Establishing time stability for multivariate qualitative methods. Case study: Sudan I and IV adulteration in food spices

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ABSTRACT

A multivariate qualitative method using UV-visible spectroscopic data and a PLS-DA chemometric treatment was proposed to identify whether paprika samples were adulterated with Sudan I and Sudan IV dyes, or their mixtures. The method was validated by calculating the main performance parameters (sensitivity and specificity) and determining the stability over time.

Three classes were defined: unadulterated samples (class 1), samples adulterated with Sudan I (class 2) and samples adulterated with Sudan IV (class 3). A total of 81 samples belonging to these classes were analyzed. There was also an additional data set consisting of 54 samples adulterated with a mixture of two dyes at two different concentration levels, which were analyzed and predicted with the established models. In addition, all 135 samples were analyzed at different times over a 6–month period to study the model's stability over time.

In general, the main performance parameters were very satisfactory. As far as training samples is concerned, sensitivity was 100% for the three classes studied. And specificity was 100% for the unadulterated class and for the adulterated with Sudan IV class, and slightly lower (96%) for the adulterated with Sudan I class. Regarding samples from the additional set, excellent specificities were obtained because no samples were assigned to the unadulterated class. In addition, sensitivity was close to 100% for the Sudan IV class and around 75% for the Sudan I class. All the main performance parameters were maintained throughout the 6 months of the study.

Keywords: multivariate classification, PLS-DA, time stability, food adulteration, individual and blends adulterants, Sudan dyes

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1. Introduction

Multivariate qualitative methods have increasingly been applied in food analysis. According to the International Union of Pure and Applied Chemistry (IUPAC) qualitative analysis is "the analysis in which substances are identified or classified on the basis of their chemical or physical properties" (Currie, 1995). Currently, and largely due to the expansion of chemometrics, and more specifically of classification techniques, qualitative analysis is used not only to identify compounds but also to classify and authenticate foodstuffs, products, specimens and materials (Szymanska et al., 2015).

Like all analytical methods, qualitative methods need to be validated by establishing their performance parameters. However, unlike the validation protocols of quantitative methods, which have been the subject of numerous studies, interest in the principles and validation of qualitative analysis has only been developing since the late 1990s (Valcárcel, Cárdenas & Gallego, 2000; Thompson, Ellison & Wood, 2002; Trullols, Ruisánchez & Rius, 2004). Additionally, studies about the validation of multivariate methods are even more recent (Szymanska et al., 2015; Lopéz, Callao & Ruisánchez, 2015; Cuadros-Rodríguez, Pérez-Castaño & Ruiz-Samblás, 2016; Galarini et al., 2011; Riedl, Esslinger & Fauhl-Hassek, 2015; Lopéz, Colomer, Ruisánchez & Callao, 2014). As an example, in 2013, AOAC International published a guideline for the validation of binary qualitative methods for detecting biological and chemical compounds (AOAC International, 2013).

In this paper, we propose to establish and validate a multivariate qualitative method for the detection of Sudan I and IV dyes and mixtures thereof in culinary spices. Samples were characterized according to their UV-visible spectrum, using the chemometric techniques of principal component analysis (PCA) for the data exploration stage and partial least squares discriminant analysis (PLS-DA) to establish the classification model for the screening stage. The UV-Vis has been shown to be suitable for detecting adulteration in spices by Sudan dyes (Di Anibal, Odena, Callao & Ruisánchez, 2009; Di Anibal, Callao & Ruisánchez, 2011; Di Anibal, Rodriguez & Albertengo, 2015; Reinholds et al., 2015; Di Anibal, Rodriguez & Albertengo, 2014). When the concentration level is low, techniques such as liquid chromatography, mass spectroscopy and others would be more suitable than UV. But, as Mishra stated, the usual concentrations present in species are higher enough to be detected by UV-Vis (Mishra et al. 2007). The method was validated by establishing the main performance parameters and determining their stability over time.

Sudan dyes I-IV are a family of synthetic azo dyes that are classified as class 3 carcinogens by the International Agency for Research on Cancer (IARC) (IARC, 1975). In the early 2000s, they were found in some food products in some European countries and, in response to the alarm caused, the European Commission generated the Commission Decision 2005/402 / CE (23 May 2005) on emergency measures regarding chili, chili products, curcuma and palm oil. In recent years, notifications concerning foods adulterated with Sudan dyes have declined considerably, so European countries only kept

the Sudan I and IV regulations in check (Commission Regulation (EC) No 594/2012). Sudan I and IV are often found together in some products, mostly in chilli, seasonings and spice mixtures (RASFF, 2005; RASFF, 2006). It should be borne in mind that each Sudan dye has a different effect on the human body, so it is important to detect adulterated foods containing more than one Sudan dye, because the simultaneous presence of these can change or increase their toxicity (Xu et al., 2010).

