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CHEMOMETRICS

Characterization of Spanish Paprika by Multivariate Analysis of Absorption and Fluorescence Spectra

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ABSTRACT
Spanish paprika was clustered on the basis of the Spanish Protected Designation of Origin "Pimentón de La Vera" by molecular absorbance and fluorescence with principal component analysis and parallel factor analysis. Rapid extraction of carotenoids, capsaicinoids, and tocopherols was optimized; the best conditions included ethanol as the extractant, an extraction time of 10 min in an ultrasonic bath, and a sample size of 0.1 g. The procedure provided good precision with a relative standard deviation of 1.2% for four samples. Molecular absorption spectra were obtained from 250 to 600 nm and fluorescence excitation and emission spectra were collected from 200 to 295 nm and 300 to 400 nm, respectively. Forty-eight "Pimentón de La Vera" paprika samples and 19 samples from other origins were characterized. A principal component analysis model was constructed from the absorption spectra and clustering was obtained based on the origin. Parallel factor analysis was performed on the fluorescence data and better characterization of the origin was obtained.

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Absorption; fluorescence; paprika; parallel factor analysis; principal component analysis

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Introduction

Paprika is a red powder made from grinding the dried pepper pods of some varieties of *Capsicum annuum*. This natural food product is commonly used as a spice and natural colorant in cookery (Palacios-Morillo et al. 2014). In Spain, La Vera (Extremadura) is one of the primary geographical areas where paprika is cultivated (Bartolomé 1988). This product is recognized under the Protected Designation of Origin by the European Union. 45

La Vera paprika is obtained from peppers that are dried by a characteristic drying system. Thus, La Vera peppers are smoked-dried (oak or holm oak wood fire), whereas peppers produced in other Spanish areas or in other countries are sun dried or hot air dried (Bartolomé, Coleto, and Velázquez 2011). This system provides the necessary heat for the optimum dehydration. It is a slow process, lasting 10–15 days and it confers on the paprika its aroma, flavor, and color stability (Pereira Jiménez et al. 2010). For this reason, in order to avoid fraudulent mixtures, it is important to have tools to differentiate products according to their origin. 50

Color is one of the main characteristics of food perceived by the consumer. It predetermines certain expectations of quality and flavor, and a product with undesired color may be rejected. The compounds responsible for the color of paprika are carotenoids. Today, 55

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approximately 650 carotenoids are known (Hornero-Méndez and Mínguez-Mosquera 2001). Carotenoids show marked biological activity, such as anticarcinogenic, antioxidant, and cancer chemopreventive activities (Csiktusnádi Kiss et al. 2000). Carotenoids are compounds of great dietary importance, not only as precursors of vitamin A but also as antioxidants in cell protection and in the prevention of degenerative diseases (Hornero-Méndez and Mínguez-Mosquera 2001). These are composed of red and yellow fractions. Within the former are capsanthin and capsorubin, the latter includes β-carotene, β-cryptoxanthin, and zeaxanthin (Giuffrida et al. 2013).

Carotenoids are polyisoprenoids with a highly conjugated system of double bonds (Cserháti et al. 2001; Rodríguez-Bernaldo de Quirós and Costa 2006). Generally, pigments are extracted with acetone for long periods (Biacs et al. 1989; Ittah, Kanner, and Granit 1993; Mínguez-Mosquera and Hornero-Méndez 1993; Giuffrida et al. 2013). Other solvents, such as diethyl ether, methanol (Weissenberg et al. 1997), and chloroform/methanol (Oliver, Palou, and Pons 1998) have also been used. To quantify pigments in peppers, liquid chromatography (LC) has been commonly employed coupled to photodiode array detectors or coupled to ion trap time of flight mass spectrometry with an atmospheric pressure chemical ionization source operated in positive and negative ionization modes (Giuffrida et al. 2013). Spectrophotometry has been used to determine the red and yellow isochromic carotenoid fractions in paprika (Hornero–Méndez and Mínguez–Mosquera 2001).

Another quality characteristic of this product is the pungency that depends on the presence of a group of alkaloids belonging to the capsaicinoids family (Giuffrida et al. 2013). The primary capsaicinoids are capsaicin and dihydrocapsaicin (90% of the total capsaicinoids) (Davis et al. 2007; Ayuso et al. 2008). These compounds absorb and fluoresce. Other important compounds in paprika include antioxidants; α-tocopherol is the most abundant (Viñas, Campillo, and Hernández Córdoba 1992). These compounds also fluoresce.

