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Synchronous fluorescence and multivariate classification analysis as a screening tool for determining Sudan I dye in culinary spices

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ABSTRACT

Spices are a globally traded commodity which has been found to be adulterated with forbidden Sudan dyes. This work proposes a screening method for determining the adulteration of paprika varieties (mild, hot and smoked) with Sudan I dye, based on constant-wavelength synchronous fluorescence spectroscopy with multivariate classification. Different wavelength-intervals ($\Delta\lambda$) were evaluated. Classification models were built with Partial Least Squares-Discriminant Analysis (PLS-DA) at two Sudan I dye concentration levels (1 and 5 mg L^{-1}) and they were tested with samples at a lower level (0.5 mg L^{-1}). Classification results were quite satisfactory when a strategy based on first-derivative spectra was used for improving classification results. $\Delta\lambda = 60$ nm was chosen as the optimum wavelength interval giving a 100% of sensitivity and specificity. These results are promising because the risk of assigning adulterated samples as safe to be consumed is highly minimized. The proposed method is feasible, rapid and simple taking advantage of Sudan I fluorescence phenomena in a direct way.

1. Introduction

Sudan dyes are synthetic azo-dyes mainly used as colorants in industrial applications. Sudan dyes are forbidden to be used in foods as they are proved to be potential carcinogens for humans. These dyes are degraded to aromatic amines which can act directly in liver cells causing toxic liver disease but also may induce cell gene mutation (Fonovich, 2013; Stiborová et al., 2009; Xu, Heinze, Paine, Cerniglia, & Chen, 2010). The bright color of Sudan dyes enhances the appearance of commercial products, so unfortunately these dyes were found in foodstuffs. Culinary spices are a globally traded commodity which has been found to be adulterated with forbidden Sudan dyes. In the last decade these dyes were found in Europe in many imported products, so as consequence the European Union has adopted regulatory measurements against the use of such dyes in foods (European Commission Decision 2004/92/EC). The continuing illicit use of Sudan dyes as food colorants has received increasing attention all over the world. Therefore there is a need for developing rapid, simple and accurate analytical methods to be used as monitoring tools for determining Sudan dyes in food products.

Several methods have been developed for determining the presence of Sudan dyes in foodstuffs, and the most common methods are based on high-performance liquid chromatography (HPLC) with different detection systems (Rebane, Leito, Yurchenko, & Herodes, 2010) as well as novel sample clean-up (Enríquez-Gabeiras, Gallego, Garcinuño, Fernández-Hernando, & Durand, 2012; Yan, Gao, & Qiao, 2012; Zhang et al., 2012). Recently other methods have been developed such as electroanalytical techniques (Wu et al., 2013; Yin et al., 2011), immunoassays (Liu, Zhang, Zhang, Gao, & Wang, 2012; Xiao et al., 2011), and spectroscopic methods such as UV–Visible (Di Anibal, Rodriguez, & Albertengo, 2014), ^{1}H-NMR (Di Anibal, Ruisánchez, & Callao, 2011), Raman (Di Anibal, Marsal, Callao, & Ruisánchez, 2012) and NIR (Haughey, Galvin-King, Ho, Bell, & Elliott, 2015). Fluorescence has also been reported for determining Sudan dyes including the use of metal nanoclusters (Chen et al., 2014), fluorescence quenching of serum albumin (Zhang, Dai, Zhang, Yang, & Liu, 2008) and hemoglobin (Zhang, Wang, & Jiang, 2009), fluorescence combined with artificial neural networks (Chen et al., 2011) and a method based on ligand-exchange using calcein as fluorescent indicator (Huang, Yang, Li, & Luo, 2013). These methods determine Sudan dyes in an indirect way and some of them have not been applied to foods. Furthermore, spectrometric methods have the advantage of providing a rapid analytical response but to obtain useful information when working with complex matrices such as foods, they must be combined with proper multivariate analysis.

