Manuscript after review

Access to published version: https://www.sciencedirect.com/science/article/pii/S0308814618315437

NON-DESTRUCTIVE RAMAN SPECTROSCOPY AS A TOOL FOR MEASURING ASTA COLOR VALUES AND SUDAN I CONTENT IN PAPRIKA POWDER

Running title: Raman Spectroscopy for measuring color quality in paprika

Olga Monago-Maraña[a,b*], Carl Emil Eskildsen[c], Nils Kristian Afseth[c], Teresa Galeano-Díaz[a,b], Arsenio Muñoz de la Peña[a,b], Jens Petter Wold[c]

[a]Department of Analytical Chemistry, University of Extremadura, Badajoz 06006, Spain

[b]Research Institute on Water, Climate Change and Sustainability (IACYS), University of Extremadura, Badajoz 06006, Spain

[c]Nofima AS – Norwegian Institute of Food, Fisheries and Aquaculture Research, PB 210, N-1431, Ås, Norway

*corresponding author. E-mail: olgamonago@unex.es

Abstract

The aim of this study was developing a non-destructive method for the determination of color in paprika powder. Non-destructive Raman spectroscopy was applied directly to paprika powder employing a laser excitation of 785 nm for the first time. The fluorescence background was estimated, by fitting a polynomial to each spectrum, and then subtracted. After preprocessing the spectra, some peaks were clearly identified as characteristic from pigments present in paprika. The preprocessed Raman spectra were correlated with the ASTA color values of paprika by partial least squares regression (PLSR). Twenty-five paprika samples were adulterated with Sudan I at different levels and the PLSR model was also obtained. The coefficients of determination (R^2) were 0.945 and 0.982, respectively, and the root mean square errors of prediction (RMSEP) were 8.8 ASTA values and 0.91 mg/g, respectively. Finally, different approaches were applied to discriminate between adulterated and non-adulterated samples. Best results were obtained for partial least squares – discriminant analysis (PLS-DA), allowing a good discrimination when the adulteration with Sudan I was higher than 0.5 %.

Keywords: Raman spectroscopy, ASTA values, Sudan I, partial least-squares regression, partial least-squares - discriminant-analysis

1. INTRODUCTION

Paprika powder is a spice that is being increasingly consumed in many areas, such as cookery or restaurant business. Paprika is also used as natural colorant in seasonings, sauces, confectionary, processed cheeses and so on. This product contains over 20 different carotenoid pigments that give its reddish color. Depending on how the paprika is manufactured and which *Capsicum* varieties of peppers are employed, the content of carotenoids may differ (Monago Maraña, Bartolomé García, & Galeano Díaz, 2016).

Color is an important quality parameter in paprika powder and is usually determined according to the American Spice Trade Association (ASTA) (ASTA, 2018), by obtaining absorbance values at 460 nm of an acetone extract of the sample (Method 20.1, revised October 2004). The ASTA color value is one of the parameters established to determine if the paprika is of high quality or not. For example, the Regulation of the Spanish Protection Designation of Origin (PDO) *"Pimentón de La Vera"* specify that the ASTA color values in paprika must be higher than 90 (MAPAMA, 2006). The samples belonging to this PDO possess some special characteristics due to the production process, which consists of a smoke drying system that gives the smoke flavor and aroma. This drying system also preserves the pigments better, while other systems induce a stronger degradation of the pigments during processing and storage (Velázquez et al., 2014).

Usually, liquid chromatography is employed to determine individual carotenoids content in paprika (Molnár et al., 2016). Capsanthin and capsorubin are the major carotenoids present in the red fraction and β-carotene is the major carotenoid in the yellow fraction.

Paprika powder loses its color during storage (Pérez-Gálvez, Mínguez-Mosquera, Garrido-Fernández, Lozano-Ruiz, & Montero-de-Espinosa, 2004). For this reason, it can be tempting to add illegal colorants, such as Sudan dyes, which are stable yellow-orange or red azo-dyes to give more persistent and intensive colors to the spices. Hence, the paprika will appear fresher and of higher quality.

Rapid detection of these illegal Sudan dyes has been attempted with spectroscopic techniques. Some authors determined the adulteration of spices and other foodstuffs with Sudan I-II-III-IV by UV-visible spectroscopy and multivariate classification showing good results in levels of concentration higher than 1.0 mg/L, 2.5 mg/L, 5 mg/L (in dissolution) or 3.6 mg/g depending on the samples and the study that they perform (Di Anibal, Rodríguez, & Albertengo, 2014; Di Anibal, Rodrí-guez, Albertengo, & Rodrí-guez, 2016). Moreover, UV-visible spectroscopy have also been used by Yuan, Liao, Lin, Deng, & He (2008) to determine Sudan dyes in chili powder samples. These authors determined Sudan I in chili samples employing gradual changes in the absorption spectra with different solvents and second order algorithms. In this case, lower concentrations of Sudan I were detected and sample pretreatment was required.

In order to determine low concentrations of these Sudan dyes, separative techniques have been widely used as shown in different reviews (Rebane, Leito, Yurchenko, & Herodes, 2010; Reinholds, Bartkevics, Silvis, van Ruth, & Esslinger, 2015). After that, other studies show that liquid chromatography coupled to various detectors can be used in the adulteration control of different foods (Rajabi, Sabzalian, Barfi, Arghavani-Beydokhti, & Asghari, 2015; Sricharoen, Limchoowong, Techawongstien, & Chanthai, 2017; Tsai, Kuo, & Shih, 2015).

