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Chemometric tools in the analysis of pharmaceutics samples: A comparison among several multivariate calibration methods

Nancy E. Ornelas-Soto[1], José Alberto Duarte-Moller*[2], Judith Amador-Hernández[3], Alma Rocío Rivera-Gomez[4], Rafael Pacheco Contreras[2], Rolando Flores Ochoa[2], Ignacio Yocupicio Villegas[2], Pedro L. López-de-Alba[5*]

[1] Centro del Agua para América Latina y el Caribe, ITESM, Monterrey, N.L., 64849 México.
[2] Universidad de Sonora. Unidad <Regional Sur. Lázaro Cárdenas del Río No.100, Col. Francisco Villa, C.P. 85880. Navojoa, Son. México
[3] Facultad de Ciencias Químicas, Universidad Autónoma de Coahuila, Saltillo, Coah., 25280 México.
[4]Tecnológico Nacional de México. Chihuahua, Ave. Tecnológico 2909, Chihuahua, Chih.31310.
[5] Universidad de La Salle Bajío. Ave. Universidad 602. Lomas del Campestre. León Gto. 37150. México

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Abstract—Bivariate calibration algorithm is compared with the results obtained by the usage of high-dimensional calibration methods such as partial least squares (PLS) and multi-way partial least-squares (N-PLS) by using UV-Vis spectrophotometric data of first and second-order. The algorithms were applied to the determination of a mixture of an analgesic and a stimulant compound and their actual concentrations of them were calculated by using spectroscopic data. The direct reading of absorbance values at 227 nm and 271 nm were employed for quantification of the compounds in the case of the bivariate method. The approaches of first-order and multi-way methods were applied with a previous optimization of the calibration matrix by constructing sets of calibration and validation with 20 and 10 samples (mixtures) respectively according to a central composite design and their UV absorption spectra were recorded at 200-350 nm. All algorithms were satisfactorily applied to the simultaneous determination of these compounds in pharmaceutical formulations with mean percentage recovery of 100.5 ± 3.67, 98.7 ± 3.42, and 100.5 ± 3.74 for bivariate, PLS-1, and N-PLS, respectively. The statistical evaluation of the bivariate method showed that this procedure is comparable with those algorithms that employ high-dimensional structured information.
The aim of the work is to compare the methods under study and it can be seen that there are no significant differences, so a simple spectrophotometer can be used up to a very specialized one. However, the advantage of bivariate calibration is its simplicity, due to the minimal experimental manipulation.

Keywords— UV–Vis spectroscopy, Bivariate and Multivariate analysis, PLS-1, N-PLS.

I. INTRODUCTION

The general tendency in the development of analytical methods is the reduction of analysis stages, which reduces also costs and increases the analysis frequency per sample. Taking into account these facts, the development of methodologies that make usage of spectroscopic techniques has been increased. The great majority of these alternative methods are based on spectroscopic techniques such as Raman, Infrared (IR), fluorimetry, and UV-Vis absorption. In particular, for the analysis of pharmaceutical preparations, have been reported a lot of spectroscopic methods to identify compounds as diverse as sedatives, analgesics, muscle relaxants, neurotransmitters, antineoplastics, Anti-infective, etc. [1-17].

The main advantages of the direct spectroscopic methods are the simplicity, rapidity, economy, elimination of pre-treatment and manipulation of the sample, and shorter analysis time. The application of chemometric methods in analytical chemistry has improved the analytical power of various spectrophotometric techniques [18-25]. The accurate quantification of several analytes simultaneously with partially overlapped absorption bands or determination of a single analyte in complex samples is no longer a problem due to the availability of software for multicomponent analysis. These

tools are especially useful for quantitative analysis using spectroscopic techniques and represent an alternative method to the well-known separation methods in chemistry. This is so because the chemometric techniques allow quantifying analytes from non-selective measurements (in presence of interferences) and, therefore, it is possible to detect discrepant samples in prediction. Thus, the combination of spectroscopic and chemometric methods can be of great help to develop alternative methods to determine if a given pharmaceutical sample has been adulterated or at the factory to have better control over the quality of the product on the line production.