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Fluorescence spectroscopy is a rapid, sensitive, and non-destructive analytical technique. The application of fluorescence spectroscopy for direct analysis of foods has increased during the last decade, probably due to the wide-spread use of multivariate analysis (Christensen, Nørgaard, Bro, & Engelsen, 2006). Sometimes conventional fluorescence is not suitable for the analysis of complex multi-component samples such as foods due to severe spectral overlap. This could be overcome by using synchronous fluorescence (SyF) where both, excitation and emission monochromators, are scanned simultaneously in such a manner that a constant wavelength interval is kept between emission and excitation wavelengths ($\Delta\lambda$). Using suitable $\Delta\lambda$, synchronous fluorescence reduces spectral overlaps by narrowing spectral bands and simplifies spectra (Patra & Mishra, 2002). Its potential can be appreciated by the increasing number of studies in the literature, for example beer analysis (Insińska-Rak et al., 2007), edible vegetable and olive oils characterization (Kunz, Ottaway, Kalivas, Georgiou, & Mousdis, 2011; Sikorska, Gliszczynä-Swiglo, Wiglo, Khmeliskii, & Sikorki, 2005), cooking meat time study (Sahar, Boubellouta, Portanguen, Kondjoyan, & Dufour, 2009), determination of phytic acid in foodstuffs (Cao, Dong, & Chen, 2011), cheese characterization (Khaled, Romdhane, & Abderrahmane, 2012) and determination of antibiotics in milk (Kaur, Saini, Singh, & Malik, 2012).

Classification methods are designed to find mathematical models able to recognize the membership of each object to its proper class on the basis of a set of measurements. Once a classification model has been obtained, the membership of unknown objects to one of the defined classes can be predicted. Multivariate classification was performed with Partial Least Squares-Discriminant Analysis (PLS-DA). This technique is a multivariate projection method that has been widely used in food adulteration issues (Marini, 2013).

The aim of this study is to evaluate the use of synchronous fluorescence (SyF) combined with multivariate classification techniques as a screening tool for determining Sudan I dye in culinary spices, specifically in mild, hot and smoked paprika. Taking into account the advantages fluorescence spectroscopy provides such as high sensitivity and selectivity, we attempt to determine adulterations at low Sudan I dye concentration levels with a direct, rapid and inexpensive method that does not require hard experimental work.

2. Material and methods

2.1. Samples and reagents

A total of 30 paprika samples distributed among 12 mild, 10 hot and 8 smoked paprika were purchased from local markets. Isopropyl alcohol (analytical grade) was obtained from Anedra (Bs. As., Argentina). Sudan I [1-(phenylazo)-2-naphtol] (dye content $\geq$95%) was obtained from Sigma Aldrich (United Kingdom). A 100 mg L^{-1} stock solution of Sudan I was prepared in isopropyl alcohol and stored at 4 °C until use.

2.2. Sample preparation

For the extraction process, 200 mg of each paprika sample was weighed in analytical balance, 10 mL of isopropyl alcohol was added and then samples were shaken in an automatic shaker (Shaker Pro Vicking) during 15 min at 150 rpm. Each extract was obtained by filtering twice, first with glass microfiber filters and then with nylon syringe filters of 0.45 μm (Microclar Argentina). The obtained extracts were used to prepare both original (non-adulterated) and adulterated samples with Sudan I dye. For original samples, an aliquot of each extract (200 μL) was taken and diluted to 10 mL in isopropyl alcohol in volumetric flasks, and for adulterated samples an appropriate amount of Sudan I dye solution was added to get a final concentration of 0.5, 1 and 5 ppm (mg L^{-1}).

2.3. Instrument

Fluorescence spectra were acquired on a Jasco spectrofluorometer (model FP6500), equipped with a xenon discharge light source (150 W). Operational parameters were excitation and emission slit widths set at 2 nm, data pitch of 1 nm, scanning speed of 1000 nm s^{-1}, time response of 0.2 s. All measurements were done at high sensitivity. A quartz cell 10 × 4 × 45 mm was used. In conventional fluorescence the excitation wavelength was 420 nm. Synchronous spectra were recorded in the region of 400–690 nm varying the wavelength interval from 20 to 60 nm in steps of 10 nm.