Furthermore, near infrared spectroscopy (NIRS) was applied directly to paprika powder samples in order to determine the ASTA color values content in paprika samples (Bae, Han, & Hong, 1998) where they built a PLS model with 8 components obtaining good results ($R^2 = 0.896$). Han et al. (2015) also determined ASTA color values with UV/NIR hyperspectral image obtaining a square correlation coefficient of 0.88.

With their unsatured and conjugated chemical structure, carotenoids and other pigments usually have very favorable Raman scattering properties. However, Di Anibal, Marsal, Callao, & Ruisánchez (2012) suggested that using conventional Raman spectroscopy directly on paprika powder is impossible due to the strong fluorescence background. Hence, the studies found in the literature mostly employ surface enhanced Raman spectroscopy to determine Sudan dyes in food (Gao et al., 2015; Jahn et al., 2015). Although surface-enhanced Raman spectroscopy is a

commonly used method for enhancing sensitivity in Raman spectroscopy, the technique is based on interactions between the analyte and nanoparticles, ant it can be difficult to obtain useful signals for quantification.

Note that all the studies mentioned above, except NIRS, require sample pre-treatment, which means more time, solvents and, consequently, the approaches are more expensive. The literature concerning the application of direct Raman spectroscopy to determine the adulteration of spices with Sudan dyes is scarce (Haughey, Galvin-King, Ho, Bell, & Elliott, 2015). Hitherto, the application of Raman using a 785 nm laser on paprika powder has not been reported yet.

As reported in the literature, the 1064 nm Raman excitation can be a good choice to determine Raman sensitive compounds in samples that exhibit strong fluorescence (Waesner & Longmire, 2001), since fluorescence for this excitation is generally lower than for shorter wavelengths. However, the use of longer laser wavelengths decreases the efficiency of Raman scattering and the CCD detector has a very weak response for Raman signals excited at longer wavelengths than 785 nm. Hence, longer laser wavelength rapidly disqualifies the CCD as a viable detector and room-temperature indium gallium arsenide (InGaAs) or liquid nitrogen-cooled germanium (Ge) detectors have to be used (Waesner & Longmire, 2001). These detectors are more expensive than CCD detectors, especially for portable instruments. Besides, the equipment with a 785 nm laser is more sensitive, faster, and generally, less than half of the price compared to instruments that employ 1064 nm lasers. Hence, it can be a challenge employ 785 nm laser combined with mathematical methods for quantifying these samples which exhibit high fluorescence signals, employing this methodology as an alternative to Fourier-transform Raman spectroscopy.

Many mathematical methods have been proposed to pre-process Raman spectra (Cordero et al., 2017; Gautam, Vanga, Ariese, & Umapathy, 2015; Liland, Kohler, & Afseth, 2016; Liu, Sun, Huang, Li, & Liu, 2015). Polynomial fitting (Lieber & Mahadevan-Jansen, 2003) and extended multiplicative scatter correction (Martens & Stark, 1991) are examples of such methods.

Polynomial fitting is based on an approximation of the broad fluorescence background as an n-order polynomial function. The polynomial is then subtracted from the raw Raman spectrum. This approach has been applied to measurements from different analytical techniques, such as liquid chromatography (Mecozzi, 2014), 2-D electrophoresis (Færgestad et al., 2007) and Raman spectroscopy (Afseth, Segtnan, & Wold, 2006; Kourkoumelis, Polymeros, & Tzaphlidou, 2012; Mclaughlin & Lednev, 2015; Qin, Chao, & Kim, 2013; J. P. Wold, Marquardt, Dable, Robb, & Hatlen, 2004) in different fields and matrices.

The main objective of this work was to analyse paprika powder with 785 nm excitation Raman spectroscopy and remove the fluorescence background by subtracting it prior to data analysis. The corrected Raman spectra were evaluated for the determination of ASTA color values in paprika samples and detection of illegal Sudan I dye concentration in adulterated paprika powder. In addition, a classification technique was assayed to establish the lowest Sudan I concentration that can be detected by Raman spectroscopy in adulterated samples.

2. EXPERIMENTAL

2.1. Chemicals and samples

Acetone (grade HPLC), sulfuric acid (99.999 %), ammonium cobalt (II) sulfate hexahydrate $(NH_4)_2Co(SO_4)_2 \cdot 6\ H_2O$, potassium dichromate ($K_2Cr_2O_7$) and Sudan I (≥ 95 %) were purchased from Sigma-Aldrich (St. Louis, MO).

The set of paprika powder samples consisted of 58 samples from different origins. A total of 32 samples were from the Spanish PDO "Pimentón de La Vera", 20 samples were from Spanish local markets not belonging to the PDO and 6 samples were from Norwegian local markets. Samples belonging to the PDO were from different years (2010 – 2016).