In the case of the bivariate method, widely used by several authors because of its simple mathematic algorithm which provides simplicity and rapidity for methodology without diminishing reliability, it has been employed recently for the analysis of several studies such as quality analysis, kinetic and photo-stability study of drugs and the simultaneous determination of the principal active drug and its degradation products [26-30]. By orher side, least squares (PLS) method has a lot of advantages over any other regression methods. It is robustly handle by more descriptor variables than some compounds, by example in those nonorthogonal descriptors and multiple biological results. It has a predictive accuracy and low risk of change correlation [31, 32].

In recent years, multi-way chemometric techniques have been introduced for the analysis of complex samples. The advantage of using data involving high-dimensional structured information like the N-PLS method is the higher stability towards interferents and matrix effects compared with first-order methodologies (PLS-1).

In this work, the bivariate calibration method which depends on a simple mathematic algorithm provides simplicity and rapidity for the analytical methodology has been compared with different multivariate calibration methods (N-PLS and PLS-1) for the resolution of the mixture of Acetylsalicylic Acid-Caffeine (ASA-CAF) en pharmaceutical samples. Since the bivariate method for the selection of wavelengths for optimum precision in simultaneous spectrophotometric determinations is needed [34], the Kaiser approximation [32] was applied. This method was developed in our laboratory [36] and uses four calibration curves, two for each component at two wavelengths (227 and 271 nm) [35, 36]

II. Material And Methods

A. Theory

At a certain wavelength, the absorbance of a mixture of two components A and B can be expressed as follows:

$$A_{AB} = \varepsilon_A b C_A + \varepsilon_B b C_B \quad (1)$$

Where A_{AB} is the absorbance of the mixture at the chosen wavelength, ε_A and ε_B, are molar absorption coefficients of the components A and B at this wavelength; C_A, C_B are the molar concentration of both components and b is the optical path length. However, in "real conditions", when the individual responses A_A and A_B are affected by the analytical and measurement errors, the calibration curve formulas for each component at one selected wavelength λ_1 can be written

$$A_{A1} = m_{A1} C_A + e_{A1} \quad (2)$$

$$A_{B1} = m_{B1} C_B + e_{B1} \quad (3)$$

Where, m_{A1} and m_{B1} are the corresponding slopes values of linear regressions; C_A, C_B are the concentrations of both components (for practical purposes the concentration units mg L^{-1} were used in this work) and e_{A1} and e_{B1} are the intercept values, which consider the differences between the model and the real system.

If the determination of the binary of the binary mixture is performed at two selected wavelengths 1 and 2 and according to the Beer-Lambert rule, we have two equations:

$$A_{AB1} = (m_{A1} C_A + e_{A1}) + (m_{B1} C_B + e_{B1}) \quad (4)$$

$$A_{AB2} = (m_{A2} C_A + e_{A2}) + (m_{B2} C_B + e_{B2}) \quad (5)$$

And if we consider $e_{AB1} = e_{A1} + e_{B1}$ and $e_{AB2} = e_{A2} + e_{B2}$ where e_{AB1} and e_{AB2}, are the sum of the intercepts of the linear calibration curves at two wavelengths, we can rewrite equations (4) and (5) as:

$$A_{AB1} = m_{A1} C_A + m_{B1} C_B + e_{AB1} \quad (6)$$

$$A_{AB2} = m_{A2} C_A + m_{B2} C_B + e_{AB2} \quad (7)$$

The simultaneous solution of equation (6) and (7) allows the evaluation of C_A and C_B values

$$C_B = \frac{m_{A2}(A_{AB1} - e_{AB1}) + m_{A1}(e_{AB2} - A_{AB2})}{(m_{A2} m_{B1} - m_{A1} m_{B2})}$$

In this fashion, using a simple mathematical algorithm and the parameters of the linear regression functions evaluated individually for each component at these wavelengths, we can obtain simply, the substrate concentration in the mixture, with minimum error using the conventional resolution that considers the molar absorptivities in a two equation-two unknown system. As we pointed out before, one of the problems in the spectrophotometric multicomponent analysis is the proper selection of the set of wavelengths to measure the absorbance values, the optimal wavelengths were determined using the Kaiser method in which the sensibility matrix "K" is defined as:

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

Where "m" are the slopes of the calibration curve of the components A and B at the corresponding wavelengths 1 and 2. The pair of wavelengths with more sensibility was selected through the slope determinant in which the absolute value was higher.