2.4. Data analysis

Partial Least Squares-Discriminant Analysis (PLS-DA) was used as multivariate classification technique. In PLS-DA, both independent variables (X) and dependent variables (Y) are simultaneously modeled to find the latent variables (LVs) in X that will predict latent variables in Y, like a classical PLS model. PLS maximizes the covariance between X and Y. This classification method is aimed at finding the variables and directions in the multivariate space which discriminate the established classes in the calibration set. An optimal number of LVs can be estimated by the minimum value of root mean squared error of cross-validation (RMSECV). PLS-DA develops a model for each class. The closer an element of a certain column in Y is to 1 and the elements of the other columns to 0, the more likely an object is a member of the particular class (Brereton, 2009). In our case, class 1 is defined by unadulterated samples and class 2 by adulterated samples with Sudan I dye.

Receiver Operator Characteristic (ROC) curves (Brown & Davis, 2006; Fawcett, 2006) are useful to evaluate the sensitivity and specificity of a classification model. ROC curves can be defined by plotting the sensitivity against specificity for different PLS-DA threshold values.

Multivariate analysis was performed with Matlab 7.0 software (MathWorks, Natick, USA) and PLS Toolbox 3.5 (Eigenvector Research Incorporated). Spectra were autoscaled before classification analysis.

3. Results and discussion

3.1. Spectra

The direct determination of Sudan dyes in culinary spices can be achieved by means of the fluorescence phenomena that such dyes have. This advantage allows us to propose a method based on synchronous fluorescence which is direct, rapid, inexpensive and it does not require hard experimental work.

Samples were firstly studied by conventional fluorescence by setting an excitation wavelength at 420 nm. Fig. 1 shows the spectra of an original (unadulterated) and adulterated sample together with pure Sudan I spectrum. It can be observed broad spectra with lack of signals, which do not allow visual differences between unadulterated and adulterated samples. The analysis of complex food matrices is difficult with conventional fluorescence, so in light of these results, synchronous fluorescence was evaluated for the following measurements.

Synchronous fluorescence spectra were measured at five wavelength-intervals (from 20 to 60 nm). This range of intervals was chosen because when selecting lower intervals than 20 nm, fluorescence detector was saturated in many spectral regions while

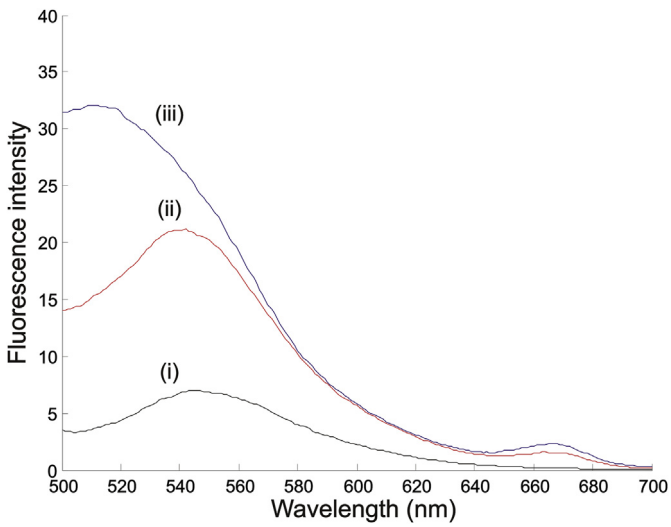

Fig. 1. Conventional fluorescence spectra of (i) pure Sudan I dye, (ii) an original sample and (iii) an adulterated sample. Sudan I concentration level is 5 ppm.

upper intervals than 60 nm did not allow visual difference between unadulterated and adulterated samples. As an example, Fig. 2 shows the spectra of both an unadulterated and adulterated mild paprika (randomly chosen) with pure Sudan I spectrum at the five different wavelength-intervals. It can be seen that the spectra of the adulterated sample is clearly different from the unadulterated one at all wavelength-intervals and most of adulterated samples present peaks belonging to the adulterant (Sudan I dye) which are not present in the unadulterated samples. This behavior was similar for the rest of paprika samples.