Five different paprika samples, with representative ASTA color values, were selected for the adulteration experiment. The five samples had the following ASTA color values: 25, 63, 85, 140 and 149. Each sample was adulterated with the illegal Sudan I dye at five concentration levels: 1mg/g, 2.5 mg/g, 5 mg/g, 10 mg/g and 25 mg/g. Hence, a total of twenty-five adulterated samples

were prepared. Each adulterated sample was prepared by mixing 4.0 g (± 0.0001 g) of paprika with various amounts of Sudan I (from 4 mg to 100 mg). The samples were manually mixed to obtain a homogeneous blend.

2.2. Reference ASTA measurements

The ASTA reference values were obtained by means of the AOAC International (2002) method 971.26 (Velázquez et al., 2014). A volume of 20.0 mL of acetone was added to 0.1000 g of paprika sample. Then samples were axially shaken (140 rpm) during three hours in a water bath at 25 ºC. After that, the samples were centrifuged during 5 min at 4000 rpm. The mixture was diluted 1:5 in acetone. Absorption spectra were acquired using an Agilent 8453 UV-Visible spectrophotometer (Agilent Technologies). The extraction solvent was used as blank for baseline correction and the Chemstation software was used for data acquisition. With the absorbance at 460 nm, ASTA values were calculated using the following equation:

$$ASTA = A_{(460nm)} * 16.1 * If/weight \quad [1]$$

where *A* is the absorbance of the extract, *If* is the deviation factor of the spectrophotometer, which is calculated by dividing the theoretical absorbance (A_t =0.600) by the real absorbance (A_s) of a standard color solution (0.01 M $K_2Cr_2O_7$ and 0.09 M $(NH_4)_2Co(SO_4)_2 \cdot 6\ H_2O$ in 1.8 M H_2SO_4) at 460 nm, and weight is the paprika sample weight in grams.

2.3. Raman measurements

A RamanRXN2™ Hybrid system (Kaiser Optical Systems, Inc., Ann Arbor, MI) was employed to perform the Raman measurements. This instrument was equipped with a non-contact PhAT-probe (Kaiser Optical Systems, Inc., Ann Arbor, MI). The excitation wavelength was 785 nm with a circular spot size of D =6 mm at 25 cm working distance operating at an average power of 400 mW. Raman spectra were collected in the range from 300 – 1800 cm^{-1} with a total of 150 scans and an accumulation time of 0.1 sec/scan.

For predicting ASTA values, Raman spectra were collected in triplicate, and the average spectrum of the three was used for further analysis.

For Raman spectra used to detect adulteration with Sudan I, each sample was measured five times and the average spectrum from each sample was used for further analysis. Five replicates were collected to ensure that the average spectrum would be representative of the sample.

2.4. Pre-processing of Raman spectra

The fluorescence background signal in the Raman spectra was removed by polynomial fitting, a method introduced by Brennan, Wang, Dasari, & Feld (1997) and refined by Lieber & Mahadevan-Jansen (2003). In the traditional approach of polynomial fitting, one polynomial of a given degree is fitted to a spectrum. The resulting baseline correction is often unsatisfactory because the polynomial fitting is severely affected by the Raman peaks in the spectrum, and not only by the baseline. The approach used here is an iterative procedure where the baseline of a given spectrum is estimated through successive polynomial fitting. It works as follows: 1. The fitted baseline is first approximated by the spectrum itself. 2. A polynomial of a given degree is fitted to the intermediate baseline. 3. The polynomial and the intermediate baseline are compared, and for each spectrum variable the lowest value of either the polynomial or the baseline is chosen. The resulting values are stored as the next approximation to the baseline. 4. The procedure of 2. and 3 are repeated for a preselected number of iterations (for instance 1000), or until the difference between the intermediate baseline and the fitted polynomial is appreciably small. 5. When the final polynomial is obtained, this polynomial is subtracted from the original spectrum.

The correction was applied from 900 to 1800 cm^{-1} with a fourth order polynomial correction. The calculations were done with Matlab R2007b (MATLAB Version 7.5, The Marhworks, Natick, Massachusetts, 2007).

2.5. Regression analysis

Partial least squares regression (PLSR) is a multivariate regression method widely used with Raman spectroscopy as described in the literature (Czaja, Mazurek, & Szostak, 2016; Su, He, &

190 Sun, 2017). For applying the classification method, the data set of samples was randomly divided
191 in a training set and a test set, resulting in 66 samples (47 non-adulterated (75 % of non-adulterated
192 samples) and 19 adulterated (75 % of adulterated samples)) for the training set, and 22 samples
193 (16 non-adulterated and 6 adulterated), for the test set.

194 In PLSR, the response variable, **y** (*Ix1*) (ASTA values) is regressed on an ill-conditioned **X** (*IxJ*)
195 (Raman spectra). This is done by defining a lower rank principal component space that maximizes
196 the covariance between **X** and **y**. In this study, leave-one-out cross-validation was used to
197 determine the rank of the principal component space (i.e. the number of principal components
198 included in the PLSR model) (Haaland & Thomas, 1988). Spectra were mean centered prior to
199 PLSR modeling.

200 The software package Unscrambler® v6.11 (CAMO A/S Olav Tryggvasonsgt, N-7011,
201 Trondheim, Norway) was employed for the building of the regression models.

