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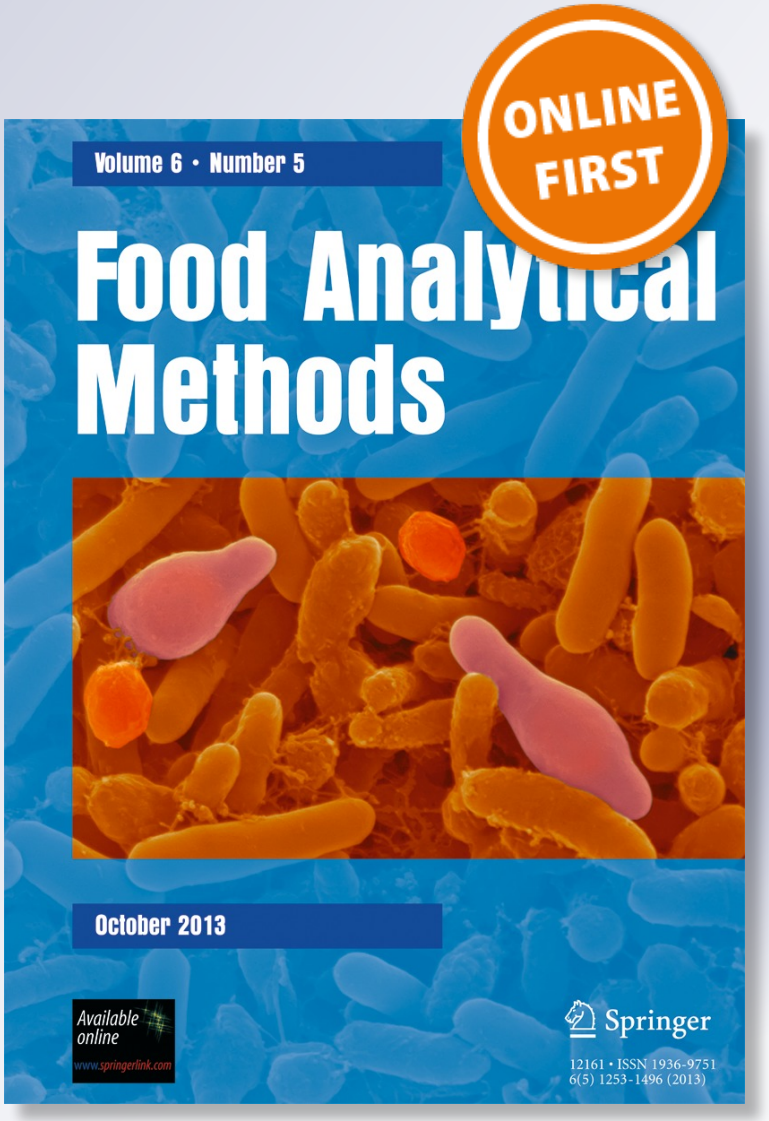

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UV-Visible Spectroscopy and Multivariate Classification as a Screening Tool to Identify Adulteration of Culinary Spices with Sudan I and Blends of Sudan I + IV Dyes

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Abstract This work propose a feasible, rapid, and simple method for detecting culinary spices adulterated either with Sudan I dye or blends of Sudan I + IV dyes at three concentration levels. The method is based on the use of UV-visible spectroscopy with multivariate analysis. Four types of spices were studied: three paprika varieties (mild, hot, and smoked) and a spice commonly consumed in Argentina called *aji molido*. Principal components analysis was firstly applied as an exploratory analysis and then, two classification techniques were used: *K*-nearest neighbors (KNN) and partial least squares-discriminant analysis (PLS-DA). Three classes were defined: unadulterated samples and adulterated samples with Sudan I or blends of Sudan I + IV dyes at 1, 2.5, and 5 ppm (mg L^{-1}). Classification techniques gave satisfactory results: between 89 and 100 % for PLS-DA and between 83 and 92 % for KNN. The sensitivity and specificity of the models were above 83 %. It has to be highlighted that none of the adulterated samples were assigned as unadulterated, which is very positive because of the implication that these results have on consumer health. The capability of detecting mixtures of Sudan dyes is a very important advantage because each Sudan dye generates different hazardous metabolites in human body so their toxicity may be enhanced by the simultaneous presence of such dyes.