LC has been commonly used to determine capsaicinoids and tocopherols in food. Capsaicinoids have been extracted from paprika or peppers with acetone (Saria, Lembeck, and Skofitsch 1981 Giuffrida et al. 2013) and ethanol (Davis et al. 2007) and have been detected by fluorescence (Daood et al. 2002; Chanthai et al. 2012; Butcher et al. 2013) or DAD (Davis et al. 2007; Ziino et al. 2008). Electrochemical techniques have also been used to characterize capsaicinoids (Manaia et al. 2012; Randviir et al. 2013). Ethyl acetate (Viñas, Campillo, and Hernández Córdoba 1992) and hexane (Gnayfeed et al. 2001) have been employed to extract tocopherols, generally with fluorescence detection (Daood et al. 1996; Ramesh et al. 2001; Daood et al. 2014).

There is considerable interest by consumers for the authenticity and quality of food. Spectroscopy and separations have been employed with chemometric methods to reduce analysis time and provide additional information (Borràs et al. 2015). These approaches have been employed for the authentication and determination of contaminants in condiments (Reinholds et al. 2015) and to discriminate food based on characteristic properties (Airado-Rodríguez, Galeano-Díaz, and Durán-Merás 2009; Palacios-Morillo et al. 2014; Di Bella et al. 2015). Principal component analysis (PCA) and parallel factor analysis (PARAFAC) are commonly used for first and second order data.

Sample preparation procedures may affect the spectroscopic properties of extracts obtained from food products. In this work, spectroscopy was coupled with chemometric tools to classify whether or not paprika samples were from the Protected Designation of Origin. The extraction procedure was optimized for the collection of absorption and fluorescence spectra.

Materials and methods

Reagents, standards, and samples

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HPLC-grade acetone and dimethylformamide were obtained from Scharlau (Barcelona, Spain). HPLC-grade acetonitrile and methanol were purchased from Sigma-Aldrich Química (Madrid, Spain). Dimethylsulfoxide was from Merck (Madrid, Spain). Ethanol (96%) and highest quality ethyl acetate were obtained from Panreac (Barcelona, Spain). Capsaicin and α-tocopherol (97%) standards were purchased from Sigma-Aldrich Química (Madrid, Spain). 110

Standard working solutions of capsaicin, α-tocopherol, capsanthin, and β-carotene were prepared by dissolving each solid in ethanol. In order to prevent oxidation and degradation, the standards were protected from light.

Paprika from Spanish Protected Designation of Origin "*Pimentón de La Vera*" ($n = 48$) and other different producers ($n = 19$) were obtained from Regulatory Council of 115 the Denomination of Origin "*Pimentón de La Vera*" and from Spanish local markets, respectively. The origin of the samples not belonging to the Spanish Protected Designation of Origin was not available although in the label reports packaging in Spain.

Extraction

The extraction of analytes from paprika was carried out using a J.P. Selecta ultrasonic bath 120 at various conditions. The solvents employed included dimethylformamide, dimethylsulfoxide, acetonitrile, ethyl acetate, acetone, methanol, and ethanol. The extraction time was varied from 3 to 15 min. Sample sizes from 0.005 to 0.1 g were employed with solvent volumes from 10 to 50 mL. Microwave heating, sonication, and vortex mixing were employed to enhance the extraction efficiency. Each sample was extracted in duplicate. In order to ensure 125 a representative signal, each 0.5 mL extraction aliquot was diluted to 3.0 mL.

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Instrumentation and software

The absorption spectrum of each sample was acquired using a Cary 50 Bio Varian spectrometer from 250 to 600 nm at 1 nm intervals. Cary 50 Bio software was used for data acquisition. An extraction solvent spectrum was used as the blank for baseline correction. 130

Fluorescence measurements were performed using a Cary Eclipse Varian spectrofluorimeter. The Cary Eclipse software was used for data acquisition. Excitation wavelengths were varied from 200 to 295 nm using 5 nm intervals. For each excitation wavelength, emission spectra were obtained from 300 to 400 nm using 1 nm intervals.