3.2. Classification results

With the aim to achieve the best discrimination between the two classes (i.e. class 1 formed by unadulterated samples and class 2 by adulterated samples), an appropriate wavelength-interval ($\Delta\lambda$) must be chosen for SyF measurements. Therefore values ranging from $\Delta\lambda = 20–60$ nm were evaluated considering the maximum Sudan dye concentration level (5 ppm). PLS-DA classification models were validated using a cross-validation approach with five deletion groups (Venetian-blind scheme) in which the first deletion group is formed by samples 1, 6, 11, … , the second deletion group by samples 2, 7, 12, … , and so on. Classification results show that when the maximum concentration level was evaluated, all wavelength-intervals gave 100% of correct classification (for both classes) except for $\Delta\lambda = 60$ nm that gives a 97% for class 1 and a 100% for class 2. This was somehow expected as the spectra containing the adulterant can be highly differentiated from the unadulterated spectra at all wavelength-interval as shown in Fig. 2.

The study follows by building a new classification model with the addition of a lower concentration level. Hence, class 2 is now formed by adulterated samples at two concentration levels: 5 and 1 ppm. During the development of a classification model, 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 reflect the variance captured by the model (fit of samples within the model). Therefore, Q residuals vs Hotelling T^2 plots were evaluated for all wavelength-intervals. As example, Fig. 3 shows a graph with defined limits at a 95% level of confidence for $\Delta\lambda = 40$ nm. It can be seen that samples 30, 60, and 90 fall outside the limits defined by Q and T^2 values, which corresponds to the same smoked paprika sample that is unadulterated (30) and adulterated at 5 and 1 ppm (60 and 90, respectively). It has to be mentioned that a similar behavior was observed for the rest of wavelength-intervals, therefore these three outliers were eliminated from all datasets.

Datasets were then divided into training set (used to construct the classification model) and test set (used to predict unknown samples). The test was generated by leaving out 6 out of 29 samples for class 1 (which represent a 20% of the total samples). For class 2 the same criterion was applied considering 5 and 1 ppm concentrations, so 12 out of 58 samples have been selected. The selection of test samples was based on the Kennard-Stone algorithm (Di Anibal, Ruisánchez, et al., 2012) that selects one by one the samples which are furthest from each other in the group (in terms of the Euclidean distance) in the multivariate space they are spread.

Before the modeling process, the optimal number of PLS-DA latent variables (LVs) retained must be chosen. This was done according to the minimum root mean square error of cross validation (RMSECV) in terms of the fractional misclassification error rate. Table 1 shows PLS-DA classification results with number of LVs used for each model, explained variance percentage and recognition and prediction ability for training and test set, respectively. It can be seen that the percentage of explained variance is higher than 83% in all cases. Regarding classification results, the maximum recognition ability (100%) was achieved with all the models ($\Delta\lambda = 20–60$ nm) for both classes. It can be remarkable that the test set gave a prediction ability of 100% for both classes ($\Delta\lambda = 30$, 40, 60 nm), 83.3% (class 1) and 100% (class 2) considering $\Delta\lambda = 20$ nm and 83.3% for the two classes with $\Delta\lambda = 50$ nm. At this point, the classification models built at all wavelength-intervals considering two Sudan I concentration levels (5 and 1 ppm) gave quite satisfactory results.