Keywords Sudan dyes · UV-visible spectroscopy · Multivariate analysis · Screening methods · Food adulteration

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Introduction

Food coloring is important because it allows manufacturers to obtain the desired aesthetic quality. Specifically, the disadvantage of using natural colored spices is that they lose their color over time, and this could lead to food adulteration by the addition of synthetic substances such as Sudan dyes.

Sudan dyes (I, II, III, and IV, Fig. 1) are a family of synthetic azo dyes that are widely used for coloring plastics, waxes, and textile products and also for scientific applications. Besides, Sudan dyes have been used in foodstuffs such as culinary spices to maintain their intense red–orange color for commercial benefits, because these dyes have attractive characteristics such as low cost, bright color, and long maintenance. Sudan dyes have been classified as category 3 carcinogens (IARC 1975) because they can generate metabolites that are converted to active mutagens and carcinogens in humans (Fonovich 2013). Therefore, the use of Sudan dyes as food additive is banned worldwide because their introduction on the food chain represents a potential health risk (Xu et al. 2007). Sudan I is the dye notified in most of the cases where adulterated foods have been found and it is a well-recognized harmful substance (Stivorová et al. 2009; Johnson et al. 2010). This dye has been found alone or in association with other Sudan dyes (Tripathi et al. 2007; Mishra et al. 2007). Moreover, Sudan I + IV dyes is the most common mixture found in spices as reported by the Rapid Alert System for Food and Feed (RASFF 2005; RASFF 2006). Since each Sudan dye is degraded to different hazardous metabolites in human body (Xu et al. 2010), it is also necessary to detect adulterated foods containing more than one Sudan dye because their toxicity may be enhanced by the simultaneous presence of such dyes.

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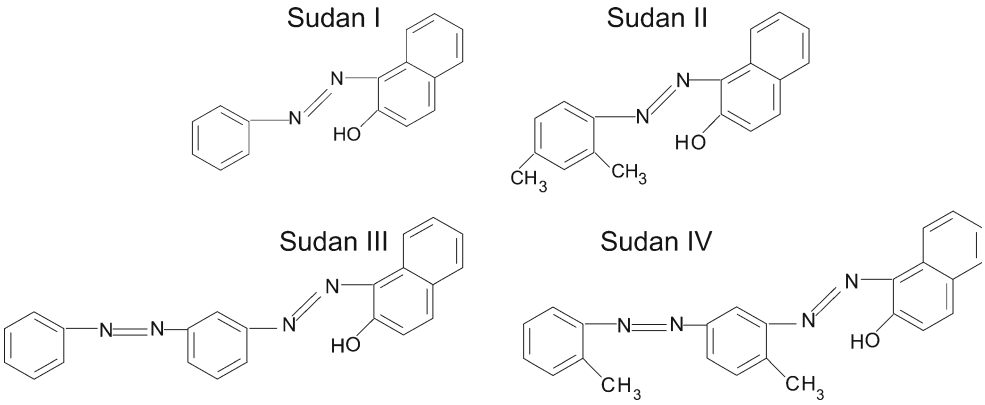

Fig. 1 Sudan dyes chemical structure

European countries have established uniform controls at the point of entry into the Community (Commission Decision 2009/669/EC) instead of the analytical report for each consignment of imported products; as the notifications concerning adulterated foods with Sudan dyes has decreased in the last few years (RASFF 2008). In such scenario, screening methods represent a good means for implementing monitoring and control or routine analysis.

The use of Sudan dyes as food additives is expected to continue, as high levels of these dyes have recently been found (Tripathi et al. 2007; Mishra et al. 2007; RASFF 2006) in other countries; particularly in non-branded or loose spices. Moreover, other food products have been recently adulterated with Sudan dyes such as poultry feed for egg yolk (Hou et al. 2010; Qiao et al. 2011) and preserved beancurds products (Yan et al. 2012). Therefore, development of accurate and fast methods for detecting Sudan dyes in foodstuff is required for ensuring food safety to protect consumer health.