B. Experimental

1. Reagents and apparatus.

All experiments were performed with analytical reagent grade chemicals. ASA and CAF were from Sigma-Aldrich (USA), monochloroacetic acid, ethanol, and potassium hydroxide Merck (México), deionized water HPLC grade was used, and a chloroacetic acid-potassium hydroxide 0.1 M buffer solution of pH 2.2. Ethanol solution was used as a solvent.

UV-spectra of samples were measured in the 200-350 nm wavelength range in steps of 1nm and performed using a Lambda 900, Perkin-Elmer spectrophotometer, and a Milton Roy 3000 spectrophotometer. For each measurement, a 1-cm-thick quartz cell was used.

2. Procedure.

For UV analysis, ASA and CAF were prepared by dissolving 100 mg of each in 100 mL of ultrapure water. A stock solution of each analyte was prepared by dilution of 10 mL of the first solution in 100 mL of ultrapure water. Subsequently, appropriate solutions of different concentrations of ASA and CAF mixtures were prepared from the stock solution

3. Bivariate calibration.

From the stock solutions, in a 25 mL volumetric flask, a series of dilutions for both components were prepared to give solutions having concentrations between 2.0 and 22.0 µg mL-1 with 5 mL of monochloroacetic acid-potassium hydroxide 0.1M buffer solution of pH 2.2 and variable amounts of ethanol were added to assure a 20 percent of ethanol in the mixture, distilled and deionized water to the desired volume. The absorbance was measured for each solution at the maximum wavelength for each analyte; 227.33 nm for ASA and 271.55 nm for CAF (in this case the set of wavelengths determined by the Kaiser matrix corresponded to the maximum wavelength for each analyte).

4. First-order data calibration method.

Appropriate solutions of different concentrations of ASA and CAF mixtures were prepared from the stock solution. A 20-samples set was built to perform the calibration process with the PLS-1 method. The calibration corresponds to a central composite design composed of three components at three levels. Similarly, to validate the chemometric proposed method, a prediction set of 10-samples was prepared. The analyte concentrations were comprised in the calibration set range (Table I). All UV absorption spectra were recorded at 200-350 nm.

Table I. Composition of calibration (Cal1-Cal20) and validation (Val1-Val10) sets.

Sample	Theoretical concentration (µg mL^{-1})	
	ASA	CAF
Cal1	8.0	0.5
Cal2	8.0	1.0
Cal3	8-0	2.0
Cal4	8.0	4.0
Cal5	12.0	0.5
Cal6	12.0	1.0
Cal7	12.0	2.0
Cal8	12.0	4.0
Cal9	16.0	0.5
Cal10	16.0	1.0
Cal11	16.0	2.0
Cal12	16.0	4.0
Cal13	20.0	0.5
Cal14	20.0	1.0
Cal15	20.0	2.0
Cal16	20.0	4.0
Cal17	8.0	0.0
Cal18	12.0	0.0
Cal19	16.0	0.0
Cal20	20.0	0.0
Val1	19.0	0.8
Val2	14.0	2.6
Val3	9.0	4.4
Val4	19.6	4.4
Val5	20.0	3.6
Val6	14.6	4.5
Val7	17.6	0.60
Val8	4.3	0.0
Val9	18.50	1.90
Val10	6.00	0.8

5. Second-order data calibration method.

A similar procedure used in section 3.2.1 was followed by building a set of calibration and validation for four different pH values (2, 3, 4, and 5). For this purpose, 20% (v/v) of phosphate buffer solutions 0.1 M were used. The UV absorption spectra recorded at 200-350 nm resulted in the construction of matrices (j x k x l) which consisted of absorbance intensity (j), the wavelength at 200 - 350 nm (k), and pH-value (l).