In order to evaluate the ability of the built classification models, samples at a lower Sudan I concentration level (0.5 ppm) were used as test set (unknown samples). The five same wavelength-intervals were taken into account. Table 2 shows the prediction results obtained with the models presented in Table 1. It can be seen that all wavelength-intervals give a prediction ability higher than 72% (original data), being the best results for $\Delta\lambda = 50$ and 60 nm (around 88%). To improve the obtained prediction results, first-derivative spectra were evaluated. The combination of synchronous and derivative fluorescence enhances minor spectral features which allow to increase differences between spectra and to resolve overlapping bands (Patra & Mishra, 2002; Sádecká & Tóthová, 2007). Table 2 also shows derivative results and it can be observed a great improvement on prediction ability for all wavelength-intervals.

When dealing with a food adulteration problem, it is important to know whether a sample is safe to be consumed or not. In this context, if adulterated samples (class 2) are wrongly classified as unadulterated (class 1) they represent a risk for consumer health. Results presented in Table 2 are shown in detail in Table 3 by means of a confusion matrix. Besides improving classification results, the first-derivative strategy reduced the number of wrongly predicted samples in class 1 (i.e. samples from class 2 assigned to class 1) for all wavelength-intervals (see bold values). This is very advantageous taking into account the implication such classification errors have on consumer health because the risk of assigning adulterated samples as safe is highly minimized. Finally, $\Delta\lambda = 60$ nm was selected as the optimum interval because gives the best classification results (100% of correct classification).