202 *2.6. Exploratory analysis and classification techniques*

203 For applying the classification method, the data set of samples employed were the same that in
204 the regression analysis. In order to perform discrimination between adulterated and non-
205 adulterated samples, different techniques were used. Principal component analysis (PCA) was
206 used for exploratory analysis of the spectral data. Like PLSR, PCA benefits from modeling the
207 matrix **X** (Raman spectra) in a lower dimensional principal component space. In PCA, **X** is
208 decomposed into scores and loadings (and residuals). The loadings describe the direction of each
209 principal component in the original **X**-space and the scores are the projections of the original data
210 onto the loading vectors (S. Wold, Esbensen, & Geladi, 1987).

211 Partial least-squares discriminant analysis (PLS-DA) was employed for supervised classification.
212 This technique requires defined classes of samples and aims to divide the data space into different
213 sub-spaces, each of which correspond to one class. Unknown samples are classified into the
214 closest class (Callao & Ruisánchez, 2018). PLS-DA was used to determine the lowest limit of

detection for Sudan I in paprika powders evaluating the probability of detection (POD) curves (López, Callao, & Ruisánchez, 2015).

In order to carry out the PLS-DA classification, the tutorial provided by Ballabio & Consonni (2013) was followed. The first step is to determine the optimal number of latent variables. For that, the venetian blinds cross-validation procedure was used. The cross-validation was done with 2, 5 and 10 data splits (i.e. for the case with 10 data splits each validation set is determined by selecting every 10^{th} samples in the data set, starting at sample 1 through 10). Background fluorescence was removed and spectra were mean centered prior to classification.

3. RESULTS AND DISCUSSION

3.1. ASTA reference measurements

Variability in sample origin and age resulted in a wide range of ASTA values (20 - 150). Samples from 2010 to 2014 (PDO samples) had ASTA values lower than 90, as the degradation of pigments is occurring over time. However, the PDO samples from the years 2015 and 2016 did still have ASTA values higher than 90. Most of the non-PDO samples had ASTA values below 90. This meant that compared with the PDO samples, the color quality of these samples was lower.

3.2. Raman spectra pre-processing and peak identification

Figure 1A shows that there was a strong fluorescence background in the Raman spectra due to the many fluorescent compounds in paprika (Monago-Maraña, Galeano-Díaz, & Muñoz de la Peña, 2017). This background signal was not reproducible between replicates and it was not correlated with the color. The sample with the highest ASTA color value produced a medium fluorescence signal, while a sample with low ASTA value gave a more intense fluorescence signal.

When the background fluorescence was removed it could be seen that the three replicates gave very similar spectra (Figure 1B), which meant that the correction preserved the Raman

information. In the corrected spectra, the main bands appeared at 1521 cm^{-1}, 1157 cm^{-1} and 1107 cm^{-1}. These peaks correspond with the three main Raman bands of carotenoids in paprika, namely C=C and C-C stretching and C-CH_3 deformation, termed ν□ ν□ and ν□ (De Oliveira, Castro, Edwards, & De Oliveira□ The major paprika pigment capsanthin has three Raman bands at 1521 cm^{-1}, 1155 cm^{-1} and 1107 cm^{-1} and β-carotene has bands at 1527 cm^{-1}, 1157 cm^{-1} and 1106 cm^{-1}. The observed peaks were clearly related to the main paprika pigments.

In the case of Sudan I, the peaks corresponding to this compound are described in the literature as: 763/722 cm^{-1} (δCCC, in-plane angular deformation), 1002/984 cm^{-1} (δCCC, in-plane angular deformation), 1169 cm^{-1} (δCH), 1227 cm^{-1} (ν_sCC, symmetric stretching vibration; δCH 1258 cm^{-1} (ν_sNN, δNH, ν_sCC, δCH), 1341 cm^{-1} (ν_sCC; δCH), 1389 cm^{-1} (ν_sC=N; δNH; □ν_sCC), 1495 cm^{-1} (ν_sCC; δCH; ν_sC-NH), 1547 cm^{-1} (ν_sC=O; γ_sC=N; ν_sC=N) and 1596 cm^{-1} (ν_sCC, δCH; δNH) (Ferreira, Garcia, Couri, Dos Santos, & De Oliveira, 2013). Figure 2 shows that some of these peaks appeared clearly in the adulterated samples: 1228, 1386, 1496 and 1598 cm^{-1}.

3.3. Regression analysis

PLSR models were built for quantification of ASTA values in paprika samples and Sudan I in adulterated paprika samples. The results from PLSR models are presented in Table 1 and Figures S1 and S2. In order to get the calibration models by means of cross-validation procedure, the training set was employed and for validating this calibration, the samples of test set were predicted.

From the results obtained in the case of the ASTA measurements calibration model, the root mean square of prediction (RMSEP) was 8.9 ASTA values and the squared correlation coefficient (R^2) was 0.94. Good results of prediction were obtained for the validation samples (Table 1). Hence, this method could most likely be employed in industry in order to obtain ASTA values rapidly and without any color extraction. In addition, these measurements could be collected in the line of production for a more exhaustive control of all samples.

Figure S1 shows the regression coefficient for the model of ASTA values determination, which is corresponding with the main peaks of carotenoids Raman spectrum, which means that these variables are influencing the model the most. These variables are: 1008.3, 1157.7, 1520.1 cm^{-1}. All these variables are the main variables of majority carotenoid present in paprika samples (capsaicin and β-carotene), as indicated above.