Unscrambler v6. 11 (CAMO A/S Olav Tryggvasonsgt, N-7011, Trondheim, Norway) soft- 135 ware was employed for principal component analysis. Statistical treatment was performed using SPSS v.19 (IBM, Statistical Package for Social Sciences) software. Analysis of fluorescence data were performed using MatLab R2008a (Matlab Version 7.6, The Marhworks, Natick, Massachusetts, 2010) and the PLS toolbox routine (Eigenvector Research, Wenatchee, WA).

Principal component analysis

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The absorption spectra were differentiated using matrix consisting of 351 columns (absorbance values from 250 to 600 nm for each sample) and sixty-seven rows (the paprika samples) for chemometric calculations. Principal components analysis (PCA) was

employed to classify paprika according to origin. Multivariate statistical analysis is a useful technique to identify common patterns in multivariate data sets. PCA is a powerful visualization for data evaluation, which may graphically represent intersample relationships and provides a way to reduce the dimension of the data. 145

Parallel factor analysis

The fluorescence spectra were treated with a PARAFAC model using the excitation-emission matrices of 48 "*Pimentón de La Vera*" paprika samples and 19 paprika samples from other producers to classify the samples on the basis of origin. The data were pretreated by the method of Airado-Rodríguez, Galeano-Díaz, and Durán-Merás (2009) to remove the Rayleigh scattering prior to PARAFAC analysis. Subsequently, the sample spectra were arranged in a three-dimensional structure with dimensions of $67 \times 101 \times 20$ (samples $\times$ number of emission wavelengths $\times$ number of excitation wavelengths) to model the set of fluorescence data by PARAFAC. This array was decomposed by PARAFAC using different number of components. In all cases, non-negative constraints for the resolved profiles for all modes were applied to obtain a realistic solution because concentrations and spectral values are positive. 150 155

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Results and discussion

Dimethylformamide, dimethylsulfoxide, acetonitrile, ethyl acetate, acetone, methanol, and ethanol were investigated as extraction solvents using times between 3 and 15 min. The selection of the most suitable solvent for extracting the analytes of interest from the sample matrix was performed based on the highest absorbance signal at 470 nm. Extracts were obtained from a precise sample size of 0.01 g in 10 mL of solvent in an ultrasound bath for 10 min. All assays were conducted in duplicate. The absorbance spectra of paprika extracts were recorded from 250 to 600 nm. Figure 1 shows the absorption spectra of paprika extracts in the various solvents. 160 165

Figure 1 shows that ethanol and acetone provided the highest signals. However, the acetone transmittance limit did not allow viewing the spectra at wavelengths below 250 nm. Moreover, acetone is more volatile than ethanol and solvent losses may result. Another advantage of ethanol is its reduced toxicity. For these reasons, ethanol was employed as the extraction solvent. 170

Generally, when the extraction time is increased, the concentration of analytes extracted is increased, although there is an enhanced chance of degradation. Extractions were performed for intervals of 3, 5, 10, and 15 min using 10 mL of ethanol and 0.01 g of paprika. The highest efficiency was obtained for 10 and 15 min; Student's *t*-test showed significant differences between these times at the 95% confidence level. Therefore, 10 min was selected as the extraction time in order to minimize the analysis time. 175

Precision of the extraction

The repeatability of the extraction stage was characterized using relative standard deviation values based on the absorbance at 470 nm using 10.0 mL of ethanol, 10 min of extraction with sonication, and 0.01 g of sample. The relative standard deviation was 3.3% for four replicate measurements. In order to improve the precision, the mass of sample (0.005, 0.01, and 0.1 g) and volume of ethanol (10, 20, and 50 mL) were optimized. Microwave-heating, sonication, 180

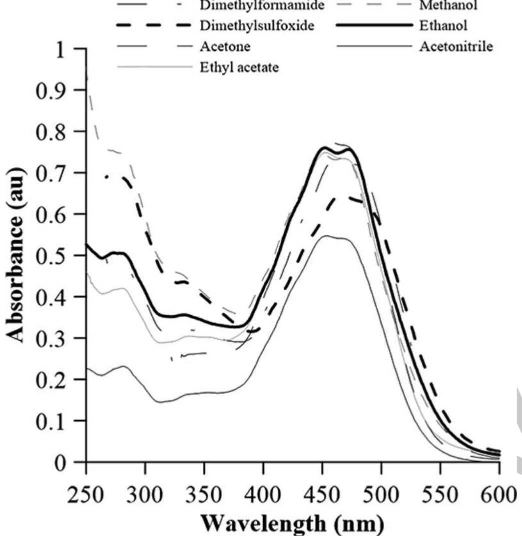

Figure 1. Absorption spectra of paprika extracts using dimethylformamide, dimethylsulfoxide, acetone, ethyl acetate, methanol, ethanol, and acetonitrile.