A wide variety of analytical methodologies have been developed for the determination of Sudan dyes in foodstuffs and the most popular are based on the use of liquid chromatography associated with different detectors and sample pretreatments (Rebane et al. 2010). These techniques shows high sensitivity but suffer from some drawbacks including time-consuming, pre-treatments, and expensive instrumentation. More recently, immunoassays methods have also been used (Anfossi et al. 2009; Xiao et al. 2011; Liu et al. 2012) but although they do not need an extensive cleanup, they are expensive. Besides, spectrometric methods coupled with multivariate analysis have alternatively been used. Previous works have demonstrated the ability of spectroscopic techniques such as UV-visible (Di Anibal et al. 2009; Yuan et al. 2008), ^{1}H-NMR (Di Anibal et al. 2011), and Raman (Di Anibal et al. 2012; Cheuung et al. 2010) to detect food adulteration with Sudan dyes in an individual way.

Multivariate analysis plays an important role in analytical chemistry because it provides a means for extracting the maximum useful information from analytical data. In food science, multivariate tools have been successfully employed in a variety of analytical problems. Specifically, classification techniques have been widely used in the food area for classification, authentication, characterization, and discrimination, for determining food origin and quality and to assure food products safety. Some examples can be cited, as the determination of illegal enhancing agents in meat (Della Donna et al. 2009), honey adulteration with fructose corn syrup (Chen et al. 2011), milk adulteration with whey (Almeida et al. 2011), minced meat with adulterants (Meza-Márquez et al. 2010), and wheat flour adulteration with bleaching agents (Yuan et al. 2011).

The aim of the present study is to evaluate the use of UV-visible spectroscopy with multivariate analysis as a screening tool to identify the adulteration of culinary spices with either Sudan I or blends of Sudan I + IV dyes at three concentrations levels that are within the range used for adulterating spices nowadays. The spices were *aji molido* (argentine spice), and three paprika varieties (mild, hot, and smoked). Principal components analysis (PCA) was applied as an exploratory analysis and two classification techniques based in different fundament were applied: *K*-nearest neighbors (KNN) and partial least squares-discriminant analysis (PLS-DA).

Materials and Methods

Reagents and Samples

Sudan I and IV dyes were purchased from Sigma Aldrich (Bs. As., Argentina). A total of 43 samples distributed among mild, hot, smoked paprika and *aji molido* were purchased from different sale-points.

Sample Preparation

Aji molido samples were firstly milled to obtain a homogeneous powder. All samples had the following extraction process: 200 mg of each sample was weighed and 10 mL of ethanol (96 %, *v*/*v*) was added, then samples were shaken in an automatic shaker (Shaker Pro Vicking) during 15 min at 150 rpm and the resulting extracts were filtered through a

0.45-μm nylon syringe filters (Microclar Argentina). The obtained extracts were used to prepare original samples (non-adulterated samples with Sudan dyes) and adulterated samples with Sudan I and blends of Sudan I + IV dyes (50 % each dye). For original samples, an aliquot of each extract (300 μL of paprika and 700 μL of *aji molido*) was taken and diluted to 10 mL in volumetric flasks (ethanol 96 % *v*/*v*), and for adulterated samples an appropriate amount of Sudan dye was also added to get a final concentration of 1, 2.5, and 5 ppm (mg L^{-1}). The stock solution of Sudan I was prepared in ethanol and the Sudan IV stock solution was initially dissolved in a small fraction of chloroform and then diluted with ethanol.

UV-Visible Analysis and Software

A UV-visible spectrophotometer (Agilent 8453, USA) equipped with a diode array detector was used to acquire spectra. UV-visible spectrum scanning was carried out in the wavelength range of 260–600 nm (each nm) which represents 341 variables.

Multivariate analysis was performed under Matlab software (Version 7.0, The Math Works Inc., Natick, USA) and PLS Toolbox 3.5 (Eigenvector Research Incorporated).