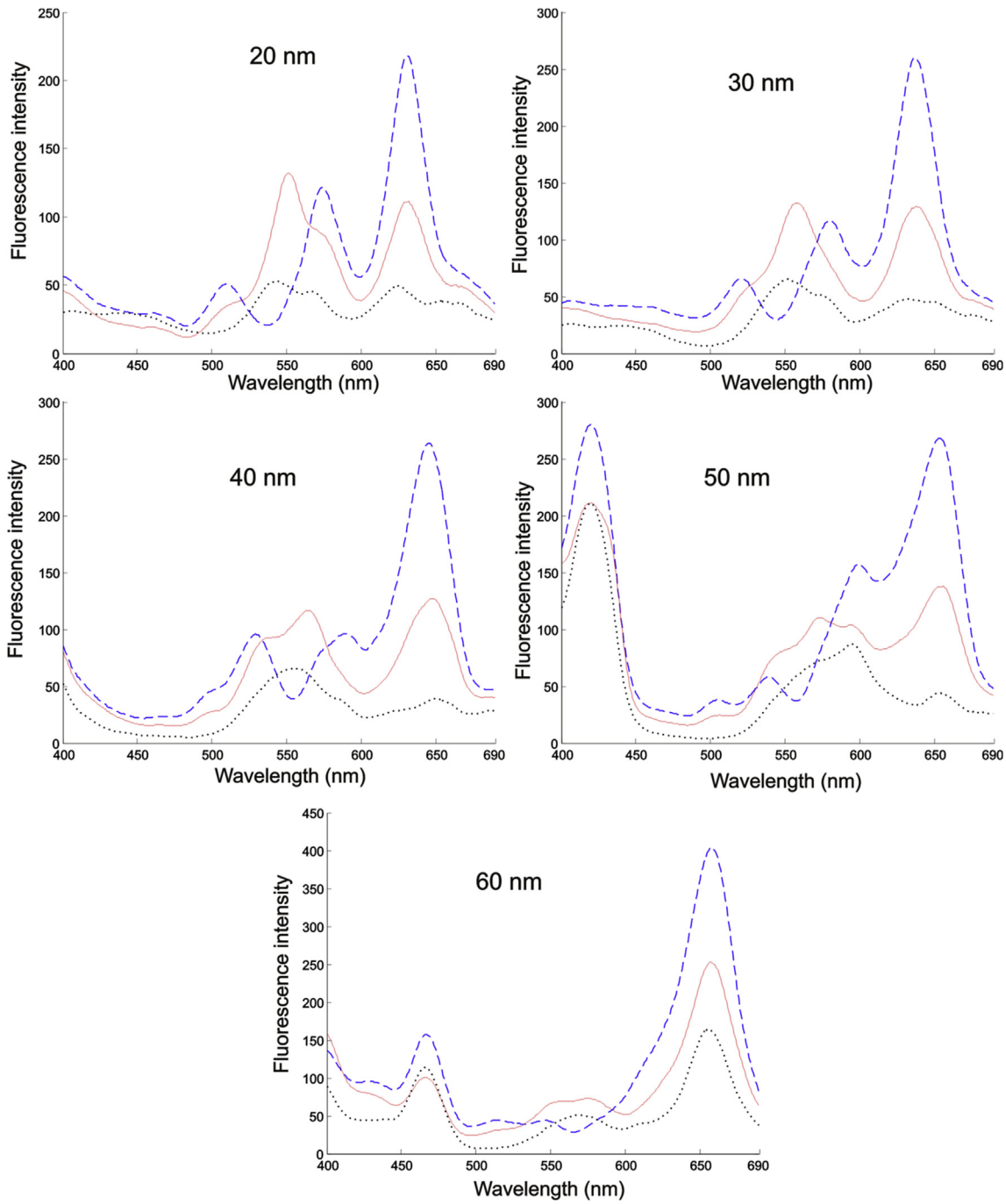

Fig. 2. Synchronous spectra of unadulterated samples (dashed lines), adulterated samples at 5 ppm (solid lines) and pure Sudan I dye (dotted line) at all wavelength-intervals.

There are two parameters to evaluate the quality of a classification model: sensitivity and specificity. The sensitivity is the ability of the model to recognize its own samples while the specificity is the ability to distinguish external samples. ROC curves combine these two parameters. Highly discriminating classifiers give ROC curves that consist of a vertical line followed by a horizontal line while models that randomly assign samples into two groups tend to have ROC curves that are along the diagonal axis and are poor classifiers without any discrimination (Brereton, 2009). The area under the ROC curve called area under curve (AUC) is a measure of a model's ability to discriminate objects of different classes. AUC values range from 0.5 to 1.0 being the higher the AUC, the better the model. Fig. 4 shows ROC curves for PLS-DA model built with first-derivative data at $\Delta\lambda = 60$ nm considering class 1 (unadulterated) and class 2 (adulterated). The crossing point between the maximum sensitivity (1) and the maximum specificity (1) correspond to the optimum PLS-DA threshold which is calculated in such a way that the number of true positives (sensitivity) and true negatives (specificity) are maximized. It can be observed perfect classification ability (100% sensitivity and specificity) because ROC curves look like squares at the upper left corner with an area close to one. This behavior shows that samples are not randomly predicted and PLS-DA is a reliable classifier. Both classes follow the same trend.

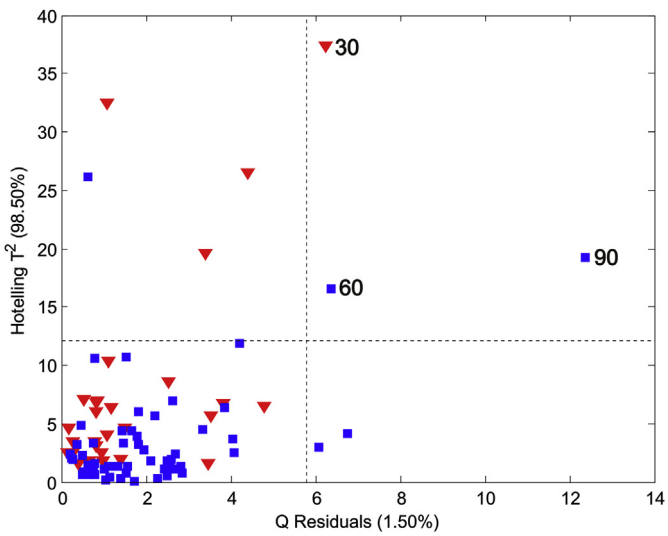

Fig. 3. Q residuals and T^2 Hotelling statistics from PLS-DA model ($\Delta\lambda = 40$ nm). Original samples (triangles) and adulterated samples (squares) at the two concentration levels (1 and 5 ppm).