In the case of Sudan I determination calibration model, the RMESP was 0.75 mg/g and R^2 was 0.98. In the case of validation samples, good results of prediction were obtained (Table 1). This result suggests that the method is suitable for effective detection of Sudan I adulterated paprika samples. It is likely that also other Sudan dyes could be detected as they present different peaks from the true pigments in paprika.

A low number of principal components (4) was required in the present study to obtain the corresponding calibration model. In general, it is favorable to have calibrations that rely on few components, as this eases model interpretation and reduces risk of overfitting.

Figure S2 shows the regression coefficient for the model of Sudan I determination, which is corresponding with the main peaks of the Sudan I spectrum, which means that these variables are influencing the model the most. These variables are: 986.1, 1002.2, 1169, 1226.7, 1259.4, 1340.4, 1391.4, 1496.7, 1549.5 and 1597.5 cm^{-1}. All these variables are the main variables of Sudan I described by Ferreira et al. (Ferreira et al., 2013).

Hence, with the Raman spectrum of one paprika sample, the ASTA value and the Sudan I concentration can be determined simultaneously. It should also be noted that the large quality variation of the samples used in this study indicates that the method is robust. This robustness was obtained by including samples from different origins and ages as Haughey et al. (2015) suggested in their study. In other similar studies, the methods were only applied to different types of paprika from local markets.

3.4. Discrimination of adulterated from non-adulterated samples

290 PCs 1 and 2 did not offer a good discrimination due to the fact that these components are related
291 with the carotenoids and noise in the matrix. There are no difference in the carotenoid content
292 between adulterated and non-adulterated samples. The clustering results from PCA are shown in
293 Figure 3A. PCs 3 and 4 gave the best discrimination between adulterated and non-adulterated
294 samples. The adulterated and non-adulterated samples are partly overlapping. The adulterated
295 samples, which overlap with the non-adulterated samples, present a concentration of Sudan lower
296 than 0.5 % of adulteration. The loadings from PC3 and PC4 contained some of the characteristics
297 peaks of Sudan I.

298 To refine the results, a new PCA was performed utilizing only the regions where Sudan I presents
299 distinct Raman bands. Different ranges were checked and the best result was obtained when only
300 the range 1573.2 – 1613.4 cm^{-1} was used. Figure 3B shows the loadings and scores corresponding
301 to the two first components. The best discrimination between the two groups was obtained by the
302 first component. The loadings of the first PC corresponded with one of the bands of the Sudan I,
303 1597 cm^{-1} (ν_sCC, symmetrical stretching vibration; δCH, in-plane angular deformation; δNH, in
304 plane angular deformation). In Figure 3B, the clustering of the samples is better than in Figure
305 3A. In this case, the adulterated samples, which overlap with non-adulterated samples, present a
306 concentration of Sudan I lower than 0.25 % of adulteration.

307 Finally, PLS-DA was employed to check the utility of Raman spectra for automated detection of
308 adulterated samples. For the supervised classification, the data set was randomly divided in a
309 training set and a test set as indicated in section 2.6. The main peaks corresponding to the pigments
310 were deleted from the spectra to get better classification results.

311 In order to carry out the PLS-DA classification, the first step was to obtain the optimal number of
312 Latent Variables (LVs) by cross validation based on the venetian procedure. Cross-validation with
313 2, 5, and 10 cross validation groups were checked and the results are shown in the Figure S3.
314 Taking into account the error rate and the non-assigned samples, the number of optimal
315 components selected was 4. Because the results obtaining in the error rate and non-assigned
316 samples for the different cross validation groups (data shown in Figure S3) are quite similar, it

317 can be concluded that the model is robust. The confusion matrices obtained for the cross
318 validation and the test samples are presented in the Table 2.

319 From these results, taking into account the assigned and non-assigned samples, it can be observed
320 that 87 % of the non-adulterated calibration samples are well-classified and the 89 % of
321 adulterated samples are well-classified. In the case of test samples, the results were also
322 satisfactory, the 100% of non-adulterated samples were well-classified and the 83 % of
323 adulterated samples were well-classified.

324 The classification parameters are summarized in the Table 3. In this case, as two classes are the
325 only classes, the specificity and sensitivity are symmetrical, this means that the specificity of non-
326 adulterated samples is the sensitivity of adulterated samples, and vice versa. In the cross-
327 validation model, the specificity and sensitivity are equal to 0.872 and 0.895, respectively. This
328 means that considering only the assigned samples, the 87 % of non-adulterated samples are well-
329 assigned as non-adulterated and 89 % of adulterated samples are well-assigned as adulterated.
330 Since sensitivity and specificity are similar, it can be deducted that the type of error is balanced,
331 that is, there is no particular trend in the model to recognize adulterated samples as non-
332 adulterated samples, or vice versa. If it is important to not misclassify non-adulterated samples,
333 the decision line can simply be adjusted to higher concentration levels of Sudan.

334 The implication in obtaining the different type of errors (false positive and false negatives) is
335 quite different considering the studied adulteration problem. The fact that assigning adulterated
336 samples as non-adulterated samples is so dangerous for consumer health. On the other hand, the
337 assignation of non-adulterated samples as adulterated implies an economic risk since these
338 samples must be withdrawn from markets.

339 To evaluate the performance parameters related to concentration, Probability of Detection (POD)
340 curves were established, estimating the decision limit, the capacity of detection and the
341 unreliability region.

