ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 77–85

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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# Simultaneous determination of binary mixtures of trimethoprim and sulfamethoxazole or sulphamethoxypyridazine by the bivariate calibration spectrophotometric method

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Received 9 July 2001; received in revised form 6 February 2002; accepted 1 April 2002

**Abstract**

The bivariate calibration algorithm was applied to the spectrophotometric simultaneous determination of trimethoprim (TMP), sulfamethoxazole (SMX) or sulphamethoxypyridazine (SMP) binary mixtures in pharmaceutical and veterinary products. The results obtained were compared with those from derivative spectrophotometry. The statistical evaluation of the method bias showed that the proposed procedure is comparable with commonly used first-derivative spectrophotometry. However, the advantage of bivariate calibration is its simplicity, due to the minimal spectra manipulation when compared with derivative techniques. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Bivariate calibration; Spectrophotometry; Simultaneous determination; Trimethoprim; Sulfamethoxazole; Sulphamethoxypyridazine

## 1. Introduction

Sulfonamides are highly effective chemotherapeutic drugs well known as antibacterial agents widely used in medicine and veterinary practice.

Many pharmaceutical products are now available containing sulfonamides in combination with another drug which function is to increase antibiotic effectivity. Some of these commercial formulations include: sulfaquinoxaline and prymethamine, sulfadiazine and trimethoprim (TMP), sulfamethoxazole (SMX) and TMP, sulphamethoxypyridazine (SMP) and TMP, etc. TMP has been one of the most widely used and studied antibacterial additives, therefore, its synergistic antibacterial effects in combination with sulfonamide is well known both in vitro and in vivo [1]. Due to the drug combination on these formulations there has been a need for creating reliable quantitative methods to determine sulfonamides and its additives in commercial samples

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PII: S0731-7085(02)00133-4

and body fluids. Development of such methods finds a wide application on assessing quality control and safety of such pharmaceuticals.

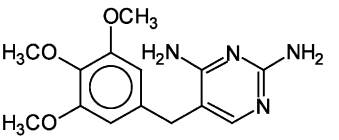
Analysis of sulfonamide mixtures has been performed by different chromatographic techniques [2–6]. Spectrophotometric methods to determine the total content of sulfonamides have been also reported. For instance, spectrophotometric techniques based on the Bratton–Marshall procedure [7] have been widely studied and thus, different automated methods by using an air-segmented continuous flow analyzer [8] and by flow injection analysis [9] were proposed. More recently, derivative spectrophotometry (on the basis of the color obtained by Bratton–Marshall reaction) has been applied to determine the total content of sulfonamides in urine and honey without pretreatment of the samples [10].

The binary mixture of SMX and TMP has been widely studied and numerous spectrophotometric methods for the simultaneous determination in authentic mixtures and pharmaceutical preparations have been developed [11–26]. However, while most of these methods are simpler than HPLC techniques, they still require a lot of data manipulation, which makes it difficult for their application as standard transferable methods. Thus, it is highly desirable to develop even simpler methodologies with minimal sample and data manipulation.

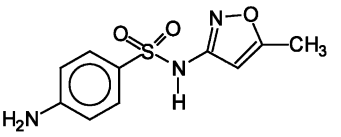
In this work, the mixtures of SMX–TMP and SMP–TMP were investigated and resolved by using the bivariate calibration spectrophotometric method [27,28]. This method is based on a simple mathematic algorithm (see method outline), in which the data used derives from four linear regression calibration equations: two calibrations for each component at two wavelengths selected using the method of Kaiser [29]. The method has been successfully applied to resolve different binary mixtures, such as: metronidazole–furazolidone and metronidazole–di-iodohydroxy-
quinoline [30], tartrazine-sunset yellow [31] and recently, for sunset yellow–allura red [32].

In the current work the mixture of SMX and TMP has been resolved in five different pharmaceutical products, Bactrim, Ectaprim, Sultiprim, Bactrim-F and Trimexazol. The mixture of SMP and TMP has been resolved in three different veterinary products, Alphaprim, Cotrisul and Sulfamiven.

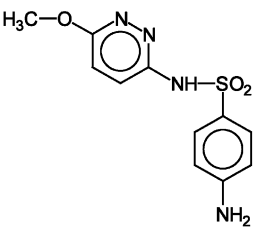
The obtained results were compared with those obtained by derivative spectrophotometry.