6. Analysis of pharmaceutical samples.

Two popular consumer products were analyzed. Ten tablets of each pharmaceutical formulation were weighed individually to obtain an average weight. Tablets were finely powdered and mixed. In the case of UV analysis, a mass corresponding to

one tablet for each formulation was weighed and placed in a 500-mL volumetric flask. 50 mL of ethanol were added and the solution was sonicated for 10 minutes. Then, the solution was diluted to volume with ultrapure water to the mark. A small aliquot of each resulting solution was taken and worked under conditions identical to the standards.

III. Results

A. Spectral characteristics of ASA and CAF.

The individual absorption spectra of ASA and CAF are presented in Fig. 1 as can be observed; the ASA and CAF spectra are completely overlapped, and, therefore, the determination of the two compounds is not possible by direct absorbance measurements. Bivariate or multivariate calibration can be used to solve this problem. To investigate the behavior of absorption spectra, several preliminary studies about the influence of physicochemical variables were realized.

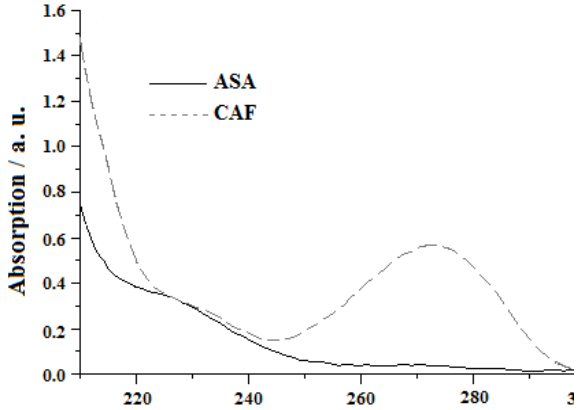

Figure 1. Individual absorption spectra of ASA and CAF

The influence of the pH on the absorption of ASA and CAF has been studied. For that, two solutions were prepared to contain 10 µg mL−1 of ASA and CAF and KCl 0.5 M each, to maintain constant, ionic strength. The pH was modified with slight additions of HCl or NaOH. The solution was to keep stirring all the time. The Absorption spectra obtained at different pH values for ASA and CAF are shown in Fig. 2A and 2A for acidic pH values and Fig. 2B and 2B for basic pH values respectively. It is worth noting that for ASA when the pH decreases at pH values lower than 3, the maximum located at 227 nm is displaced to 237 nm. The absorption intensity decreases with the increment of the pH value until it reaches a minimum between pH values 4 and 11.0. While for CAF, significant changes in absorption intensity are not observed over a wide pH range (3-10). Absorption spectra of ASA and CAF in different solvents were recorded. It could be observed that ethanol increased slightly the signal in the case of ASA while for CAF, no effect is observed.

Under the optimum physicochemical conditions, the Absorption intensity was measured at λmax = 227 nm and λmax = 271 nm for ASA and CAF respectively. The calibration curve was obtained for different standard samples containing between 2.9 and 22.0 µg mL^{-1} for ASA and 2.0 and 22.0 for CAF. The calibration graphs, A versus [ASA] and [CAF] were linear for all concentrations tested. Statistical parameters and analytical characteristics for the individual determination of ASA and CAF are summarized in Table II.

The resolution of the mixture was performed and the recovery experiments were carried out in ten synthetic mixtures of known concentrations of ASA and CAF (Table VI) and the recovery percentage was evaluated for each component in the mixture, in Table VII the results are shown where each sample recovery value is the average of triplicate measurements. As can be observed, satisfactory results were obtained with recovery values close to 100% and with low relative standard deviations (RSD).

