by the instrument during multicomponent determinations, namely, the relative fit error

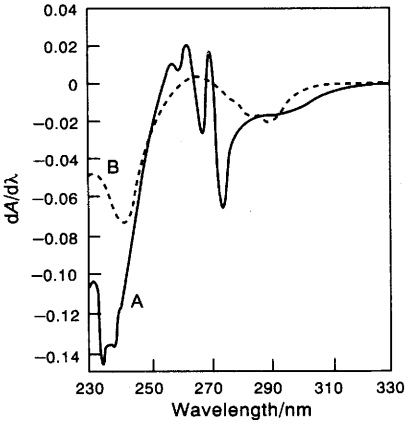

Fig. 2 First-derivative spectra of A, sulfamethoxazole 84.2 mg l^{-1} and B, trimethoprim 18.5 mg l^{-1} in 0.1 mol l^{-1} methanolic hydrochloric acid

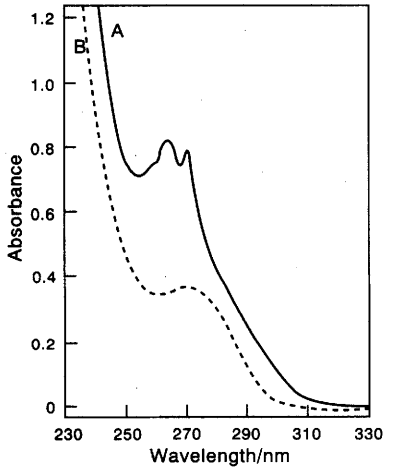

Fig. 1 Zero-order absorption spectra of A, sulfamethoxazole 84.2 mg l^{-1} and B, trimethoprim 18.5 mg l^{-1} in 0.1 mol l^{-1} methanolic hydrochloric acid

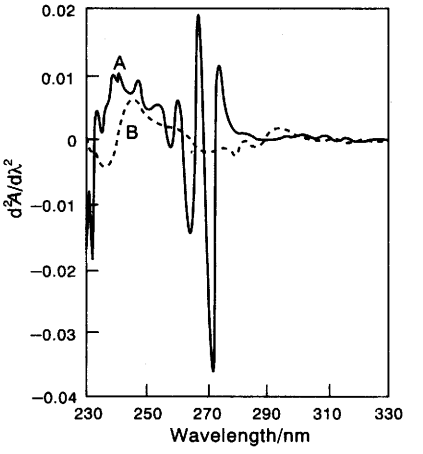

Fig. 3 Second-derivative spectra of A, sulfamethoxazole 84.2 mg l^{-1} and B, trimethoprim 18.5 mg l^{-1} in 0.1 mol l^{-1} methanolic hydrochloric acid

(RFE) and the independence of standards (IOS). The second derivative also yields more exact and precise results as shown in the tables below.

Influence of Acidity

The zero-order and first- and second-derivative spectra for the two drugs, in methanol containing various concentrations of hydrochloric acid (0.001, 0.01, 0.1 and 1 mol l^{-1}) and potassium hydroxide (0.001, 0.01 and 0.1 mol l^{-1}), were scanned. Very few spectral changes were observed for TMP, while a very strong effect was observed for SMX. Some of these spectra can be seen in Fig. 4, showing the dramatic effect of acidity on the characteristics of the absorption spectrum for SMX. In the zero-order spectrum the absorbance of the peak at 270 nm decreases radically, and a fine structure, with two different peaks, appears with increasing acidity. However, the effect on amplitude is less marked in the first-derivative spectrum and even less in the second-derivative one.

In order to assess the effect of acidity changes in the vicinity of the nominal concentration of 0.1 mol l^{-1} methanolic hydrochloric acid, spectra were obtained for a 80 mg l^{-1} solution of SMX prepared in 0.08, 0.1 and 0.12 mol l^{-1} methanolic hydrochloric acid, *i.e.*, a ±20% variation in the nominal 0.1 mol l^{-1} value. Despite the important effect on the zero-order spectrum [Fig. 5(*a*)], for this range of variation, the effect in the first-derivative spectrum is small [Fig. 5(*b*)] and is virtually negligible for the second-derivative spectrum (not shown). Two practical conclusions can be drawn from this, *i.e.*, that there is one more reason for not using zero-order or first-derivative spectra, because of their dependence on acidity, and that in all instances the same batch of methanolic hydrochloric acid should be used for the preparation of all solutions involved, in order to ensure maximum reliability in the results.

Optimum Wavelength Interval

For this determination, a synthetic mixture with known concentrations of the two drugs was analysed in the multicomponent quantitative mode *versus* separate standards containing the same concentrations of the respective drugs, and the wavelength range was varied in 5 nm increments. The percentage recovery was calculated and the RFE and IOS were monitored for zero order, first and second derivatives.