342 The POD curves showed that for concentrations close to zero of Sudan I, the chance of giving a
343 positive output (adulterated sample) was lower than 5 %. CCα (decision limit) had a very low
344 value (almost zero) which is characteristic of the P(x) POD curves that are exponential. CC_{β}
345 (detection capability) was set for concentrations at or above 0.5 % of adulteration (5 mg/g) which
346 meant that the probability of giving a negative output was also lower than 5 % at or above this
347 concentration of adulteration.

348 The unreliability region is between the two limits. In between those two limits, the probability of
349 making a wrong decision is higher than 5 %. In this regard, unreliability could be related to
350 uncertainty in quantitative analysis. But, unreliability cannot be considered as dispersion around
351 a value as the response in qualitative analysis is not quantifiable.

352 Hence, the limit of detection could be stablished around this value, which means that it is 2 times
353 lower than the limit proposed by Haughey et al. (2015) in the study with chilli powder where they
354 discriminated between adulterated and non-adulterated samples in a percentage between 1-5 %
355 of adulteration.

356 **4. CONCLUSIONS**

357 This study shows that Raman spectroscopy, with a 785 nm laser excitation, can be applied directly
358 on paprika powder for the determination of ASTA values and Sudan I content simultaneously.
359 Mathematical pre-treatment of the Raman spectra was done by fitting a polynomial to each
360 spectrum and then subtracting it, to remove the fluorescence background signal and this was key
361 for proper interpretation and modelling of the spectra. The method is quick, non-destructive and
362 easy to use. No pre-treatment of the paprika powders is required. The method therefore easily
363 lends itself to industrial use.

364 **Acknowledgments**

365 Financial support was provided by the Junta de Extremadura (Ayuda GR18041-Research Group-
366 FQM003 and Project IB16058) and Ministerio de Economía, Industria y Competitividad of Spain
367 (Project CTQ2017-82496-P), both co-financed by the European Social funds. Olga Monago

Maraña is grateful to the Ministerio de Educación, Cultura y Deporte of Spain for a FPU grant (Resolución de 18 de noviembre de 2013, de la Secretaría de Estado de Educación, Formación Profesional y Universidades, BOE nº 279, de 21/11/13, reference number FPU13/00249) and a stay research FPU grant (Resolución de 22 de diciembre de 2017, BOE nº 14, de 17/01/17, reference number EST16/00333). The work was also funded by the Norwegian Agricultural Food Research Foundation (Oslo, Norway) through the project FoodSMaCK - Spectroscopy, Modelling & Consumer Knowledge (project number 262308/F40).

References

Afseth, N. K., Segtnan, V. H., & Wold, J. P. (2006). Raman spectra of biological samples: A study of preprocessing methods. *Applied Spectroscopy*, *60*(12), 1358–1367.

ASTA, A. S. T. A. (2018). The voice of the U.S. spice industry in the Global Market. Retrieved November 26, 2017, from http://www.astaspice.org/food-safety/astas-analytical-methods-manual/

Bae, M.-J., Han, E.-S., & Hong, S.-H. (1998). Use of near infrared spectroscopy in quality control of red pepper powder. *Journal of Near Infrared Spectroscopy*, *6*, A333–A337.

Ballabio, D., & Consonni, V. (2013). Classification tools in chemistry. Part 1: linear models. PLS-DA. *Analytical Methods*, *5*(16), 3790–3978.

Brennan, J. F., Wang, Y., Dasari, R. R., & Feld, M. S. (1997). Near-infrared Raman spectrometer systems for human tissue studies. *Applied Spectroscopy*, *51*(2), 201–208.

Callao, M. P., & Ruisánchez, I. (2018). An overview of multivariate qualitative methods for food fraud detection. *Food Control*, *86*, 283–293.

Cordero, E., Korinth, F., Stiebing, C., Krafft, C., Schie, I. W., & Popp, J. (2017). Evaluation of shifted excitation Raman difference spectroscopy and comparison to computational background correction methods applied to biochemical Raman spectra. *Sensors*, *17*, 1–17.

Czaja, T., Mazurek, S., & Szostak, R. (2016). Quantification of gluten in wheat flour by FT-Raman spectroscopy. *Food Chemistry*, *211*, 560–563.

De Oliveira, V. E., Castro, H. V., Edwards, H. G. M., & De Oliveira, L. F. C. (2010). Carotenes and carotenoids in natural biological samples: A Raman spectroscopic analysis. *Journal of Raman Spectroscopy*, *41*(6), 642–650.

Di Anibal, C., Rodríguez, M. S., & Albertengo, L. (2014). 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. *Food Analytical Methods*, *7*(5), 1090–1096. http://doi.org/10.1007/s12161-013-9717-2

Di Anibal, C. V., Marsal, L. F., Callao, M. P., & Ruisánchez, I. (2012). Surface Enhanced Raman Spectroscopy (SERS) and multivariate analysis as a screening tool for detecting Sudan I dye in culinary spices. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, *87*, 135–141.

Di Anibal, C. V., Rodrí-guez, S., Albertengo, L., & Rodrí-guez, M. S. (2016). UV-Visible spectroscopy and multivariate classification as a screening tool for determining the adulteration of sauces. *Food Analytical Methods*, *9*(11), 3117–3124.