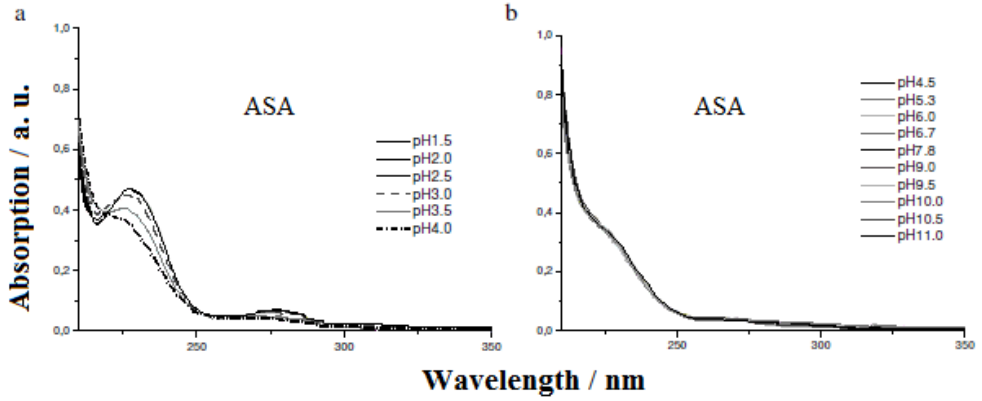

Figure 2. pH absorption spectra for ASA

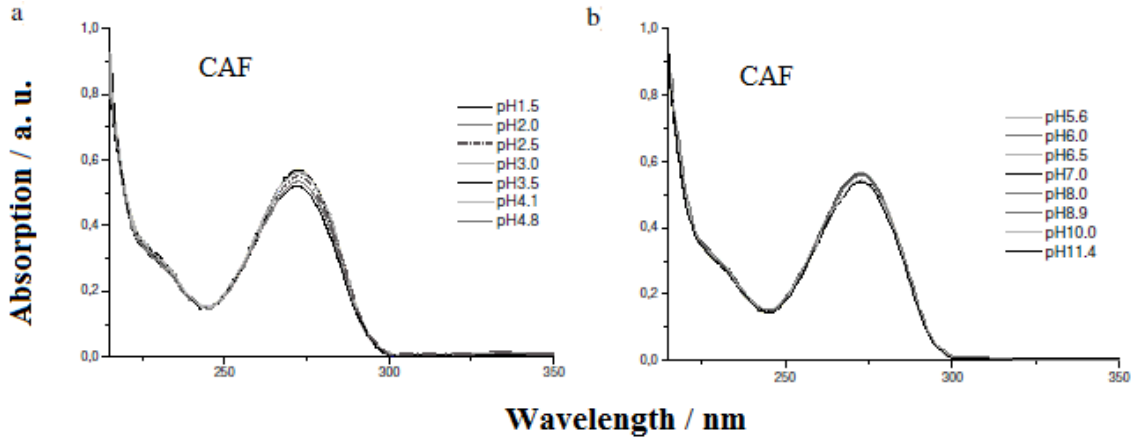

Figure 3. pH absorption spectra for CAF

Table II. Analytical characteristics for ASA and CAF.

Analyte	λ_{max} (mn)	Analytical Range[a]	LD[b]	LQ[c]	RSD (%)[d]	%E[e]
ASA	227.33	2.9 - 22.0	0.10	0.33	0.11	0.25
CAF	271.55	2.0 - 22.0	0.05	0.17	0.19	0.43

[a] µg mL^{-1}
[b] Detection Limit (µg mL^{-1})
[c] Quantification Limit (µg mL^{-1})
[d] Relative Standard Deviation
[e] Relative error over the medium value

B. Bivariate analysis.

For the binary mixture, the concentration range for each compound was taken according to the linear range evaluated in a single-component calibration. From the absorption spectra for ASA and CAF, ten wavelengths were chosen and the slope values of the linear calibration regression were estimated for each compound at these wavelengths (Table III), and the Kaiser method was applied to select the two wavelengths set for the proposed bivariate procedure, see Table IV. With the obtained data, a sensitivity matrix was created and the respective determinants were calculated. From the Kaiser sensitivity chart, the optimum absorption wavelengths for the mixture of ASA and CAF were the same as the maximum

absorption wavelength for the individual compounds. With this information a calibration curve for ASA and CAF was run at 227.33 and 271.55 nm respectively, results are presented in Table V.