Trimethoprim [2,4-Diamino-5-(3,4,5trimethoxybenzyl) pyrimidine]




Sulfamethoxazole [$N^1$-(5-Methyl-3-isoxazoly) sulfanilamide]




Sulphamethoxypyridazine

## 2. Experimental

### 2.1. Apparatus

A Milton Roy (Rochester, NY, USA) Spectronic 3000 diode array spectrophotometer with 0.35 nm resolution, coupled to a Milton Roy 486 PC and User Data version 2.01 software for spectral data acquisition, storage and manipulation was used. All data treatment operations were carried out using a Hewlett Packard Vectra

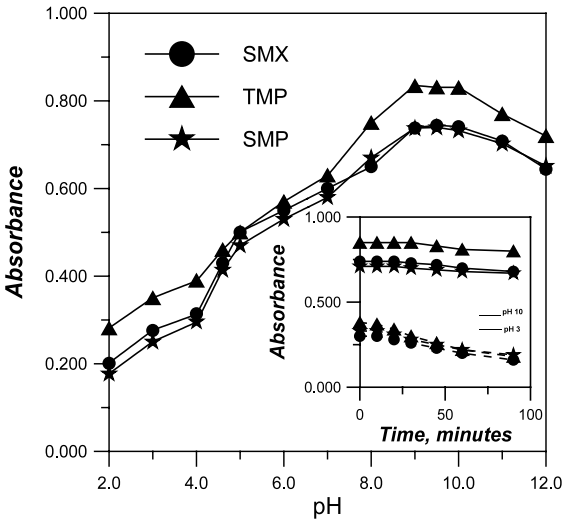



Fig. 1. Effect of the pH on sensitivity and stability of analytical signals from TMP ($\lambda = 259$ nm), SMP ($\lambda = 251$ nm) and SMX ($\lambda = 257$ nm).

486/66 VL microcomputer equipped with the GRAMS/32™ software package, version 5.01 (Galactic Industries, Salem, NH, USA). All calculations for the bivariate method were performed using a simple GWBASIC program. (The GWBASIC program is available upon request to the corresponding author).

### 2.2. Reagents

All chemicals were of analytical-reagent grade. SMX, TMP, and SMP were obtained from Sigma (México), ethanol was from J.T. Baker. The ammonium buffer solution (0.5 mol $l^{-1}$, pH 10) was prepared from Sigma reagents.

Stock solutions contained respectively 1.000 g $l^{-1}$ of SMX, TMP and SMP in ethanol were prepared and stored at 4 °C. Working solutions were prepared daily by appropriate dilution. Pure water of Milli-Q class (Labconco, Kansas City, MO, USA) was used throughout.

### 2.3. Procedures

#### 2.3.1. General procedure

Samples were prepared in 25 ml volumetric flasks by adding between 0.3 and 15 mg $l^{-1}$ of SMX and SMP, and between 0.5 and 40 mg $l^{-1}$ of TMP, 2.0 ml of ethanol, 5 ml of ammonium buffer solution (pH 10.0) and volume adjusted with purified water (Milli-Q).

Spectra of the solutions were recorded between 200 and 330 nm for SMX–TMP, and 200 and 350 nm for SMP–TMP. A 25 ml solution containing 5 ml of buffer and 2 ml of ethanol was used as reference. Absorbance of this solution at 268 nm did not exceed 0.03 uA as measured against water.

Absorbance values were measured at the optimum wavelengths found by the Kaiser method for each mixture (240 and 257 nm for SMX–TMP, 240 and 259 nm for SMP–TMP) and concentration of each component was determined in the samples.