The alarm caused by the discovery of Sudan dyes in food products raised considerable scientific interest in developing analytical methods to determine them. This interest is still alive today (Petrakis et al., 2017; Bazregar et al., 2018; Kılınç, Çelik & Bilgetekin, 2018; Liu, Qi & Zhang, 2018). Of the different methods developed, those based on the multivariate treatment of spectroscopic data are worth mentioning here because of their connection with the present study (Li et al., 2016; Di Anibal, Marsal, Callao & Ruisánchez, 2012; Lohumi et al.,2017; He et al., 2015; Haughey et al., 2015). A few of them have focused on the determination of dye mixtures but none of them have established the validity of the models over time. This is the most important contribution of this study and it can be extrapolated to multivariate qualitative methods, for any type of sample and objective.

2. Material and methods

2.1. Samples

A total of 27 spices of different commercial brands were purchased from local markets, distributed as follows: 14 mild and 13 hot paprika. An extraction process with acetonitrile was applied to these spices. Details about previous studies and experimental treatment can be found elsewhere (Di Anibal, Odena, Callao & Ruisánchez, 2009). These extracts are a set of unadulterated samples named Class 1.

The two adulterated sample sets, one adulterated with Sudan I (class 2) and the other with Sudan IV (Class 3), were obtained by spiking the unadulterated samples with an appropriate amount of one Sudan dye in such a way that the final concentration was 5 mg/g. This is the usual concentration found in adulterated spices (Mishra et al., 2007).

Two additional adulterated data sets, with mixtures of both Sudan dyes (I and IV), were prepared. The first additional set consisted of samples adulterated with 5mg/g of each dye (final concentration of both 10mg/g), to keep the amount of each dye used to establish the corresponding class model. The second consisted of samples adulterated with 2.5mg/g of each dye, so that the final concentration of both was 5mg/g, just as it was for establishing the class model. Finally, the UV-visible spectrum was acquired for 135 samples.

To accurately describe the system under study, a sufficient number of samples should be available to produce independent training and test sets. In this study, because of the relatively small number of samples for each class (27), they were all placed in the training set, which was validated by a leave-one-out cross-validation procedure (Foca et al., 2009).

2.2 Instrumentation and software

UV-visible measurements were made by an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies Inc., Palo Alto, CA, United States) equipped with a diode array detector (DAD) and ChemStation software. Each spectrum was registered in the range between 300 and 600 nm with a spectral resolution of 1 nm (301 variables).

Multivariate analysis was performed with Matlab software (Version 7.0, The MathWorks, Inc., Natick, USA) and PLS Toolbox version 7.0.2 (Eigenvector Research Incorporated). Data was pre-processed with baseline correction, smoothing by Savitzky Golay and mean centering before the chemometric treatment.

3. Background

Principal components analysis (PCA) (Esbensen & Geladi, 2009; Li Vigni, Durante & Cocchi, 2013) is an unsupervised exploratory analysis that can be used to visualize sample distribution in the multivariable space, check any trends, clustering or identify possible outliers previously to the classification step.

PCA seeks to reduce the dimensionality of a data set consisting of a large number of variables and defines new variables as linear combinations of the original variables. Redundant information is summarized while as much of the variation as possible is retained in the data set

Partial Least Squares-Discriminant Analysis (PLS-DA) is the classical PLS regression technique adapted to a supervised classification task (Bevilacqua et al., 2013). It aims to establish a linear regression between a matrix of independent variables X (spectra) and a matrix of dependent variables (classes). Dependent variables Y designate the class to which a sample belongs with a binary response, where 1 indicates membership and 0 does not. This means that samples of class 1 were encoded as (1,0,0), class 2 as (0,1,0) and class 3 as (0,0,1).

The prediction of the model for each sample is expressed in terms of a value around 1 or zero. A threshold is set between 0 and 1. Values higher than the threshold mean that the sample belongs to the class considered and values lower than the threshold that they do not.