Table III. Application of the Kaiser method for the determination of the optimum wavelengths

λ (nm)	m_{ASA}	m_{CAF}
222.17	0.0416	0.0338
227.33	0.0459	0.0274
232.49	0.0410	0.0230
235.44	0.0344	0.0194
243.54	0.0127	0.0131

257.55	0.0039	0.0293	**275.23**	0.0063	0.0460
266.76	0.0051	0.0444	**276.70**	0.0062	0.0442
271.55	0.0060	0.0478			

Table IV. Kaiser sensibility matrix for ASA and CAF

λ/λ	**K (10^{-4})**									
(nm)	**222**	**227**	**232**	**235**	**243**	**257**	**266**	**271**	**275**	**276**
222	0.00	-4.12	-4.29	-356.00	1.16	10.87	16.75	17.86	17.01	16.29
227		0.00	-0.68	-0.52	2.53	12.38	18.98	20.30	19.39	18.59
232			0.00	0.04	2.45	11.12	17.03	18.22	17.41	16.70
235				0.00	2.04	9.32	14.28	15.28	14.60	14.00
243					0.00	3.21	4.97	5.28	5.02	4.80
257						0.00	0.24	0.11	-0.05	-0.09
266							0.00	-0.23	-0.45	-0.50
271								0.00	-0.25	-0.31
275									0.00	-0.07
276										0.00

Table V. Linear regression calibration functions for bivariate calibration method

Active Ingrediente	**$\lambda_{227.33}$ (nm)**	**$\lambda_{271.55}$ (nm)**
ASA	A = (0.0459) C_{ASA} - 0.0092	A = (0.0459) C_{ASA} - 0.0010
CAF	A = (0.0274) C_{CAF} - 0.0006	A = (0.0459) C_{CAF} - 0.0012

Table VI. Determination of ASA and CAF in synthetic mixtures

Sample	**ASA (μg mL^{-1})**	**CAF (μg mL^{-1})**
1	2.9	22.0
2	5.0	19.8
3	7.2	17.4
4	9.4	15.1
5	11.5	12.7
6	13.7	10.4
7	15.8	8.1
8	18.0	5.7
9	20.2	3.4
10	21.8	2.1

C. PLS-1 analysis.

In parallel, this same binary system was resolved by using a first-multivariate calibration method. To perform the analysis of the binary mixture of ASA and CAF, a chemometric approach, based on partial least squares was evaluated. The independent calibration curves for each component were used to establish the analytical range of concentration. According to the results shown in the analysis of the influence of pH, the pH value chosen for the study was 3 which provides a better definition of the ASA spectrum (see Figure 2A). For calibration and prediction purposes, the whole wavelength range was used. To determine the correct number of loading vectors to be used for the modeling of the data, a cross-validation calculation for all samples in the calibration set was performed to calculate the PRESS (prediction residual error sum of squares) [36]. An optimum number of loading vectors of two was found.

The PLS model was applied to the data set of problem samples (prediction set of Table I). The samples analyzed were composed of binary mixtures of variable amounts of the components randomly selected. The statistical parameters and the results obtained in the analysis of this test set of synthetic samples (10 samples) are summarized in Table VII. The recoveries obtained are indicating a satisfactory resolution of the binary mixtures investigated. The mean recovery is around 100%.

D. N-PLS analysis.

The three-way data used were obtained by recording absorption spectra of the mixtures with concentration for ASA

and CAF reported in Table I at 4 pH-values 2, 3, 4, and 5 (Fig. 4). These pH values were chosen because in acidic media there is more variability in the spectra of the mixture components than in the basic medium which provides better differentiation between ASA and CAF (see Figures 2 and 3). A calibration set of 20 samples was constructed by using a central composite design and three blanks were also included (Table I). Spectra were recorded in the range of 200–350 nm.