The best results were obtained when the following wavelength ranges were used: zero order, 240–340 nm; first derivative, 250–320 nm; second derivative, 250–320 nm. These ranges were selected chiefly on the basis of the recoveries found, with the RFE and IOS values as secondary criteria.

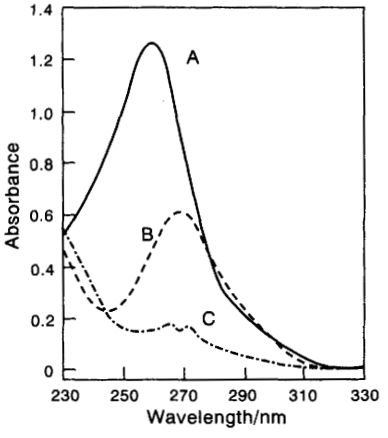

Fig. 4 Effect of acidity on absorption spectra of sulfamethoxazole solutions (16.8 mg l^{-1} in methanol). A, 0.1 mol l^{-1} HCl; B, 0.01 mol l^{-1} HCl; C, 0.1 mol l^{-1} KOH

Linearity and Interactions

As a part of the validation of the analytical method, we must calculate simultaneously the linearity of the calibration for each analyte as well as possible interactions between the two analytes and between analytes and matrix. For this purpose, a central composite design[1,2] was chosen.

The main advantage of this kind of experimental design, as compared with previously used factorial designs, is that less experimental units are required. Assuming a full second-order model, for a 3^k-factorial design, in this instance $3^3 = 27$ experiments would be necessary, as compared with just 15 experiments necessary to obtain the same information with a central composite design.

In this design, SMX and TMP solutions, as well as an extract of a typical pharmaceutical excipient (containing starch, lactose, carboxymethylcellulose, magnesium stearate and sodium lauryl sulfate in appropriate amounts), were mixed in various proportions, in the range 60–140% of the analytical proportions. In order to establish the nominal analytical concentrations, the composition of a 'typical' tablet in this experiment was chosen to be 400 mg of SMX, 80 mg of TMP and 180 mg of excipient, and the analytical concentrations of SMX and TMP to be 80 and 16 mg l^{-1}, respectively. Fifteen different mixtures were evaluated in this way by using zero-order and first- and second-derivative multicomponent measurements. The results are presented in Table 1.

The recoveries (100 × found/added) for all measurements, the average recovery (n = 15) and relative standard deviation (s_r, %) were calculated. The results are presented in Table 2.

Data were later processed by means of the SPSS/PC+ statistical package, version 2.0 (SPSS, Chicago, IL, USA).[14]

Correlation calculations were performed between all variables, using the concentrations 'found' for SMX and TMP,

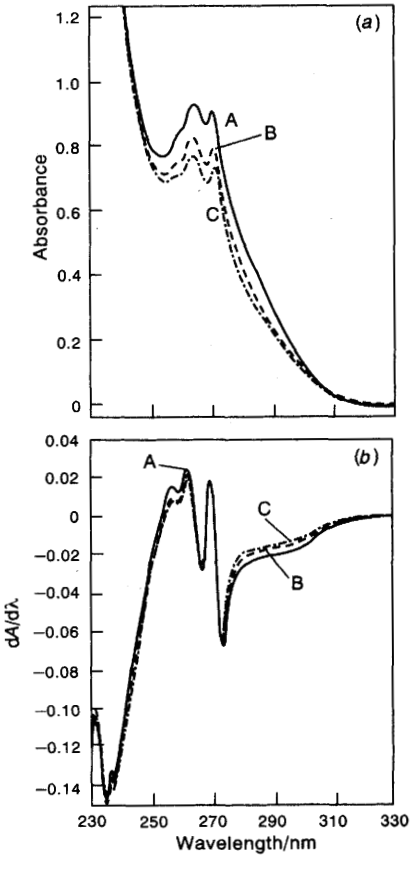

Fig. 5 Effect of slight acidity changes on (*a*) the zero-order spectrum, and (*b*) first-derivative spectrum of a sulfamethoxazole methanolic solution (84.2 mg l^{-1}). A, 0.08 mol l^{-1} HCl; B, 0.1 mol l^{-1} HCl; C, 0.12 mol l^{-1} HCl

Table 1 Central composite design used for studying linearity and interactions. Added and found concentrations are in mg l^{-1}. Zero-order and first and second derivative results are shown. The values in the excipients (Ex) column correspond to the amounts of excipient extract present, expressed as relative with respect to the nominal amount (180 mg of excipient per tablet)