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and vortex mixing were also evaluated. Relative standard deviation values between 1.2 and 4.9% were obtained for four measurements. The optimum precision of 1.2% was obtained using 0.1 g of sample, 20.0 mL of ethanol, and 10 min of extraction with sonication. Therefore, these conditions were used in subsequent measurements. 185

Absorption and principal component analysis

The absorbance spectra of the paprika extracts were recorded from 250 to 600 nm in duplicate. The absorbance signals over in this wavelength range are primarily due to the total carotenoid concentration (Hornero-Méndez and Mínguez-Mosquera 2001). Figure 2 shows averaged 190

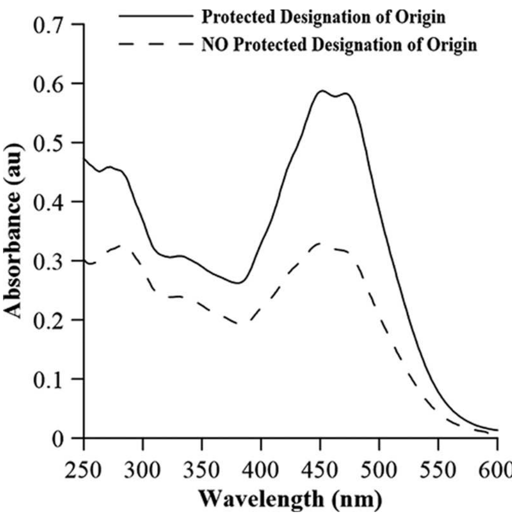

Figure 2. Mean absorption spectra of protected design of origin "*Pimentón de la Vera*" (solid line) and paprika of unknown origin (dashed line).

spectra of Protected Designation of Origin "*Pimentón de La Vera*" paprika and paprika of unknown origin. There were clear differences between the spectra. Moreover, the Student's *t*-test confirmed that the means were significantly different at the 95% level. These results showed that carotenoid concentrations may allow differentiation of samples based on their origin. 195

Principal component analysis was subsequently employed to classify paprika as being Protected Designation of Origin "*Pimentón de La Vera*" or some other material. Principal components (PCs) were employed to explain the variance of the original data and reduce the dimensionality of the data matrix. In this work, the three first principal components explained the 99.8% of total variance. 200