Multivariate Analysis

Principal Components Analysis

PCA is an unsupervised analysis that was applied as a first step for detecting trends and patterns in the measured data. PCA projects high-dimensional data onto lower-dimensional space, so all redundant information is summarized which simplifies the graphical interpretation of the data.

K-Nearest Neighbors

KNN is a simple distance-based classification technique. In this method, the training set is directly used to classify new samples, so an unknown sample is classified according to the majority of the class membership of its k nearest neighbors in the training set. The method works as follow: (a) calculate distances between an unknown (test) object (u) and all the objects in the training set; (b) select from the training set k objects most similar to object u, according to the calculated distances (k is usually an odd number), and (c) classify object u with the group to which a majority of k objects belongs. Usually Euclidean distance is employed but other distances can also be used (Mahalanobis, weighted Euclidean, and Manhattan). Neighbors number is selected by the optimization through the lowest prediction error or by a cross-validation approach. For many applications this technique proved to be much better than others, even more complex chemometric approaches (Oliveri et al. 2009).

Partial Least Squares-Discriminant Analysis

Although partial least squares was developed as a regression technique, it can also be used for classification in the form of PLS-DA. The idea is to find compressed variables (latent variables) that sequentially describe the most variance in both the independent variables X (spectra) and the dependent variables Y (classes). Hence, the X and Y data are simultaneously modeled to find the latent variables in X that will predict latent variables in Y, like a classical PLS model. In PLS-DA, a model is developed for each class. Prediction values for each class range from 0 (not belonging) to 1 (belonging), so it is necessary to establish a threshold between these two values. This threshold is calculated by assuming that the class predicted values follow a Gaussian distribution, which is estimated by using the mean and standard deviation of the predicted values for each class. The number of PLS-DA latent variables (LVs) can be selected by means of the root mean squared error of cross-validation (RMSECV) (Bakeev 2010).

Dataset

Data set was divided into training set (samples used to build the classification rule) and test set (samples used to test the ability of the classifier). Training and test set were obtained by random selection. Classes were defined as follows: class 1 correspond to original (unadulterated) samples, class 2 correspond to adulterated samples with Sudan I, and class 3 correspond to adulterated samples with blends of Sudan I and IV (50 % each dye). Adulterated classes contain the three studied concentration levels (1, 2.5, and 5 ppm). Test set was form by selecting 12 out of 43 samples for class 1 and 36 out of 129 for both adulterated classes (12 from each concentration level), which represent a 28 % of the total samples number. Finally, the data was mean-centered before the multivariate analysis.

Results and Discussion

The studied concentration levels (1, 2.5, and 5 ppm) were selected lower than previous studies (Di Anibal et al. 2009), taking into account that the concentrations used to adulterate culinary spices are in the studied range (ASTA 2005; Mishra et al. 2007).

Spectra Characterization

The spectra of Sudan I, Sudan IV standards, and Sudan I + IV blend at 5 ppm (mg L^{-1}) are shown in Fig. 2a. Both Sudan dyes have different spectral shape although they are partially overlapped. The maximum absorbance of Sudan I is