	Added concentrations			Found concentrations					
				Zero order		First derivative		Second derivative	
Solution no.	[SMX]	[TMP]	[Ex]	SMX	TMP	SMX	TMP	SMX	TMP
1	79.44	16.40	100	79.2	16.6	80.9	16.3	78.8	16.4
2	47.66	16.40	100	49.1	16.0	47.0	16.4	47.6	16.2
3	111.22	16.40	100	114.8	15.9	111.1	16.7	110.3	16.3
4	79.44	9.84	100	79.0	9.9	80.5	9.7	78.6	9.8
5	79.44	22.96	100	79.5	23.3	80.1	23.1	79.2	22.9
6	79.44	16.40	0	77.3	16.8	79.3	16.4	78.0	16.4
7	79.44	16.40	200	78.4	16.7	79.9	16.4	79.0	16.4
8	63.55	13.12	50	63.1	13.1	64.2	13.0	63.1	13.2
9	63.55	19.68	50	66.9	19.3	65.6	19.6	63.2	19.6
10	95.33	19.68	50	93.0	20.0	94.6	19.6	94.6	19.4
11	95.33	13.12	50	91.4	13.8	94.3	13.2	94.6	13.0
12	63.55	13.12	150	65.2	12.9	63.9	13.2	63.1	13.2
13	63.55	19.68	150	62.7	19.6	63.8	19.5	64.1	19.5
14	95.33	19.68	150	92.4	19.7	93.1	19.6	93.9	19.2
15	95.33	13.12	150	92.4	13.7	94.7	13.2	95.0	13.0

Table 2 Recoveries of the multicomponent determination performed on artificial mixtures, and results of multi-linear regression

	Zero order		First derivative		Second derivative	
	SMX	TMP	SMX	TMP	SMX	TMP
Average recovery*	0.997	1.009	1.007	0.997	0.993	0.997
s†	0.028	0.023	0.023	0.009	0.006	0.009
s_r‡ (%)	2.768	2.288	2.236	0.900	0.593	0.904
A§	3.15	0.33	2.57	−0.10	0.73	0.24
B§	0.9553	0.9880	0.9737	1.0029	0.9836	0.9818
r¶	0.9878	0.9925	0.9921	0.9989	0.9996	0.9987

* Average recovery of 15 measurements.
† s = standard deviation of the recovery values.
‡ s_r = relative standard deviation of the recovery values.
§ A and B = regression coefficients; found concentration (mg l^{-1}) = A + B × added concentration (mg l^{-1}).
¶ r = partial correlation coefficient.

Table 3 Results of replicate analyses (n = 5) of two brands of commercial tablets by the USP XXII and multicomponent (MC) methods (zero order, first and second derivative). Results in mg per tablet

		Sulfamethoxazole			Trimethoprim		
Method	Brand	MC	s_r* (%)	RD† (%)	MC	s_r (%)	RD (%)
Zero	A	454.7	9.0	15.6	65.1	12.2	−17.4
	B	825.9	1.9	4.0	154.9	1.9	−2.4
First	A	379.9	1.7	−3.4	80.2	1.2	1.9
	B	818.2	2.0	3.0	160.7	0.4	1.3
Second	A	385.6	0.8	−1.9	78.5	0.4	−0.4
	B	794.2	1.3	0.0	158.5	0.6	−0.13
USP	A	393.2	0.4	—	78.8	0.9	—
	B	794.0	0.8	—	158.7	1.1	—

* s_r = relative standard deviation.
† RD = relative difference between both methods.

i.e., F[SMX] and F[TMP], the respective concentrations added, [SMX], [TMP] and [Ex] (amount of excipient extract), as well as their squares $[SMX]^2$, $[TMP]^2$ and $[Ex]^2$ and the cross-products [SMX][TMP], [SMX][Ex] and [TMP][Ex]. The partial correlation coefficients (r) were calculated. From the values found, an interesting enhancement was found in the values of r for the pairs of variables F[SMX]-[SMX] and F[TMP]-[TMP] when the order of derivation was increased from zero to two.

Multilinear regression was performed, using the found concentrations of SMX and TMP as dependent variables and the respective added concentrations [SMX], [TMP] and [Ex] as well as their squares $[SMX]^2$, $[TMP]^2$ and $[Ex]^2$ and the cross-products [SMX][TMP], [SMX] [Ex] and [TMP][Ex] as independent variables. This was carried out by means of the 'enter method' (which considers all independent variables simultaneously) as well as of the 'stepwise method', where individual variables are added by the program according to the degree of enhancement they provide in the regression fit, measured by the 'F' statistic for a given significance level (5% being the default value).