*2.3.1.1. Synthetic mixture*. Two series of solutions containing TMP (1.0–36.0 mg $l^{-1}$)–SMX

Table 1
Analytical characteristics and statistical parameters for single-component determination of SMX, sulphamethoxypyridazine and thrimethropim

| Component | SMX | TMP | SMP |
|---|---|---|---|
| $\lambda_{max}$ | 257 nm | 289 nm | 251 nm |
| Linearity range, μg $ml^{-1}$ | 0.3–15.0 | 0.5–40 | 0.3–15.0 |
| Equation | $A = 0.0081$ (SMX) + 0.0066 | $A = 0.0238$ (TMP) + 0.0057 | $A = 0.0681$ (SMP) − 0.0082 |
| Regression coefficient | 0.9994 | 0.9990 | 0.9998 |
| R.S.D. (%) | 0.57 ($n = 10$) | 0.86 ($n = 10$) | 0.36 ($n = 10$) |
| Error (%) ($\alpha = 0.05$) | 0.45 | 0.68 | 0.28 |
| Detection limit[a], μg $ml^{-1}$ | 0.34 | 0.56 | 0.19 |

[a] Detection limit = $3s_B/m$; $s_B$ = standard deviation of blank; $m$ = slope of calibration.

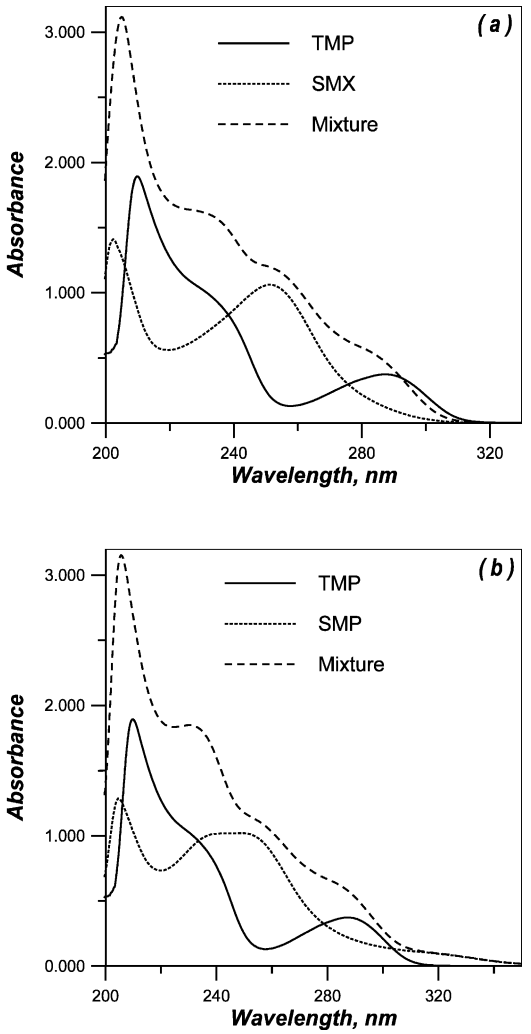



Fig. 2. Absorption spectra (a) 16 mg $l^{-1}$ trimethoprim (TMP), 15 mg $l^{-1}$ sulfamethoxazole (SMX); and their mixture. (b) 16 mg $l^{-1}$ trimethoprim (TMP), 15 mg $l^{-1}$ sulphamethoxypyridazine (SMP); and their mixture.

(0.5–14.0 mg $l^{-1}$), and TMP (2.0–36.0 mg $l^{-1}$)–SMP (1–14.0 mg $l^{-1}$) were prepared for the bivariate calibration. The accurate volumes of stock solutions of SMX and TMP, or SMP and TMP, were added to 25 ml volumetric flasks, followed by 2.0 ml of ethanol and 5 ml of ammonium buffer solution (pH 10.0) and then volume adjusted with Milli-Q water.

*2.3.1.2. Analysis of pharmaceutical and veterinary formulations*. Tablets: ten tablets were pulverized and homogenized, 100 mg of the powder were dissolved in ethanol, filtered and diluted to 100 ml with ethanol to give stock solution. Working solutions were prepared using stock solutions following the previously described procedure.

Suspensions: appropriate volumes of the sample were suspended in ethanol filtered and diluted to 100 ml with ethanol to give 100 mg $l^{-1}$ solutions. Appropriate aliquots of these solutions were used following the general procedure.

## *2.4. Outline of the Bivariate Calibration Method [27]*

The linear calibration regression function for the spectrophotometric determination of an analyte A at a selected wavelength ($\lambda_i$) is given by

$$A_{Ai} = m_{Ai} \cdot C_A + e_{Ai}$$

where $m_{Ai}$, is the slope of linear regression, $C_A$ is the concentration of analyte A (for practical reasons the concentration units of mg $l^{-1}$ were used in this work) and $e_{Ai}$ is the intercept value, which reflects the differences between the ideal and the real system.