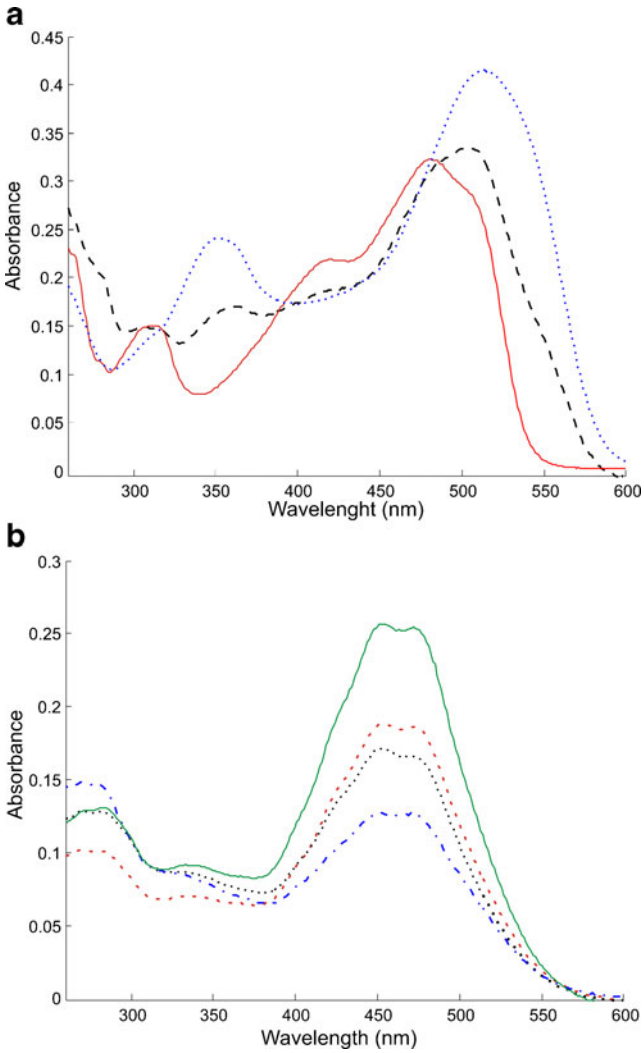

Fig. 2 **a** UV-visible spectra of Sudan I (*solid line*), Sudan IV (*dotted line*), and Sudan I + IV blend (*dashed line*); **b** spectra of mild paprika (*solid line*), hot paprika (*dashed line*), smoked paprika (*dotted line*), and *aji molido* (*dashed-dotted line*) samples

positioned at 481 nm whereas for Sudan IV is at 513 nm, and they also have other maximums in the UV zone. Finally, considering the spectrum of a mixture of the two dyes, it can be seen the additive contribution of Sudan I and IV dyes.

Figure 2b shows the spectra of four random unadulterated spices (mild paprika, hot paprika, smoked paprika, and *aji molido*). It can be observed that within each group of paprika samples (mild, hot, and smoked) the spectra are slight different. Instead, *aji molido* samples present similar spectral shape and lower absorbance values than paprika samples.

Figure 3 shows the spectra of a paprika sample with Sudan I and Sudan I + IV at the three concentration levels. It can be seen that both adulterated samples follow the same spectral trend as standards Sudan dyes and they shift slightly towards a longer wavelength respect to the unadulterated one.

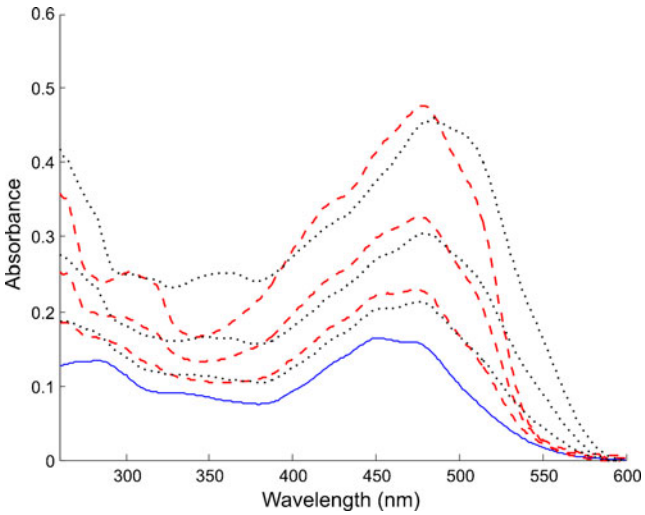

Fig. 3 **a** UV-visible spectra of adulterated sample with Sudan I (*dashed line*), Sudan I + IV blend (*dotted line*) at the three studied concentration levels (1, 2.5, and 5 ppm), and original sample (*solid line*)

Multivariate Analysis: PCA

As it was previously mentioned, PCA was applied before classification techniques in order to see the three classes distribution in the multivariate space defined by few PCs. Figure 4 shows PCA scores plot for the first three principal components, which represent 98.93 % of the original information (PC1=79.65 %; PC2=16.71 %, and PC3=2.57 %).