For the second derivative, at a significance level of 5%, only the variables [SMX] and [TMP] were found to be representative. It can be concluded, therefore, that no significant interactions exist between the two substances and between the two substances and the excipients, and also that second-order polynomials are not necessary for the calibration curves; hence, straight lines can be used for this purpose. Regression parameters are listed in Table 2.

Accuracy and Precision of Analytical Determinations

Accuracy assays were performed on commercial tablets (five analytical replications) by comparing the results obtained with the official USP method and with those of the proposed method using zero-order and first- and second-derivative spectrophotometry. The results found are presented in Table 3.

The results found according to the proposed method differ by less than 2% with respect to those found by the official USP method. The difference is always less for TMP than for SMX (under 0.4% for the former). This difference is small enough to be considered negligible for most routine purposes. The precision found in the analysis of real samples (s_r under 1.5% in all instances) is sufficient for this purpose.

It should be noted that the acidity must be strictly controlled as it affects strongly the spectrum for SMX.

Table 4 Comparison of two sources of variability. Multicomponent instrumental response for SMX–TMP mixtures (80 mg l^{-1} SMX and 16 mg l^{-2} TMP) with cell refilling (CRN = cell refilling noise; s_r, n = 10) and without cell refilling (PIN = purely instrumental noise, s_r, n = 10)

	Zero order		First derivative		Second derivative	
	SMX	TMP	SMX	TMP	SMX	TMP
CRN (%)	1.24	2.08	1.29	0.49	0.40	0.45
PIN (%)	0.33	0.28	0.18	0.17	0.27	0.28

Study of Sources of Variability

Two main sources of variability were considered, namely, instrumental noise and cell-refilling noise.

Instrumental noise was calculated by analysing the same solution ten times (SMX, 80 mg l^{-1}; and TMP, 16 mg l^{-1} in 0.1 mol l^{-1} methanolic hydrochloric acid), determining the results *versus* separate standards and calculating the s_r of these results.

Cell-refilling noise was calculated by analysing the same solution mixture ten times, as mentioned above, changing the contents of the cell each time. The results are presented in Table 4.

It can be seen that the cell noise is higher for zero-order determinations. This can be explained because the action of emptying, refilling and re-positioning the cells introduces an additive component to the apparent absorbance. This component originates in the slight differences in reflections at the cell walls as it is handled and re-positioned. This additive noise is cancelled out by the derivation process, thereby lowering the dispersion in the results.

Also, it can be seen that the dispersion in this instance is significantly higher than purely instrumental noise, about 50% higher for the second derivative, but up to seven times higher for zero-order multicomponent measurements, for the same reasons pointed out.

Therefore, in order to minimize errors, replications should include cell refilling, although complete analytical replications are usually preferable in all analytical determinations.

Another variable to be considered is temperature, which has a strong effect in derivative spectrophotometry.[15,16] The instrument used in this work was not fitted with a constant-temperature cell-holder, but every care was taken to ensure that standard and unknown solutions were at the same temperature during measurements.

Selection of Order of Derivation

According to the results of the experiments presented above, second-derivative spectrophotometry was chosen for multicomponent determinations. This selection was made on the grounds of better independence from the amount and composition of excipients, better recoveries and precision, as well as better accordance with the results of the official USP method. Also, the clear enhancement in the partial correlation coefficients found was taken into consideration.

It should be emphasized that despite the fact that, theoretically, noise level increases with increasing order of derivation, better precision can often be found with high-order derivatives because of the better independence from matrix-additive interference it provides.

Conclusions

This work proves that the application of derivative spectrophotometry to overdetermined multicomponent systems allows determinations to be carried out in difficult cases when the extreme spectral overlap would prohibit determinations based on zero-order spectra. For these types of determination, the wavelength sets taken into account should be chosen for optimum performance, *i.e.*, analytical recovery and S/N ratio. However, the instrument used in this work only allows the selection of a wavelength range instead of discontinuous sets (*i.e.*, specific wavelengths within the range cannot be deleted), which may limit to some extent the adoption of the optimum spectral conditions.

Also, in this particular instance, the importance of strict control of the acidity of standards and samples, in order to avoid undesired effects due to spectral shifts, has been demonstrated.

The usefulness of experimental designs for optimization of analytical methods is widely accepted; however, its application to method validation is less frequent. The use of experimental designs such as the central composite design provides an easy and objective procedure for the detection and evaluation of mutual interactions. Multivariate methods present clear advantages over the univariate ones as they allow the detection of second-order effects.

Linearity and interferences were evaluated together, by analysis of the analytical response (*i.e.*, concentrations found) as a function of the exact concentrations of the analytes involved. Linear regression provided an easy way for carrying out this evaluation, which showed that mutual interferences were absent, and that linearity for the two analytes is sufficient for analytical purposes.

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