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Near-Infrared Spectroscopy in Saffron Quality Control: Determination of Chemical Composition and Geographical Origin

AMAYA ZALACAIN,*,† STELLA A. ORDOUDI,§ EVA M. DÍAZ-PLAZA,† MANUEL CARMONA,† INMACULADA BLÁZQUEZ,† MARIA Z. TSIMIDOU,§ AND GONZALO L. ALONSO†

Cátedra de Química Agrícola, ETSI Agrónomos, Castilla-La Mancha University, 02071 Albacete, Spain, and Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

Near-infrared reflectance spectroscopy has been applied for the first time to saffron spice to determine the chemical composition and geographical origin of 111 samples from the there main producers' countries: Iran, Greece, and Spain. The validation procedures with the results obtained by UV−vis and HPLC-DAD measurements demonstrated that this technique is appropriate to determine the following parameters: moisture and volatile content, coloring strength, $E_{1cm}^{1\%}$ (250 nm), and $E_{1cm}^{1\%}$ (330 nm), established on the ISO 3632 Technical Specification Normative and used to certify saffron quality in the international market. Also, it can be used to estimate the content of the five main crocetin glycosides, the compounds responsible for saffron color, the best correlations being for *trans*-crocetin di-(β-D-gentibiosyl) ester ($R^2 = 0.93$), *trans*-crocetin (β-D-glucosyl)-(β-D-gentibiosyl) ($R^2 = 0.94$), and picrocrocin ($R^2 = 0.92$), the compound accepted as responsible for saffron bitterness. Finally, a discriminant analysis among the three geographical origins reveals that Iranian samples are the most different, whereas Greek and Spanish samples are more similar. All of these results reveal that NIRS spectroscopy has an enormous potential for its application to saffron quality control as the results are obtained in 2 min and without any sample manipulation.

KEYWORDS: NIRS; saffron stigmas; crocetin glycosides; picrocrocin; geographical origin

INTRODUCTION

Technological improvements and novelties in the food industry had an impact on monitoring of the quality of raw materials, processes, and finished products by means of powerful analytical tools. This trend was supported by regulatory requirements, which the traded products have to comply with, and consumer interest in food quality and safety. Introduction of spectroscopic techniques in the routine control of food products became, thus, a challenging area for manufacturers of analytical instruments. This is the case for near-infrared spectroscopy (NIRS), which, although known for a long time, was avoided by the spectroscopists because of difficulties in the interpretation of overlapping bands as acute peaks do not appear in this region of the spectrum; consequently, band assignment to specific functional groups is rather complex due to the numerous overtones and combinations. Treatment of spectral information was made feasible only after advances in data processing technology and computer programming (*1*).

An interesting area of food quality control is that of commercial spices. Among the latter, saffron, the dried stigmas of *Crocus sativus* L., are known to be the most expensive in the international market. Saffron commercial quality is determined by specifications recommended by the ISO/TS 3632 (*2*). Loss of quality can be due to different reasons: (a) inadequate harvesting conditions—the flower should be collected as soon as possible to avoid degradation; (b) inadequate dehydration conditions—in India, Iran, and Morocco the flower is frequently dehydrated under direct sunlight, whereas in Greece and Spain dehydration takes place indoors at higher than ambient temperature; (c) poor storage conditions; or (d) simply by the action of the producer or dealer to mix stigmas with other noncolored parts of the plant, generally the style. To establish saffron quality, a large number of parameters such as microscopic characteristics, presence of flower waste, moisture content, ash content, and nitrogen content can be determined. Of these, the most important ones are the aroma, bitterness, and coloring strength. UV−vis spectrometry is proposed by the mentioned normative to estimate the three latter characteristics at specific wavelengths expressed as $E_{\lambda max}$ values. For example, absorbance at 440 nm is used for crocetin glycosides quantification. As

* Author to whom correspondence should be addressed (e-mail Amaya.Zalacain@uclm.es).
† Cátedra de Química Agrícola, ETSI Agrónomos.
§ Aristotle University of Thessaloniki.

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Table 1. Summary of the Different Samples Analyzed, Geographical Origins, and Their Quality According to the ISO/TS 3632 (2003)

	no. of samples	ISO category		
		I	II	III
Iran	18	2	4	12
Greece	22	22	2	
Spain	71	64	4	3
total	111	88	11	15

this procedure does not allow separation and quantification of individual members, other techniques such as thin-layer chromatography or high-performance liquid chromatography (HPLC) have been also used to study saffron carotenoids or picrocrocin, the compound considered to be responsible for the bitter taste of the spice (*3*−*8*). However, application of HPLC requires a long process of sample preparation, including solvent extraction of the carotenoids, which are usually strongly bonded to other plant constituents, and therefore the analysis results may not present the real carotenoid content. Moreover, solvents and also other factors such as high temperature or light may cause changes in the carotenoid conformation, leading to the formation of cis isomers (*9*, *10*). FT-Raman spectroscopy has been also used to study the chemical composition of many carotenoids (*11*, *12*). NIRS has been used for exploring the authenticity of many foods, such as vegetable oils (*13*), olive oil (*14*), coffee (*15*), honey (*16*), cheese (*17*), maize (*18*), and beer (*19*), but not for saffron. NIRS could serve as a potential tool for detecting adulteration or for routine quality control if proper calibration and validation procedures with data acquisition protocols are established. In this study, the possibility of applying NIRS to the determination of saffron chemical composition is examined. NIRS data were compared with those obtained using UV−vis and HPLC-DAD methodologies. Geographical origin discrimination using NIRS data was also examined because the price of this spice strongly depends on its country of production.