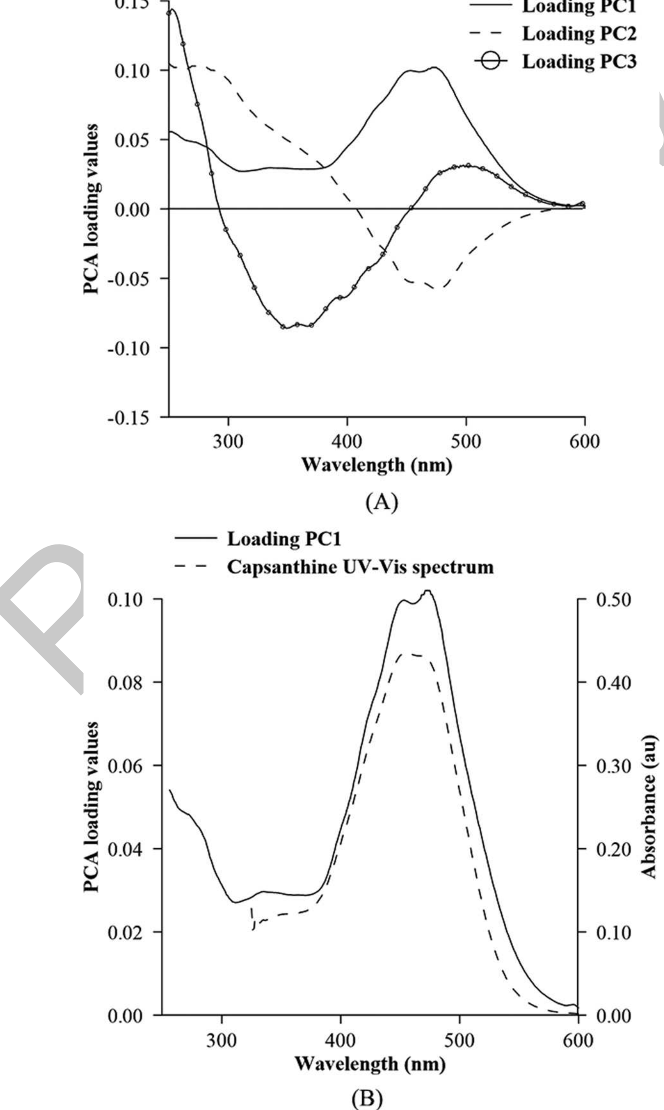

Figure 3. (A) Loadings for principal component 1, principal component 2, and principal component 3 from absorption spectra. (B) Absorption spectrum of 2.00 μg mL^{-1} capsanthin and loading for principal component 1 based on the absorption spectrum.

The loading of each variable in the extracted principal components is shown in Figure 3A. Figure 3B shows that the absorption spectrum of capsanthin was highly correlated with principal component 1 that explains 91% of the data variability. No significant correlations with the other compounds were found with principal components 2 and 3. Principal component 2 was not related with the spectra of the red and yellow carotenoids. In other words, the distribution of fractions in the samples was not a primary source of variance. However, the maximum loadings of the third principal component were at 508 nm. The largest difference between red and yellow fractions spectra were obtained at

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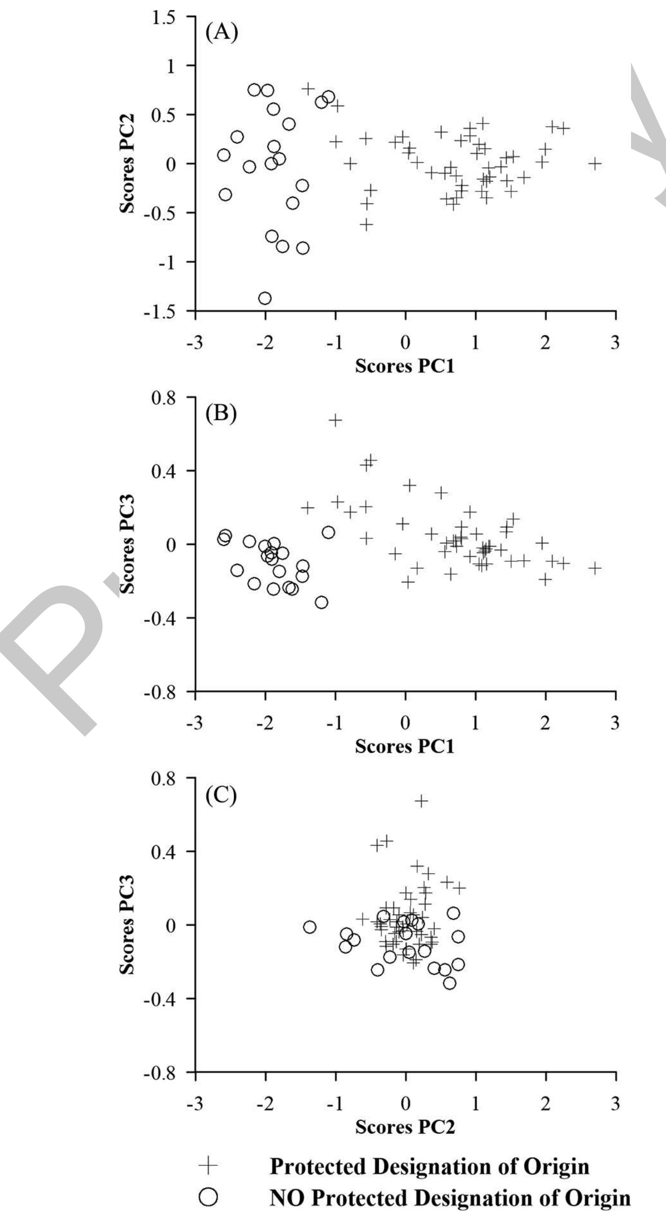

Figure 4. Two-dimensional representation of scores corresponding to the three principal components.