Færgestad, E. M., Rye, M., Walczak, B., Gidskehaug, L., Wold, J. P., Grove, H., … Martens, H. (2007). Pixel-based analysis of multiple images for the identification of changes: A novel approach applied to unravel proteome patters of 2-D electrophoresis gel images. *Proteomics*, *7*(19), 3450–3461.

Ferreira, G. R., Garcia, H. C., Couri, M. R. C., Dos Santos, H. F., & De Oliveira, L. F. C. (2013). On the azo/hydrazo equilibrium in Sudan i azo dye derivatives. *Journal of Physical Chemistry A*, *117*(3), 642–649. http://doi.org/10.1021/jp310229h

Gao, F., Hu, Y., Chen, D., Li-Chan, E. C. Y., Grant, E., & Lu, X. (2015). Determination of Sudan I in paprika powder by molecularly imprinted polymers-thin layer chromatography-surface enhanced Raman spectroscopic biosensor. *Talanta*, *143*, 344–352.

Gautam, R., Vanga, S., Ariese, F., & Umapathy, S. (2015). Review of multidimensional data

processing approaches for Raman and infrared spectroscopy. *EPJ Techniques and Instrumentation*, *2*(1), 2–8.

Haaland, D. M., & Thomas, E. V. (1988). Partial Least-Squares methods for spectral analyses. 1. Relation to other quantitative calibration Methods and the Extraction of Qualitative Information. *Analytical Chemistry*, *60*(11), 1193–1202.

Han, K., Lee, H., Kang, J., Choi, E., Oh, S., Lee, Y., & Cho, B. (2015). A Simple Method for Evaluation of Pepper Powder Color Using Vis/NIR Hyperspectral System. *Korean Journal of Horticulture Science and Technology*, *33*, 403–408.

Haughey, S. A., Galvin-King, P., Ho, Y. C., Bell, S. E. J., & Elliott, C. T. (2015). The feasibility of using near infrared and Raman spectroscopic techniques to detect fraudulent adulteration of chili powders with Sudan dye. *Food Control*, *48*, 75–83.

Jahn, M., Patze, S., Bocklitz, T., Weber, K., Cialla-May, D., & Popp, J. (2015). Towards SERS based applications in food analytics: Lipophilic sensor layers for the detection of Sudan III in food matrices. *Analytica Chimica Acta*, *860*, 43–50.

Kourkoumelis, N., Polymeros, A., & Tzaphlidou, M. (2012). Background estimation of biomedical Raman spectra using a geometric approach. *Spectroscopy: An International Journal*, *27*(5), 441–447.

Lieber, C. A., & Mahadevan-Jansen, A. (2003). Automated method for subtraction of fluorescence from biological Raman spectra. *Applied Spectroscopy*, *57*(11), 1363–1367.

Liland, K. H., Kohler, A., & Afseth, N. K. (2016). Model-based pre-processing in Raman spectroscopy of biological samples. *Journal of Raman Spectroscopy*, *47*(6), 643–650.

Liu, J., Sun, J., Huang, X., Li, G., & Liu, B. (2015). Goldindec: A novel algorithm for Raman spectrum baseline correction. *Applied Spectroscopy*, *69*(7), 834–842.

López, M. I., Callao, M. P., & Ruisánchez, I. (2015). A tutorial on the validation of qualitative methods: From the univariate to the multivariate approach. *Analytica Chimica Acta*, *891*, 62–72.

MAPAMA. (2006). BOE Nº4, Jueves 5 enero 2006. ORDEN APA/4178/2005, de 22 de diciembre, por la que se ratifica el Reglamento de la Denominación de Origen Protegida Pimentón de La Vera y su Consejo Regulador., 778–786.

Martens, H., & Stark, E. (1991). Extended multiplicative signal correction and spectral interference subtraction: New preprocessing methods for near infrared spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, *9*(8), 625–635.

Mclaughlin, G., & Lednev, I. K. (2015). In situ identification of semen stains on common substrates via Raman spectroscopy. *Journal of Forensic Sciences*, *60*(3), 595–604.

Mecozzi, M. (2014). A polynomial curve fitting method for baseline drift correction in the chromatographic analysis of Hydrocarbons in environmental samples. *APCBEE Procedia*, *10*, 2–6.

Molnár, H., Kónya, É., Zalán, Z., Bata-Vidács, I., Tömösközi-Farkas, R., Székács, A., & Adányi, N. (2016). Chemical characteristics of spice paprika of different origins. *Food Control*, *83*, 54–60.

Monago-Maraña, O., Galeano-Díaz, T., & Muñoz de la Peña, A. (2017). Chemometric Discrimination Between Smoked and Non-Smoked Paprika Samples. Quantification of PAHs in Smoked Paprika by Fluorescence-U-PLS/RBL. *Food Analytical Methods*, *10*, 1128–1137.

Monago Maraña, O., Bartolomé García, T. de J., & Galeano Díaz, T. (2016). Characterization of

Spanish Paprika by Multivariate Analysis of Absorption and Fluorescence Spectra, *49 (8)*(May), 1184–1197.

Pérez-Gálvez, A., Mínguez-Mosquera, M. I., Garrido-Fernández, J., Lozano-Ruiz, M., & Montero-de-Espinosa, V. (2004). Correlación entre unidades ASTA-concentración carotenoide en pimentones. Predicción de la estabilidad del color durante el almacenamiento. *Grasas y Aceites*, *55*(3), 213–218.