Table 1
Classification results for training and test set.

Δλ	N° LVs	Explained variance	Class 1		Class 2	
			Training	Test	Training	Test
20	4	83.25	100	83.3	100	100
30	4	83.74	100	100	100	100
40	4	85.87	100	100	100	100
50	4	85.74	100	83.3	100	83.3
60	4	86.42	100	100	100	100

Class 1 correspond to unadulterated samples and class 2 adulterated samples (5 and 1 ppm).

Table 2
Percentage of prediction ability for adulterated samples at 0.5 ppm.

Δλ	% Prediction ability	
	Original data	Derivative data
20	72.4	86.2
30	72.4	86.2
40	79.3	86.2
50	86.2	96.5
60	89.6	100

Results are presented for original and first-derivative data.

Table 3
Confusion matrix of the results shown in Table 2.

Δλ	Number of samples	True class	Predicted in		
			Class 1	Class 2	NAC[a]
20	29	2	7	21	1
			2	**25**	**2**
30	29	2	7	21	1
			2	**25**	**2**
40	29	2	5	23	1
			4	**25**	**0**
50	29	2	4	25	0
			1	**28**	**0**
60	29	2	1	26	2
			0	**29**	**0**

Original data and first-derivative data (bold values).
[a] NAC: Not in any class.

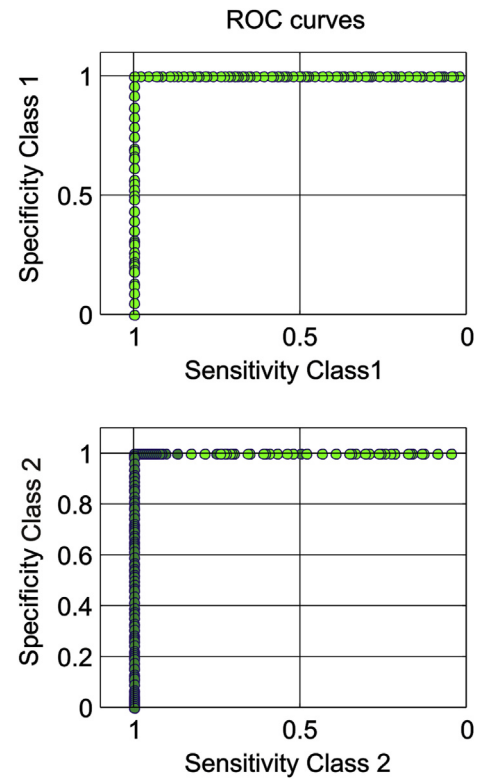

Fig. 4. ROC (Receiver Operating Characteristics) curves of PLS-DA model for first-derivative data at $\Delta\lambda = 60$ nm.

4. Conclusions

This work proposes a direct, rapid, feasible and reliable screening tool for determining Sudan I dye in three varieties of paprika spices based on synchronous fluorescence and multivariate classification. Synchronous fluorescence is more appropriate than conventional fluorescence when a complex matrix such as paprika is analyzed. Different wavelength-intervals (Δλ) were evaluated. A strategy based on first-derivative spectra demonstrated to be useful for improving classification results, and excellent classification results (100%) were obtained when using $\Delta\lambda = 60$ nm. The accuracy of this optimal classification model was assessed by means of ROC curves. It has to be highlighted that this methodology determined a lower adulterant concentration level respect to our previous works (Di Anibal, Callao, & Ruisánchez, 2011; Di Anibal, Rodriguez, & Albertengo, 2014).

The developed method can be used as a practical screening tool to distinguish food samples suspicious to be adulterated with Sudan I dye that could be applied to achieve on-site detection in situations requiring a rapid response such as those found in international commerce. In addition, this methodology may also be valuable to determine the adulteration of other foods such as sauces and related-foods containing spices as well as food adulteration with mixtures of Sudan dyes, considering the advantages that synchronous fluorescence provides for multi-component matrices.

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