The threshold is usually calculated using Bayesian statistics, which assume that the predicted values of Y follow a normal distribution similar to that of future samples and select the value of Y in which the number of false positives and false negatives should be minimized for future predictions. Details of the PLS-DA technique can be found in the literature (Barker & Rayens, 2003).

Performance parameters. To evaluate the quality of the classification model, sensitivity and specificity values were evaluated. Sensitivity is the proportion of samples belonging to a class that were correctly identified as belonging to the class. And the specificity is the proportion of samples not belonging to a class that were identified as foreign by the model (Callao & Ruisánchez, 2018).

Sensitivity = 100 × [TP/(TP + FN)]
Specificity = 100 × [TN / (TN + FP)]

where TP = true positive, number of positive samples that are correctly identified as positive samples;
FN = false negative, number of positive samples that are misclassified as negative samples;
FP = false positive, number of negative samples that are incorrectly identified as positive samples;
TN = true negative, number of negative samples that are correctly identified as negative samples.

4. Results and Discussion

Figure 1a shows the spectra of the standards of Sudan I, Sudan IV and Sudan I + IV in acetonitrile at a concentration of 5mg/g. It can be observed that the spectra of the two dyes clearly overlapped, although Sudan I had higher intensity values and Sudan IV appeared to be slightly shifted to the right. Figure 1b shows the spectra of two randomly chosen unadulterated samples (mild paprika and hot paprika). It can be seen that there are no significant differences between them and that the absorbance region is the same as the dye region.

Before submitting the data set to any chemometric analysis, two data pre-treatments were applied to the spectra. A Savitzky-Golay smoothing using a window with eleven points and a second-order polynomial was applied to reduce instrumental noise. Then, the baseline was corrected with a polynomial of order 0.

First, an exploratory analysis by Principal Component Analysis (PCA) was performed on all the samples from the three classes studied. Fig. 2 shows the scores for the first two PCs. The total variance explained by the first two components was 97,56% (PC1 = 87.37% and PC2 = 10.19%). It can be seen that the unadulterated samples are well separated from those adulterated with Sudan I and Sudan IV. PC1 differentiates between unadulterated samples (negative scores) and samples adulterated with Sudan I (positive scores), while those samples adulterated with Sudan IV have negative and positive scores. On the other hand, PC2 distinguishes between the unadulterated samples and the samples adulterated with Sudan I (negative scores) from the samples adulterated with Sudan IV (positive scores). The plot shows that the samples do not show tendencies regarding the paprika type (mild paprika or hot paprika).

Two unadulterated samples (no. 4 and no. 20) present higher PC1 scores than the rest of their class since they were the only ones with a positive PC1 score, overlapping with Sudan I samples. The corresponding adulterated samples were also the ones with the highest PC1 scores in Sudan I and Sudan IV classes. It should be pointed out that these samples are a special variety of mild and hot paprika called “De la Vera”. They had be kept on further analysis.

Fig. 3 shows the PCA loadings for the first two PCs. The PC1 loading has spectral characteristics similar to the Sudan I dye spectrum while the PC2 loading correlates with the Sudan IV dye spectrum as it has intensity values between 550 and 600 nm like Sudan IV. The following PCs loadings (not shown) are correlated to both Sudan dyes spectra as well as to the samples spectra.

PLS-DA was the technique used to establish the model for the three classes: unadulterated samples (class 1), samples adulterated with Sudan I (class 2) and samples adulterated with Sudan IV (class 3). PLS-DA classification was performed with three latent variables (LVs), which were chosen using leave-one-out cross-validation to minimize the root mean square error for cross validation (RMSECV) for each class.

Fig. 4 shows the PLS-DA assignation of samples to each of the predefined classes. The horizontal line corresponds to the threshold and the symbols are the predicted value for each sample in the different classes. All unadulterated samples were predicted to belong to class 1 (unadulterated, Fig 4a, prediction values higher than the threshold) and only one sample was wrongly assigned to class 2 (adulterated with Sudan I, Fig 4b, prediction values higher than the threshold). This assignment to class 2 is a false positive because an unadulterated sample was classified as being adulterated with Sudan I. It can be regarded as an error without serious consequences, because this sample would be submitted to a confirmatory analysis. All samples from class 2 and class 3 are all properly classified in their own class (Fig 4b and 4c, prediction values higher than the threshold). On the other hand, the mild and hot paprika samples called "De la Vera" (no. 4 and no. 20), unadulterated and spiked samples, were assigned correctly even though they behaved slightly differently from the others observed in the PCA plot. These results, expressed in terms of sensitivity and specificity, are shown in the first column of Table 1 (indicated as t0). It can be seen that they were 100% for the three classes, except for class 2, which as explained above, has a specificity of 96%.