If the measurements of the binary mixture (A, B) are performed at two selected wavelengths (1 and 2), we have a two equations set:

Table 2
Sensitivities evaluated for SMP and TMP determination in single-component solutions at nine selected wavelengths ($m_{SMP}$, $m_{TMP}$ = slope value of linear regression calibration for SMP and TMP)

| Wavelength, nm | $m_{SMP} \times 10^3$ | $m_{TMP} \times 10^3$ |
|---|---|---|
| 240 | 6.64 | 4.59 |
| 254 | 6.85 | 0.99 |
| 259 | 6.66 | 0.79 |
| 265 | 5.79 | 1.01 |
| 270 | 4.65 | 1.34 |
| 277 | 3.14 | 1.85 |
| 289 | 1.70 | 2.33 |
| 297 | 1.25 | 1.85 |
| 312 | 0.84 | 1.99 |

Table 3
Application of the method of Kaiser for the selection of the wavelengths set for the SMX–TMP the absolute values of determinants of sensitivity matrices ($K \times 10^{-4}$)

| $\lambda_1/\lambda_2$ | 240 | 250 | 257 | 260 | 270 | 280 | 289 | 290 | 300 |
|---|---|---|---|---|---|---|---|---|---|
| 240 | 0 | 23.5 | 29.4 | 28.0 | 13.3 | 9.3 | 6.8 | 7.3 | 5.4 |
| 250 | | 0 | 5.5 | 4.9 | 2.8 | 10.7 | 13.8 | 13.4 | 8.6 |
| 257 | | | 0 | 0.4 | 6.5 | 13.1 | 15.7 | 15.6 | 9.6 |
| 260 | | | | 0 | 6.0 | 12.5 | 15.1 | 15.0 | 9.2 |
| 270 | | | | | 0 | 6.1 | 8.6 | 8.7 | 5.5 |
| 280 | | | | | | 0 | 2.5 | 2.8 | 2.1 |
| 289 | | | | | | | 0 | 0.3 | 0.7 |
| 290 | | | | | | | | 0 | 0.5 |
| 300 | | | | | | | | | 0 |

Table 4
Application of the method of Kaiser for the selection of the wavelengths set for the SMP–TMP: the absolute values of determinants of sensitivity matrices ($K \times 10^{-4}$)

| $\lambda_1/\lambda_2$ | 240 | 254 | 259 | 265 | 270 | 277 | 289 | 297 | 312 |
|---|---|---|---|---|---|---|---|---|---|
| 240 | 0 | 26.9 | 27.3 | 21.6 | 13.8 | 3.1 | 7.2 | 6.2 | 2.8 |
| 254 | | 0 | 1.2 | 1.1 | 4.6 | 9.5 | 14.3 | 11.45 | 0.5 |
| 259 | | | 0 | 2.1 | 5.3 | 9.8 | 14.2 | 11.4 | 0.6 |
| 265 | | | | 0 | 3.1 | 7.5 | 11.8 | 9.5 | 0.3 |
| 270 | | | | | 0 | 4.4 | 8.6 | 0.6 | 0.2 |
| 277 | | | | | | 0 | 4.2 | 3.5 | 0.9 |
| 289 | | | | | | | 0 | 0.2 | 1.6 |
| 297 | | | | | | | | 0 | 1.3 |
| 312 | | | | | | | | | 0 |

Table 5
Linear regression calibration formulae used for bivariate algorithm ($A_i = m_I\ C + e_i$)

| Binary mixture | Component | Calibration equations | |
|---|---|---|---|
| | | $\lambda = 240$ nm | $\lambda = 257$ |
| SMX–TMP | SMX | $A = 0.0682C - 0.009$ ($r = 0.9992$) | $A = 0.0701C + 0.0059$ ($r = 0.9996$) |
| | TMP | $A = 0.0491C + 0.012$ ($r = 0.9991$) | $A = 0.0081C + 0.0066$ ($r = 0.9995$) |
| | | $\lambda = 240$ nm | $\lambda = 259$ nm |
| SMP–TMP | SMP | $A = 0.0663C - 0.005$ ($r = 0.9992$) | $A = 0.0666C - 0.006$ ($r = 0.9992$) |
| | TMP | $A = 0.0488C + 0.019$ ($r = 0.9992$) | $A = 0.0079C + 0.004$ ($r = 0.9992$) |