For calibration and prediction purposes, the whole wavelength range was used. The set of calibration samples was investigated with N-PLS. As expected from the sample composition, the number of factors found was two calculated according to the Haaland and Thomas criterion [36,347] The statistical parameters and the results obtained in the analysis of the test set of synthetic samples (10 samples, Table I), are summarized in Table VII.

Table VII. Recovery for synthetic mixtures of ASA and CAF

	Bivariate		PLS-1		N-PLS	
	ASA	CAF	ASA	CAF	ASA	CAF
Factors	2	2	2	2	2	2
REP (%)	1.32	0.98	3.92	4.73	1.54	1.8
Root mean square of prediction (RMS)	0.39	0.29	1.02	1.47	0.25	0.07
Correlation Coefficient (R)	0.9998	0.9998	0.9910	0.9942	0.9998	0.9999
LOD[a]	0.10	0.05	1.16	0.37	0.17	0.12
Mean recovery (%)	97.50	98.62	102.33	101.38	100.96	100.15
SD [b]	0.72	1.24	2.25	3.13	0.59	1.34

[a] (μg/mL)
[b] Standard Deviation of 10 samples

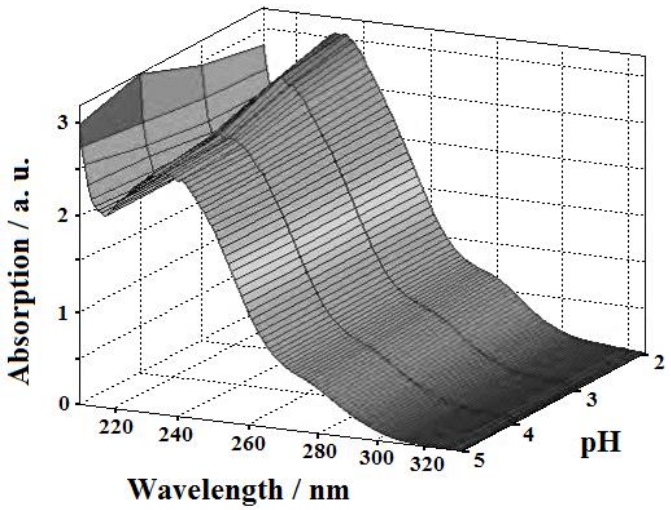

Figure 4. Absorption spectra of mixtures with concentration for ASA and CAF

Samples	API	Mass per Tablet (mg) Label amounts	Bivariate	Mean recovery (%) (RSD)	
				PLS-1	N-PLS
Sample 1	ASA	500	99 (±4.46)	101 (±2.83)	102±3.95
	CAF	50	97 (±3.40)	95 (±2.99)	98 ±3.31
Sample 2	ASA	600	101 (±4.40)	98 (±5.34)	99 ±4.44
	CAF	65	105(±2.44)	101 (±2.51)	103 ±3.2

Table VIII. Consumer Products Assay

E . Comparison of the chemometric methods in the analysis of synthetic samples.

As seen, Table VII shows that the recovery average values

for CAF and ASA were very similar in all chemometric methods employed nevertheless for bivariate calibration these were slightly lower than those for PLS-1 and N-PLS. Similar standard deviation (SD) values were observed for both bivariate and N-PLS methods for the two analytes, while SD values were thrice and twice higher for ASA and CAF respectively when PLS-1 was applied. This suggests a better reproducibility for bivariate and N-PLS methods. On the other hand, SD values for CAF are higher than those for ASA with all algorithms employed. Values of relative error of prediction (REP %) are slightly higher for CAF than those for ASA in the cases of PLS-1 and N-PLS, the worst results of REP% were obtained when applying PLS-1. The above mentioned may be owing to the low concentration of CAF compared to the other one (1:10) and because its UV spectrum is overlapped completely by the ASA spectrum [37-39].