Qin, J., Chao, K., & Kim, M. S. (2013). Development of a Raman chemical image detection algorithm for authenticating dry milk. *Sensing for Agriculture and Food Quality and Safety*, *8721*, 872102-1-872107–10.

Rajabi, M., Sabzalian, S., Barfi, B., Arghavani-Beydokhti, S., & Asghari, A. (2015). In-line micro-matrix solid-phase dispersion extraction for simultaneous separation and extraction of Sudan dyes in different spices. *Journal of Chromatography A*, *1425*, 42–50.

Rebane, R., Leito, I., Yurchenko, S., & Herodes, K. (2010). A review of analytical techniques for determination of Sudan I-IV dyes in food matrixes. *Journal of Chromatography A*, *1217*(17), 2747–2757.

Reinholds, I., Bartkevics, V., Silvis, I. C. J., van Ruth, S. M., & Esslinger, S. (2015). Analytical techniques combined with chemometrics for authentication and determination of contaminants in condiments: A review. *Journal of Food Composition and Analysis*, *44*, 56–72.

Sricharoen, P., Limchoowong, N., Techawongstien, S., & Chanthai, S. (2017). Ultrasound-assisted emulsification microextraction coupled with salt-induced demulsification based on solidified floating organic drop prior to HPLC determination of Sudan dyes in chili products. *Arabian Journal of Chemistry*, In press.

Su, W.-H., He, H.-J., & Sun, D.-W. (2017). Non-Destructive and rapid evaluation of staple foods quality by using spectroscopic techniques: A review. *Critical Reviews in Food Science and Nutrition*, *57*(5), 1039–1051.

Tsai, C. F., Kuo, C. H., & Shih, D. Y. C. (2015). Determination of 20 synthetic dyes in chili powders and syrup-preserved fruits by liquid chromatography/tandem mass spectrometry. *Journal of Food and Drug Analysis*, *23*(3), 453–462.

Velázquez, R., Hernández, A., Martín, A., Aranda, E., Gallardo, G., Bartolomé, T., & Córdoba, M. G. (2014). Quality assessment of commercial paprikas. *International Journal of Food Science and Technology*, *49*(3), 830–839.

Waesner, F., & Longmire, M. (2001). Dispersive and Fourier Transform Raman. *Spectroscopy*, *16*(2), 68–77.

Wold, J. P., Marquardt, B. J., Dable, B. K., Robb, D., & Hatlen, B. (2004). Rapid quantification of carotenoids and fat in atlantic salmon (Salmo salar L.) by Raman spectroscopy and chemometrics. *Applied Spectroscopy*, *58*(4), 395–403.

Wold, S., Esbensen, K. I. M., & Geladi, P. (1987). Principal Component Analysis. *SpringerReference*, *2*, 37–52.

Yuan, J., Liao, L., Lin, Y., Deng, C., & He, B. (2008). Determination of Sudan I in chilli powder from solvent components gradual change-visible spectra data using second order calibration algorithms. *Analytica Chimica Acta*, *607*(2), 160–167.

508

Table 1. Statistical parameters of the PLS models constructed with Raman spectra.

	ASTA	**R^2**	**RMSEP* (ASTA values)**	**Nº comp.**
Calibration	n = 66	0.943	8.9	1
Validation	n = 22	0.954	7.5	
	Sudan I	**R^2 (val)**	**RMSEP* (mg/g)**	**Nº comp.**
Calibration	n = 88	0.981	0.75	4
Validation	n = 22	0.986	1.01	

509 *RMSEP: root-mean-square error of prediction.

510

Table 2. Confusion matrices obtained in cross validation (with 2 groups split in venetian blinds) and test samples.

Cross validation				
	non-adult.	**Adult.**	**not-ass.**	**%CC**
non-adult	41	6	0	87
adult	2	17	0	89
Test samples				
	non-adult.	**Adult.**	**not-ass.**	**%CC**
non-adult	16	0	0	100
adult	1	5	0	83

511 % CC: percentage of correctly classified samples; adult.: adulterated; non-adult.: non-adulterated.

Table 3. Classification parameters (non-error rate, error rate, class specify and sensitivity, ratio of not assigned samples) obtained cross validation (with 2 groups split in venetian blinds) and on the test set.

			Non-adulterated		**Adulterated**		
	NER	**ER**	**Specificity**	**Sensitivity**	**Specifity**	**Sensitivity**	**Not ass.**
Cross validation	0.883	0.116	0.872	0.895	0.895	0.872	0.0
Test set	0.917	0.083	1	0.833	0.833	1	0

512 NER: non-error rate; ER: error rate.

513

514 **Figure captions**

515 Figure 1. Raman spectra from different paprika samples with different ASTA values (A). The
516 same spectra after subtraction of fitted polynomial (B).

517 Figure 2. Pre-processed Raman spectra of a paprika sample adulterated with different
518 concentrations of Sudan I.

519 Figure 3. A) Loadings for principal components 1, 2, 3 and 4 (left). Score values of PC 4 versus
520 PC 3 (right). B) Loading of the PCs 1 and 2 (left). Score values of PC 1 versus PC 2 (right).

521

522

523

524