It can be seen that along PC1 the original samples (unadulterated) can be generally differentiated from adulterated samples (with Sudan I or Sudan I + IV dyes). The original samples have the most PC1 negative scores values while the adulterated ones have both, negative and positive scores values. On the other hand, PC3 allows to distinguish between adulterated samples with Sudan I (negative scores values) and adulterated samples with Sudan I + IV (positive scores values) which means that the information provided by this component

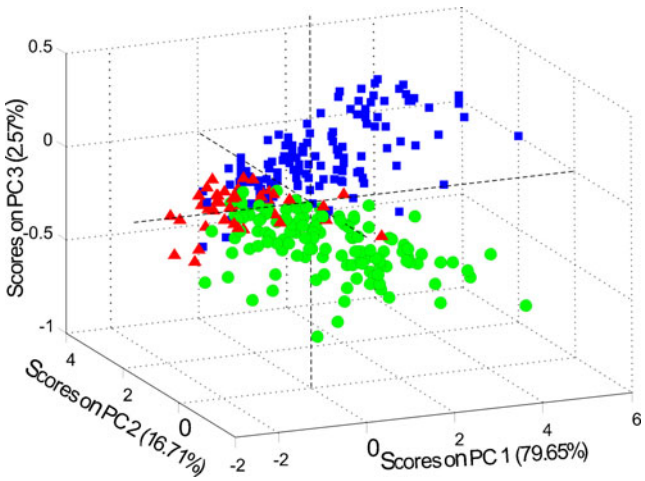

Fig. 4 PCA scores plot of original samples (*triangles*), samples spiked with Sudan I dye (*circles*), and Sudan I + IV dyes (*squares*)

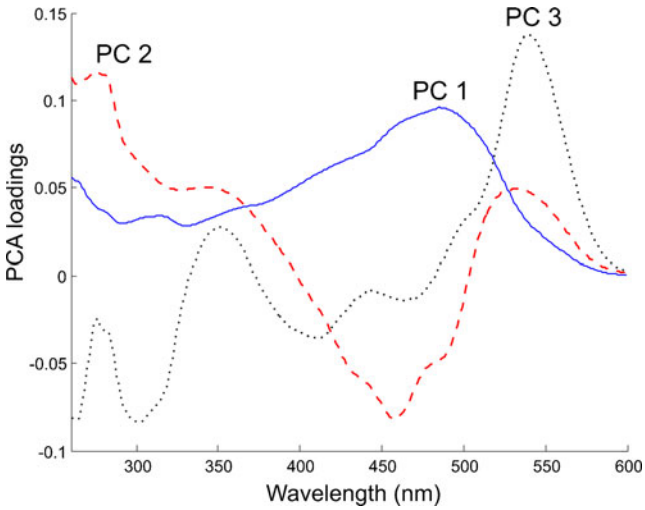

Fig. 5 PCA loadings plot for PC1, PC2, and PC3

is related with the adulterant. Finally, most samples containing Sudan I dye have PC2 negative scores values.

In addition, PCA loadings (Fig. 5) and the correlation coefficients between the spectra of Sudan I, Sudan IV, the blend of Sudan I + IV, and unadulterated samples (mean spectrum) were examined. PC1 loading has a similar shape to Sudan I dye spectrum with the maximum correlation coefficient value. On the other hand, PC3 is highly correlated to Sudan IV dye spectrum (highest correlation value). Finally, the assignation for PC2 is more dubious as the loading of this component resemble in its negative contribution to the unadulterated samples although the maximum correlation values are for both Sudan IV and the blend. These results suggest that sometimes it is difficult to assign each PC to each pure component, mostly taking into account the complex food matrices we are working with.