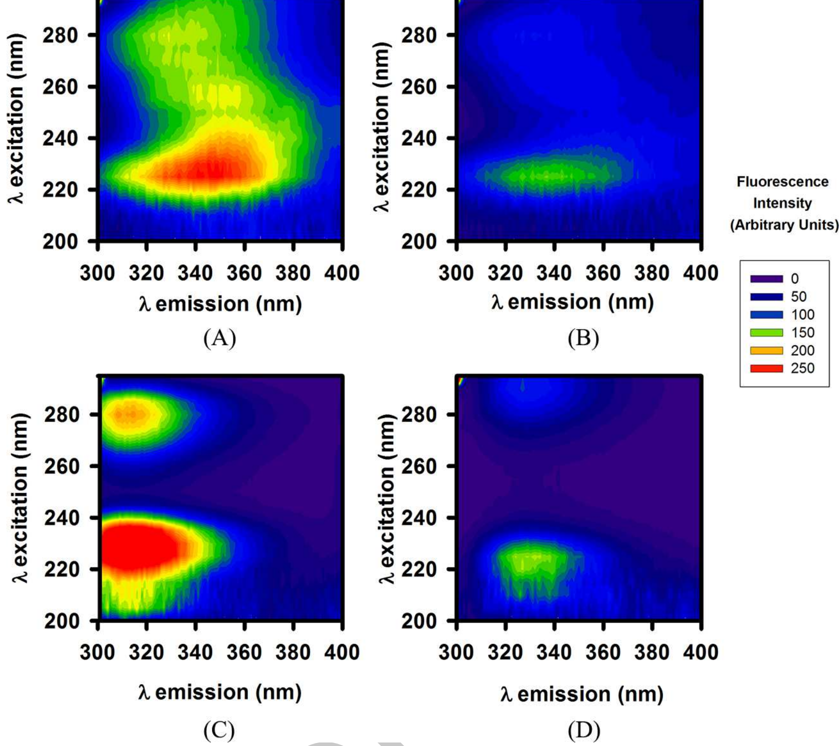

Figure 5. Excitation-emission spectra of (A) paprika belonging to protected design of origin "*Pimentón de La Vera*", (B) paprika of unknown origin, (C) 1.00 μg mL^{-1} capsaicin, and (D) 3.00 μg mL^{-1} α-tocopherol.

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this wavelength as reported by Hornero-Méndez and Mínguez-Mosquera (2001). Therefore, this principal component may be related to the concentrations of both fractions. 210

Figure 4 shows the distribution of data in the planes formed by the first two principal components and from the first and third principal components. Two groups along the principal component 1 axis were present in both figures, although there were some overlaps. Only three of the forty-eight Protected Designation of Origin samples were too close to the samples of unspecified origin. The samples that overlapped were sweet paprika obtained from *Bola* peppers with lower concentrations of carotenoids than other varieties for Protected Designation of Origin paprika production (Mínguez-Mosquera and Hornero-Méndez 1994). 215

As principal component 1 was related to the carotenoid concentration, Protected Designation of Origin samples that appeared at positive principal component 1 scores contain carotenoid concentrations that exceeded the mean. The paprika of unspecified origin provided negative PC1 scores and therefore contained lower carotenoid concentrations. These results show that the origin influences the color of paprika and samples may be differentiated by this characteristic. 220

Fluorescence and parallel factor analysis 225

The fluorescence excitation-emission matrices of paprika extracts were recorded as described in the Experimental section. Numerous fluorescent compounds may be present

in paprika, including capsaicinoids or tocopherols. The excitation-emission spectra of the paprika extracts from samples of Protected Designation of Origin "*Pimentón de La Vera*" and other commercial samples were collected. Representative excitation-emission matrices of each type are shown in Figure 5.

These excitation-emission spectra were compared with standard spectra of expected components in the samples, including capsanthin, β-carotene, α-tocopherol, and capsaicin. Capsanthin and β-carotene did not fluoresce in this wavelength range. Figure 5 shows that capsaicin and α-tocopherol fluoresced in this region and hence they were expected to contribute to the total fluorescence signal of the samples.