$$A_{AB1} = m_{A1} \cdot C_A + m_{B1} \cdot C_B + e_{AB1}$$

$$A_{AB2} = m_{A2} \cdot C_A + m_{B2} \cdot C_B + e_{AB2}$$

where $e_{AB1}$ and $e_{AB2}$ are the sum of the intercepts of the linear calibration at two wavelengths ($e_{ABi} = e_{Ai} + e_{Bi}$). The values of $C_A$ and $C_B$ can be evaluated as follows:

$$C_B = \frac{m_{A2}(A_{AB1} - e_{AB1}) + m_{A1}(e_{AB2} - A_{AB2})}{m_{AB1} - m_{A1}m_{B2}}$$

$$C_A = \frac{A_{AB1} - e_{AB1} - m_{B1}C_B}{m_{A1}}$$

This simple algorithm allows the resolution of binary mixtures by measuring the absorbance of

the mixture at two selected wavelengths and using the parameters of the linear regression functions evaluated for each component at the same wavelengths. The method of Kaiser [29] is used for the selection of the optimum wavelengths

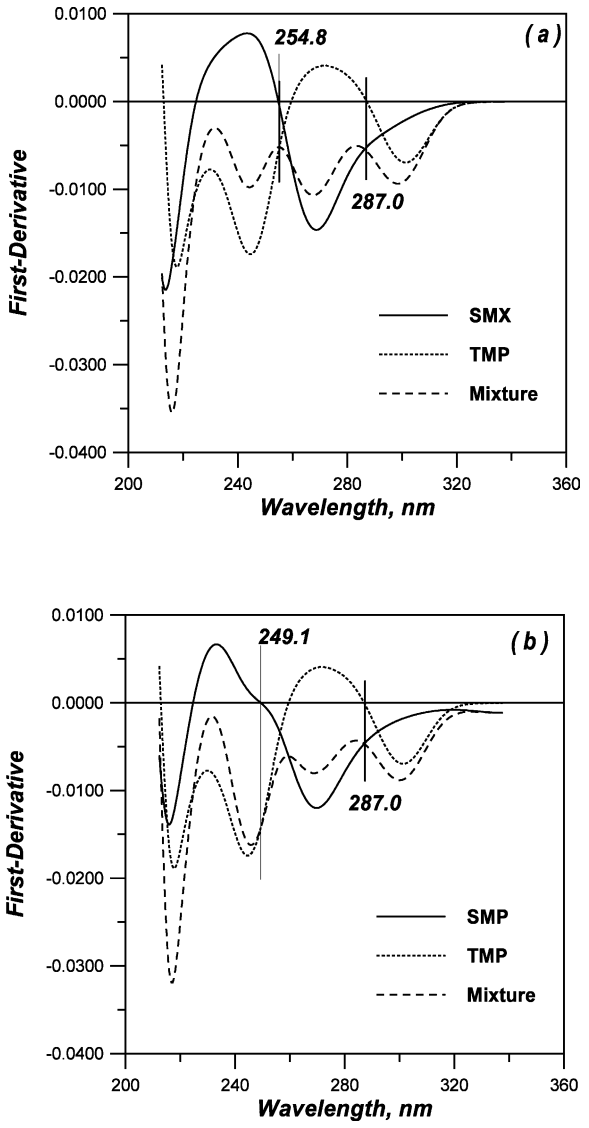



Fig. 3. First derivative spectra (a) 16 mg $l^{-1}$ trimethoprim (TMP), 15 mg $l^{-1}$ sulfamethoxazole (SMX); and their mixture. (b) 16 mg $l^{-1}$ trimethoprim (TMP), 15 mg $l^{-1}$ sulphamethoxypyridazine (SMP); and their mixture.

set, which assured the best sensitivity and selectivity of the determination. A series of sensitivity matrices $K$ are created for each binary mixture and for every pair of pre-selected wavelengths:

$$K = \begin{bmatrix} m_{A1} & m_{B1} \\ m_{A2} & m_{B2} \end{bmatrix}$$

where $m_{A1}$, $m_{A2}$ are the slopes, which are considered as the sensitivity parameters of the component A at two selected wavelengths (1, 2) and $m_{B1}$, $m_{B2}$ are the parameters for the component B. The resolution of these matrices is calculated and the values obtained are used as the optimization criterion; the wavelengths set selected is that with the highest absolute matrix determinant value.