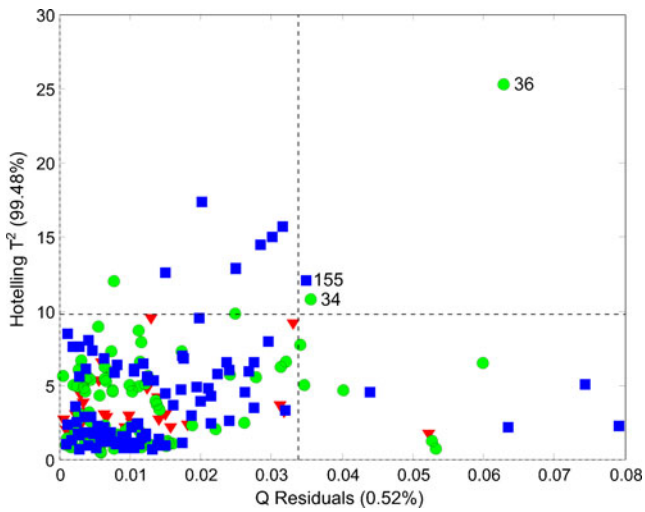

Fig. 6 Q residuals and T^2 Hotelling statistics from PLS-DA model. Original samples (*triangles*), samples with Sudan I dye (*circles*), and with Sudan I + IV dyes (*squares*)

Table 1 RMSECV values for PLS-DA and prediction rates for KNN

RMSECV in PLS-DA				Prediction rate in KNN			
LVs	C1	C2	C3	K=	C1	C2	C3
1	0.14630	0.56920	0.38710	**1**	**83.3**	**91.7**	**86.1**
2	0.14630	0.23550	0.18130	3	83.3	83.3	86.1
3	0.13500	0.10030	0.02809	5	75	83.3	80.6
4	**0.01667**	**0.01557**	**0.02671**	7	75	86.1	75
5	0.05621	0.02273	0.03933	9	66.7	86.1	72.2
6	0.11500	0.00560	0.03790	11	66.7	91.7	72.2

Final selected values are depicted in bold

Classification Techniques

In order to discuss the classification results, the following null hypothesis was considered: "S*ample is not adulterated with Sudan dyes"*. Therefore, two types of errors can be obtained: false positives and false negatives. In this context the implication of each error has different impact. The most serious risk for consumer health is associated with false negatives results because adulterated samples could be considered safe for consumption. Otherwise, false positives results have lesser impact because they only represent an economical risk: unadulterated samples are considered as adulterated samples. As consequence, these samples must be unnecessary withdrawn from sell points and submitted to a confirmatory analysis.

Also, there are two parameters for evaluating the classification model quality: sensitivity and specificity. Sensitivity is defined as the proportion of samples belonging to a class that were correctly identified by the model while specificity is the proportion of samples not belonging to a class which were identified as being foreign by the model.

During the model development, it is important to detect if there are samples with extreme behavior which could worsen the classification performance (outliers). Outliers can be detected by inspection of Q residuals and Hotelling T^2 values obtained during PLS-DA model construction (Bakeev 2010). Q values represent a measure of variance which is not captured by the model (fit of samples outside the model) while T^2 values reflects the variance captured by the model (fit of samples within the model). Figure 6 shows Q residuals values plotted against T^2 values with limits defined at a 95 % level of

Table 2 PLS-DA and KNN classification results

	% Model ability	% Prediction ability	
	PLS-DA	PLS-DA	KNN
Class 1	94	92	83
Class 2	98	100	92
Class 3	96	89	86

Table 3 Class assignation, sensitivity, and specificity results for PLS-DA and KNN

		Sample class	No. of samples	Classified in				Sensitivity	Specificity
				C 1	C 2	C 3	NAC	%	%
PLS-DA	Model	C 1	31	29	0	0	2	94	100
		C 2	91	0	89	0	2	98	100
		C 3	92	0	0	88	4	96	100
	Prediction	C 1	12	12	1	0	0	92	100
		C 2	36	0	36	0	0	100	96
		C 3	36	0	1	32	3	89	100
KNN	Prediction	C 1	12	10	1	1	0	83	100
		C 2	36	0	33	3	0	92	88
		C 3	36	0	5	31	0	86	92

NAC not in any class, *C1* class 1, *C2* class 2, *C3* class 3

confidence for the training data. It can be seen that three samples fall outside the limits defined by Q and T^2 values (samples 34, 36, and 155), so these samples were considered as outliers and a new classification models was built.

Regarding the classification parameters, the optimal number of PLS-DA latent variables was chosen using leave-one-out cross-validation to minimize the RMSECV for each class, and the final value is selected considering the optimal value for each class (four LVs). Otherwise, in KNN the number of neighbors (k) was chosen according to the minimum percentage prediction error ($k=1$; Table 1).