Large differences in fluorescence signal were observed from extracts of "*La Vera*" paprika compared to extracts from the other types of paprika. Hence, the use of these signals was explored to classify samples on the basis of origin using multivariate data analysis by PARAFAC. Excitation-emission spectra were obtained for 67 samples, 48 from the Protected Designation of Origin "*Pimentón de la Vera*" and other 19 samples from other producers.

First, the number of components for constructing the PARAFAC model was selected. The criterion used was the core consistency diagnostic (Andersen and Bro 2003). Core consistency percentages of 100, 94.5, 91.1, and 9.1% were obtained for one, two, three, and four component models, respectively. It was clear that three components were the optimal in this case for constructing the model. The three dimensional structures of the three PARAFAC components obtained from this PARAFAC model are shown in the Figure 6. The loading

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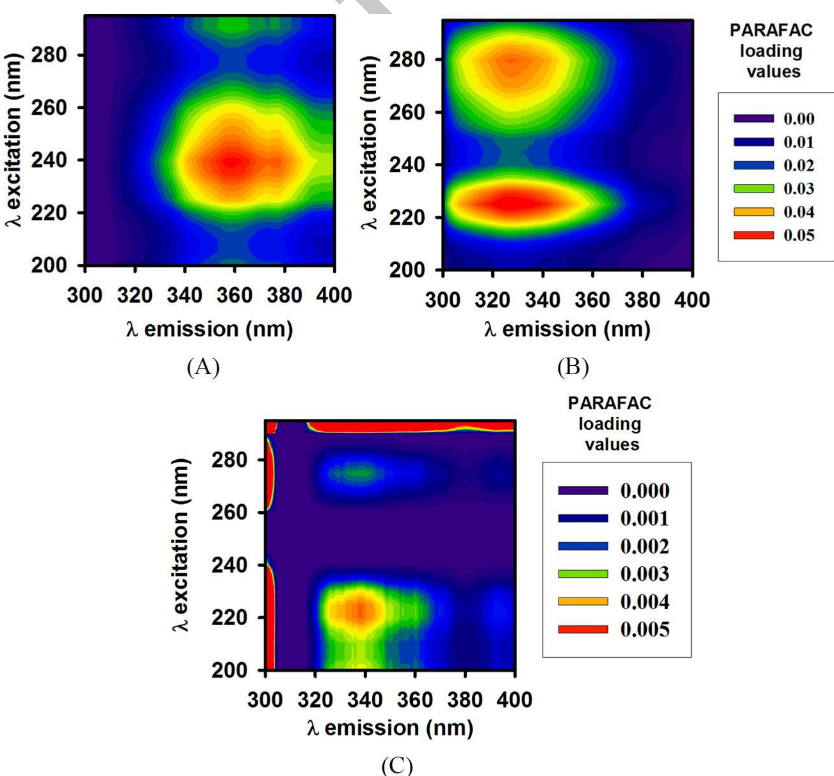

Figure 6. Three dimensional structures of the (A) first, (B) second, and (C) third parallel factor analysis components obtained by multiplying the corresponding vectors.

corresponding to the first component did not match capsaicin or α-tocopherol. However, the loading corresponding to the second component was very similar to the capsaicin or α-tocopherol fluorescence spectra. A grouping of the samples was not observed based on the sweet, hot/sweet, and hot types of paprika. Therefore, the loading corresponding to the second component was not related to capsaicin, which is present in hot paprika at higher concentrations than in sweet or hot/sweet paprika.

The score values corresponding to each PARAFAC component were plotted against each other to study systematic information contained in fluorescence data with respect to the origin of the sample. As shown in Figure 7, two clusters of the paprika were obtained