## 3. Results and discussion

Optimum experimental conditions were studied previously for the individual and mixture determination of the analytes [33]. The effect of pH on the absorption spectra of TMP, SMX and sulfamethoxypyridzine was studied at the corresponding maximum absorption wavelength and a value of pH 10.0 was selected based on sensitivity and stability of the analytes (Fig. 1)

Analytical characteristics for individual determination of the three compounds were evaluated at the maximum absorption wavelength and the results are summarized in Table 1. Optimum linear concentration range for each compound was obtained from the Ringbom plot (no shown) concerning each individual calibration giving the following ranges, between 0.5–14 mg $l^{-1}$ for SMP and SMX, and 0.5–36 mg $l^{-1}$ for TMP.

Fig. 2(a–b) show the absorption spectrum for individual components as well as their corresponding binary mixtures. As shown, there is a high spectra overlap, which makes it difficult for the simultaneous determination of the analytes in a mixture, without any sample or data manipulation. For this reason, the bivariate calibration method was applied on the simultaneous determination of the analytes in mixtures.

Table 6
Calibration formulas for TMP, SMX and sulphamethoxypyridazine (SMP) in the binary mixtures obtained using the zero-crossing method from the derivative spectra

| Binary Mixture | Component | $\lambda$ (nm) | Calibration equation | $r^2$ ($\alpha = 0.05$) |
|---|---|---|---|---|
| SMX–TMP | SMX | 287.0 | $^1D = -3.33 \times 10^{-4}$ [SMX] $- 4.0 \times 10^{-5}$ | 0.9998 |
| | TMP | 254.8 | $^1D = -1.18 \times 10^{-4}$ [TMP] $- 6.3 \times 10^{-4}$ | 0.9993 |
| SMP–TMP | SMP | 287.0 | $^1D = -2.87 \times 10^{-4}$ [SMP] $+ 9.8 \times 10^{-6}$ | 0.9998 |
| | TMP | 249.1 | $^1D = -1.29 \times 10^{-4}$ [TMP] $- 9.2 \times 10^{-5}$ | 0.9994 |

Table 7
Recovery results for TMP, SMX and SMP in the binary mixtures

| Mixture | Analyte | Average recovery, % R ± S.D. ($n = 10$, $P < 0.05$) | |
|---|---|---|---|
| | | Bivariate method | Derivative spectrophotometry |
| SMX–TMP | SMX | 101.0 ± 2.0 | 102.1 ± 2.3 |
| | TMP | 101.5 ± 1.5 | 102.5 ± 3.1 |
| SMP–TMP | SMP | 101.3 ± 1.1 | 98.1 ± 2.9 |
| | TMP | 99.4 ± 0.7 | 103.1 ± 1.9 |

Table 8
Determination of TMP, SMX and SMP in the pharmaceuticals and veterinary products

| Pharmaceutical | Analyte | Approximate Content (mg) | Average content (mg ± R.S.D.) ($n = 3$, $p < 0.05$) | |
|---|---|---|---|---|
| | | | Bivariate method | Deriv. Spectrophotom. |
| Bactrim | TMP | 80 | 78.8 ± 5.9 | 77.6 ± 6.8 |
| | SMX | 400 | 395.1 ± 11.1 | 395.9 ± 8.9 |
| Ectaprim | TMP | 80 | 78.2 ± 2.5 | 77.4 ± 4.6 |
| | SMX | 400 | 394.2 ± 2.9 | 395.1 ± 3.2 |
| Sultiprim | TMP | 80 | 82.5 ± 1.7 | 82.8 ± 3.5 |
| | SMX | 400 | 369.3 ± 1.9 | 370.7 ± 2.2 |
| Bactrim-F | TMP | 160 | 181.5 ± 10.9 | 184.6 ± 12.5 |
| | SMX | 800 | 781.4 ± 6.8 | 780.1 ± 7.3 |
| Trimexazol | TMP | 160 | 178.1 ± 1.7 | 176.8 ± 3.4 |
| | SMX | 800 | 809.7 ± 7.3 | 808.2 ± 6.9 |
| *Veterinary Products* | | | | |
| Alphaprim | TMP | 12[a] | 12.5 ± 1.1 | 12.9 ± 2.3 |
| | SMP | 44[a] | 43.8 ± 1.6 | 44.3 ± 1.9 |
| Cotrisul | TMP | 40[a] | 38.9 ± 1.9 | 36.8 ± 4.6 |
| | SMP | 200[a] | 197.8 ± 3.6 | 191.3 ± 8.9 |
| Sulfamiven | TMP | 40[a] | 38.6 ± 2.2 | 38.2 ± 1.9 |
| | SMP | 200[a] | 195.6 ± 4.3 | 196.4 ± 7.8 |