As far as the prediction of the additional data set is concerned, all samples adulterated with a mixture of 5mg/g of each Sudan dye were properly assigned to their own class. As they are mixtures, they were assigned to both class 2 (Sudan I, Fig. 4b) and class 3 (Sudan IV, Fig. 4c). Samples adulterated with a mixture of 2.5mg/g of each Sudan dye were all properly assigned to class 3 (Fig. 4c), but 13 samples out of 27 were not assigned to class 2 (Fig. 4b, prediction values lower than the class threshold). Therefore, the implication of this result is not so serious for the health of the consumer because samples adulterated with a mixture of two Sudan dyes will be regarded as samples adulterated with only one Sudan dye. Even though the models were established with samples adulterated at 5 mg / g, they can successfully identify samples adulterated with a lower proportion (2.5 mg / g). None of these samples were assigned to class 1 (unadulterated, Fig. 4a), so no false negative errors were obtained. These results, expressed in terms of sensitivity and specificity, are shown in the first column of Table 1 (indicated as t0).

To study the stability of the multivariate models over time, unadulterated samples, samples adulterated with Sudan I and Sudan IV, and the additional data set were measured over a period of six months. Six-time delay data sets were measured. The first five measurements were made at 15-day intervals while the sixth was made 60 days after

the fifth. During the time intervals, samples were kept in the refrigerator at low temperature.

Table 1 shows the sensitivity and specificity results of each class at different times (indicated as t1 to t6), obtained from the PLS-DA classification model established using the zero time measurements. Considering that the quality parameters obtained with the established model at time zero (t0) are the reference values, it can be stated that the classification models were stable over time since they present no significant variations.

As far as the training set is concerned, the sensitivity of Class 1 and Class 3 had a constant value of 100% while the sensitivity of class 2 was lower for t3, t5 and t6. The majority of class 2 errors were samples being predicted as adulterated with both dyes (class 2 and 3) and not assigned only to their class. As mentioned above, the consequences of a double assignment error are not as serious since they are still recognized as an adulterated health risk. These lower sensitivity values (93 and 96%) are also reflected in the lower specificity values of Class 1 (96 or 98%) and the 98% specificity values for class 3. This slight decrease in the specificity of class 1 (unadulterated class), although not continuous and with no clear trend, is more important since the products would not be withdrawn from the food chain and, therefore, there would be a health risk.

As far as the additional data set is concerned, the specificity of class 1 was 100%. Therefore, no sample containing Sudan dye was assigned to class 1 (unadulterated samples). This is important since no false negatives were obtained in the samples adulterated with mixtures of dyes. The sensitivity of class 3 was lower after time 4 but, despite the small variations, no trend was observed. Various samples were not assigned to this class (adulterated with Sudan IV) at different times and there was no relationship between them. The consequences of these errors are minimal since the samples were assigned to class 2, which is also adulterated class (with Sudan I).

Finally, the lowest quality parameter was the sensitivity of class 2 (74% at t0) and, as expected, values were similar when samples from the additional data set measured over time (from t1 to t6) were predicted. Some samples were not assigned to the class by any of the measurements made over time, but no relationship was observed between them. Only one sample was correctly assigned by the first measurement (t0) but not by subsequent measurements. For the other incorrectly assigned samples, no general trends were observed.

5. Conclusions

PLS-DA multivariate analysis and UV-visible spectrometry are powerful tools for identifying banned Sudan dyes at the referenced concentration level, both individually and in mixtures of different proportions, in commercial spices intended for human consumption. Samples adulterated with single dyes were properly detected at concentrations that are within the usual range for commercial benefit. Some of the samples adulterated with lower concentrations of mixtures of both dyes were not assigned as adulterated with Sudan I, although they were assigned as adulterated. This type of error has no consequences on health as the products would be withdrawn from the food chain.

306
307 The study of the validity of the PLS-DA classification model over time has shown that the
308 main quality parameters of the method were maintained over the time studied (6
309 months). No trends have been found in the slight fluctuations of these parameters, so
310 stability can be ensured. It should be noted that studies of this type are not usually
311 performed for multivariate qualitative methods, so we believe that our findings may lead
312 to greater use of this type of method.
313
314 This methodology proposed is a rapid, simple and affordable detection tool for this type
315 of samples.
316
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