Overall, the results obtained for the two analytes are satisfactory for all the chemometric methods employed. The mean recovery values for each analyte are plotted in Fig. 5, using a box–whisker plot. Recoveries of around 100% are found for both analytes with the three multivariate methods used, and the results obtained using the bivariate algorithm were in fairly good agreement with those obtained by the N-PLS method applied to the UV-spectra.

The relationship between the real and found concentrations of ASA and CAF in the mixtures is represented by R2 and it seems that slightly better results were obtained using the bivariate and N-PLS procedures than using the common PLS-1 procedure.

Finally, the evaluation of method bias was carried out using statistical t-tests, with a confidence level of 95%, and statistically significant differences were detected for recoveries and precisions of CAF and ASA in the synthetic samples when bivariate and PLS-1 procedures were compared while there are no differences between bivariate and N-PLS procedures.

F. Analysis of pharmaceutical samples.

The evaluated bivariate calibration model was applied to the determination of ASA and CAF in two popular OTC consumer products with excellent results reported in Table VII. This table presents also the recoveries obtained by PLS-1 and N-PLS algorithms. Adequate and similar recoveries for ASA and CAF with the three algorithms were obtained and are c.

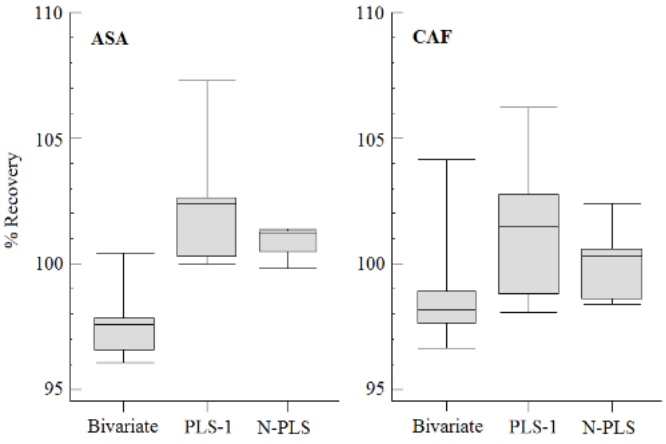

Figure 5. Mean recovery values for each analyte

As can be seen in the table, the results obtained for the case of caffeine with the bivariate method show better recoveries than with the PSL 1 method. and slightly lower than with the p-PSL method. If we refer to the recoveries of acetylsalicylic acid, in the case of the bvariant method, better results are observed than even with the other two methods under study. And observing the deviations in the recovery for both caein and acetylsalicylic acid are similar for all methods.Regardless of the mathematical model used in the mixture analysis, there are no statistically significant differences in the results, however, with two simple simultaneous equations, such as the bivariate calibration method, produce satisfactory results.

IV. Conclus1ions

In this work, three chemometric methods were compared for the rapid, economic and easy simultaneous determination of ASA and CAF through the UV-spectroscopic technique. The previous treatment of the sample was reduced and subsequently, the time analysis was shorter. The proposed methods were applied to determine ASA and CAF mixtures in pharmaceutical formulation samples no matter the UV spectra of the components of the mixture overlap greatly. Bivariate, First-order and second-order multivariate methods, such as PLS-1 and N-PLS allowed the resolution of the mixture, and satisfactory results were obtained.

Statistical comparison among bivariate, PLS-1, and N-PLS algorithms was applied to the simultaneous determination of ASA and CAF in synthetic samples by UV spectrophotometric technique. The first showed a good analytical performance obtained for the single component determination of ASA and CAF. The resolution of binary mixtures carried out for pharmaceutical samples was consistent with those reported by the manufacturer with all algorithms used. In this case, the bivariate procedure gave similar results that those for PLS-1 and N-PLS. The results suggest that bivariate calibration is a good alternative to PLS-1 and N-PLS with the clear advantage that the sample pretreatment is minimum and complicated mathematical treatment of higher dimensional spectral data is not required.

In short, the combination of spectroscopic and chemometric methods can be of great help to develop alternative methods to determine if a given pharmaceutical sample is fake or has been adulterated or at the factory to have better control over the quality of the product on the line production.

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