[a] mg in 1 ml.

For the application of the bivariate calibration, two optimum calibration wavelength sets were selected using the method of Kaiser. While in principle, wavelength selection for evaluation with Keiser's method could include the full range of wavelengths with $\Delta\lambda$ increments as small as 0.5, 1 or 2 nm, minimization on the number of data used is suggested to keep the simplicity of the method. Besides, previous reports [30–32] have shown that is not necessary to evaluate consecutive wavelengths with similar sensitivity values, since no statistically significant difference of the methods was observed. A particular case arises when one or both of the analytes present broad bands or flat bands with no well-defined maximum, in such case similar results are expected within the range of wavelengths of the band (see spectrum of SMP in Fig. 2(b)). For these reasons, in this work nine wavelengths for the SMX–TMP and SMP–TMP systems were chosen based on sensitivity levels and the slope values of the linear regression for each component were estimated at each wavelength (see Table 2, i.e. SMP–TMP mixture). With the obtained data, the Kaiser sensitivity matrix was created and the respective determinants calculated. The sensitivity value charts obtained for each mixture are shown in Tables 3 and 4, showing that the highest values were at 240 and 257 nm for SMX–TMP and 240 and 259 nm for SMP–TMP. These optimum wavelengths were chosen to determine the single component calibration functions (regression coefficient, $r > 0.999$) and the $m_i$, $e_i$ values were applied in the bivariate algorithm (Table 5).

Resolution of the binary mixtures was also made by using first derivative spectra. Instrumental conditions were optimized and the first derivative spectra were obtained by smoothing zero order spectra with 21 experimental points followed by derivatization with $\Delta\lambda$ 10.85 nm using the Savitsky–Golay method [34] as shown in Fig. 3. To select the analytical signals the zero crossing measurement technique was applied. The selected wavelengths and the calibration function for each component in the two mixtures studied are summarized in Table 6.

Validation of the methods was carried out by resolution of two sets of ten synthetic mixtures prepared following the general procedure within the working concentration range as determined by the Ringbom plot (no shown). For each mixture recovery experiments were carried out using the bivariate and first derivative calibration equations (Tables 5 and 6). Mean recovery results obtained are given in Table 7. Evaluation of the method bias was carried out using statistical tests ($F$- and $t$-tests, $p = 0.05$), and no statistically significant differences were detected for recoveries and precisions of SMX and SMP. For TMP the bivariate procedure gave better results

The proposed method was applied to the direct simultaneous determination of SMX and TMP in five pharmaceutical formulations: Bactrim, Ectaprim, Sultiprim containing 80 mg of TMP and 400 mg of SMX, Bactrim-F, and Trimexazol containing 160 mg of TMP and 800 mg of SMX. Determination of SMP and TMP was also applied in three veterinary products: Alphaprim containing 44 mg of SMP and 12 mg of TMP in 1 ml, Cotrisul and Sulfamiven containing 200 mg of SMP and 40 mg of TMP in 1 ml. The obtained results showed no statistically significant differences between the bivariate calibration procedure and derivative spectrophotometry and the results agreed with the product label contents (Table 8).

## 4. Conclusions

The obtained results show that the proposed algorithms effective and suitable for the simultaneous determination of SMX and TMP or SMP and TMP in pharmaceutical products with comparable accuracy when compared to classical derivative spectrophotometry. The advantage of the bivariate calibration is its simplicity, which offers a step forward for development of standard transferable methods.

## Acknowledgements

The authors gratefully acknowledge financial support from CONACYT (México), project 21118-E.

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