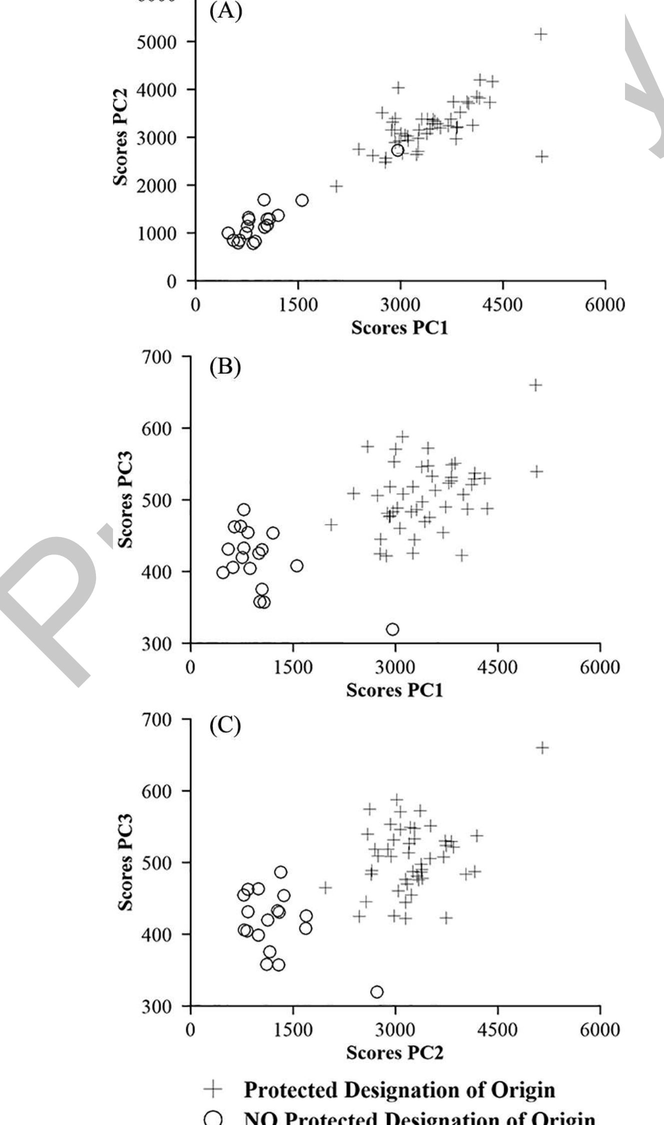

Figure 7. Two-dimensional representation of parallel factor analysis scores corresponding to the three component optimized model.

Table 1. Comparison of spectrophotometry and principal component analysis with fluorescence and parallel factor analysis.

Technique and chemometric tool	Results
Spectrophotometry and principal component analysis	Loading values are highly correlated with the absorption spectrum of capsanthin. Samples are clustered in two groups along the principal component 1 axis.
Fluorescence and parallel factor analysis	Three-dimensional structures of three components obtained by parallel factor analysis were examined. Loading corresponding to the second component was similar to the capsaicin or α-tocopherol excitation-emission spectra. Two clusters of paprika were separated according to origin along the principal component 1 and principal component 2 axes and the principal component 1 and principal component 3 axes. Better differentiation of paprika was obtained compared to spectrophotometry and principal component analysis.

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according to origin. The clusters were along the PC1/PC2 and the PC1/PC3 axes. Only one of the non-Protected Designation of Origin samples was included in the group of Protected Designation of Origin samples. This sample was a smoked-dried paprika. Therefore, the unknown component or components responsible of principal component 1 may be employed to distinguish these samples, as shown in Figures 7A and C. In addition, if the second component is related to the concentration of α-tocopherol, then the Spanish paprika from the Protected Designation of Origin contains higher α-tocopherol concentrations than the other samples. However, the third component did not allow differentiation of the samples. Table 1 provides a summary of the advantages and limitation of the spectroscopic techniques and chemometric tools.

Conclusions

Absorbance and fluorescence spectra were coupled to chemometric tools and used to determine whether or not paprika samples were from Protected Designation of Origin "*Pimentón de La Vera.*" The extraction conditions were optimized for carotenoids, capsaicinoids, and tocopherols. The optimum precision was obtained using 20 mL of ethanol as the extracting solvent, a sample size of approximately 0.1 g, and 10 min for extraction with sonication. Absorption spectra of each sample were obtained in order to construct principal component analysis models for the two sets of samples. Fluorescence spectra were collected followed by parallel factor analysis. The Protected Designation of Origin samples were clustered by both methods, but the separation was better by fluorescence. This methodology may be used as a rapid tool to authenticate "*La Vera*" paprika. These studies may continue through the characterization of mixtures of "La Vera" paprika with other paprika and the evolution of absorbance and fluorescence signals as a function of time. In addition, supervised pattern recognition methods as soft independent modeling of class analogy may be investigated to provide improved classification.

Funding

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