Table 2 shows the classification performance for PLS-DA and KNN. It can be observed that global classification accuracy was satisfactory: for PLS-DA model between 94 and 98 %, PLS-DA prediction step between 89 and 100 %, and the prediction rate for KNN was between 83 and 92 %.

PLS-DA gives three types of results: samples wrongly assigned, samples assigned to more than one class and samples not assigned to any class. Otherwise, KNN always assigns samples to a certain class. A deep look at the classification results can be seen in Table 3. It has to be mentioned that no false negatives were obtained with both classification techniques. This has a great significance from the point of view of its implication, as the risk involved when adulterated samples are being consumed as unadulterated is highly minimized. Otherwise, few false positives were obtained. This situation corresponds to unadulterated samples (class 1) that were wrongly classified as adulterated or samples classified in more than one class: its real class (class 1) and another class (2 or 3). Such types of errors involve an economical risk, as these samples must be immediately withdrawn until its real state is confirmed with other technique such as HPLC. Finally, there are some misclassifications between adulterated classes, where samples are either assigned to another adulterated class or not assigned to any class.

Concerning the studied concentrations, PLS-DA misclassified samples belong to the lowest level (1 ppm) and KNN gives more misclassifications at 1 ppm with only one sample that belongs to 2.5 ppm. Therefore, adulterated samples at 5 and 2.5 ppm and most samples at 1 ppm are correctly classified with both classification techniques. It should be highlighted that PLS-DA misclassified samples are coincident with KNN samples, so although PLS-DA and KNN are based on a different fundament both techniques give concordant results.

Regarding sensitivity (Table 3), quite satisfactory results were obtained with PLS-DA: between 93 and 99 % considering the three classes. In this case, few samples that do not belong to any class influence these percentages. For KNN, sensitivity percentages are slightly lower: between 83 and 92 %, where samples wrongly assigned to another class are responsible of such values. In view of these results, PLS-DA outcomes are more advantageous because wrongly class assignations are minimized. Furthermore, excellent specificity values were obtained for class 1 (unadulterated samples) with both PLS-DA and KNN, as all adulterated samples were correctly rejected (100 % specificity, no false negative results). For adulterated samples with Sudan I or blends of Sudan I + IV, respectively (classes 2 and 3), PLS-DA gave almost 100 % of specificity, which means that false positives (related with unadulterated samples) and samples adulterated with either individual or blend of dyes are quite likely to be excluded from the corresponding class. In case of KNN, wrong assignations between adulterated classes (classes 2 and 3) decrease the specificity, although it is still considered satisfactory.

Conclusions

The use of UV-visible spectroscopy and multivariate analysis represent a fast, simple, and affordable screening tool for the identification of Sudan I and blends of Sudan I + IV dyes in three varieties of paprika and *aji molido* samples at three concentration levels. This methodology can be implemented as an alternative to the classical methods for determining Sudan dyes in foods. Adulterated samples with single or blended dyes were detected in concentrations that are within

the usual range to obtain commercial benefits. The capability of detecting mixtures of Sudan dyes is a very important advantage because each Sudan dye generates different hazardous metabolites in human body so their toxicity may be enhanced by the simultaneous presence of such dyes.

Two classification techniques were used: PLS-DA and KNN. Considering the null hypothesis, no false negatives were obtained with both techniques which is quite satisfactory because this type of result implies a risk for consumer health. The sensitivity and specificity of the models were satisfactory but PLS-DA gives slightly better results than KNN.

This work opens the possibility for implementing portable UV-visible spectrometers that makes accessible the analysis to be used in situ. This methodology would be very useful when a rapid response is required like situations commonly found in international commerce, where the screening of food products is mandatory to evaluate their safety or their accomplishment according to a specific legislation.

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Conflict of Interest Carolina Di Anibal declares that she has no conflict of interest. Maria Susana Rodriguez declares that she has no conflict of interest. Liliana Albertengo declares that she has no conflict of interest. This article does not contain any studies with human or animal subjects.

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