MATERIALS AND METHODS

Samples. This study involved the analysis of 111 well-defined samples of saffron from leading producers (Iran, Greece, and Spain) (**Table 1**). The samples were obtained directly from the producers with the guarantee of their origin and freedom from fraud. A pool of crocetin glycosides standard of 99.5% purity were obtained according to the methodology proposed by Escribano and co-workers (*20*).

ISO 3632/TS (2003) Determinations. *Moisture and Volatile Contents.* Saffron stigmas were ground and passed through a 0.5 mm mesh. Moisture and volatiles were determined by successive weighing of 1 g of powdered sample introduced in an oven set at 103 ± 2 °C for 16 h. The content was calculated by the following ratio: (initial mass − constant mass/initial mass) × 100.

Coloring Strength [$E^{1\%}_{1cm}$ (440 nm), $E^{1\%}_{1cm}$ (250 nm), and $E^{1\%}_{1cm}$ (330 nm)] *(UV−Vis Determinations).* Saffron samples were analyzed according to the ISO 3632 trade standard (*2*). Measurements of $E^{1\%}$ of an aqueous saffron extract at 440, 330, and 250 nm, respectively, were carried out using a 1 cm pathway quartz cell. The reported values are the average values of two replicates.

Identification and Quantitation of Crocetin Glycosides and Picrocrocin by LC-DAD-MS. A 50 ppm aqueous extract of each sample have been prepared according to ISO 3632/TS (*2*). Twenty-five microliters of the extract filtered through a PVDF filter of 0.45 μm (Millipore, Bedford, MA) was injected into an Agilent 1100 HPLC chromatograph (Palo Alto, CA) equipped with a 5 μm Nucleosil C18 column (200 × 4 mm) thermostated at 30 °C. Solvents of chromatographic grade were water (A) and acetonitrile (B). The gradient system was 20% B for 5 min to 75% B in 15 min, at a flow rate of 0.8 mL/min, then 100% B for 3 min at a flow rate of 0.8 mL/min, and 10 min of 100% B at 1 mL/min. Double on-line detection was carried out by a diode array spectrophotometer and a quadrupole mass spectrometer with electrospray ionization (ESI) (Agilent 1100, Palo Alto, CA). The probe of the mass spectrometer was connected to the UV cell outlet. The DAD detector was set at 250, 330, and 440 nm. Both the auxiliary and the sheat gas were nitrogen with a flow rate of 12 L/min. The drying gas temperature was set at 350 °C and the nebulizer pressure at 30 psi. The capillary voltage was ±2500 V and the capillary temperature 195 °C. Spectra were recorded in positive and negative ion mode between *m*/*z* 100 and 1500. Identification was carried out with Agilent Chemstation software for LC-MS. Data reported represent the average of two sample replicates.

NIRS Analysis. Approximately 2 g of each powdered saffron sample, ground and passed through a 0.5 mm mesh, was placed into quartz sample plates of 3 cm diameter (Perkin-Elmer, Norwalk, CT). Crocetin glycoside standard in solid state was submitted to the same procedure. Samples were analyzed by Perkin-Elmer Spectrum One FT-NIR equipment coupled with a near-infrared reflectance accessory (NIRA). Data collection was acquired over a wavelength range of 4000−10000 cm^{-1}, and the resolution was set at 16 cm^{-1}. All samples were scanned in duplicate.

Data Processing. Multivariate analysis was used for quantitative and qualitative analysis. The principal component regression was used in the present study as a quantitative test. Equations for NIRS prediction were developed by Spectrum Quant+ software (Perkin-Elmer) with the principal component analysis option and two passes to eliminate the outliers. The equation selected as the best for each chemical fraction was obtained using the lowest standard error of validation to minimize the risk of overfitting when the model accuracy was evaluated. Samples with large residuals were omitted, and validation was performed again. Discriminate analysis was performed using the SPSS version 11.5 statistical package for Windows (SPSS, Chicago, IL) to classify samples on the basis of geographical origin.

NIRS fingerprint of saffron was recorded and compared with that of a pure crocetin glycosides standard to establish possible differences.

RESULTS AND DISCUSSION

Sample Classification on the Basis of ISO 3632/TS (2003). Saffron is very appreciated by European consumers as a food colorant and also for its aromatic and flavoring properties. Within Europe, packaging and distribution of saffron are the responsibility of small- and medium-size enterprises. Such a market fragmentation results in high competition and low technological development. Saffron adulteration and also misbranding is, thus, frequent (*21*). Classification of the samples examined in this study was based on the limits set by the ISO 3632/TS normative (*2*).

The majority of the samples, 79.2%, were found to belong to category I. Iranian samples were the exception because only 2 of 18 were classified in category I (**Table 1**). Saffron color is due to crocetin glycosides, which were analyzed by LC-DAD-MS. The main five crocetin glycosides were identified together with picrocrocin, 4-(β-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde, although there are no commercial standards available. The clear fragmentation patterns obtained by the soft ionization offered by the ESI interface and its comparison with previous papers (*5*, *8*, *22*−*24*) allowed the structural assignation of **Table 2**. The major crocetin glycoside was *trans*-crocetin di-(β-D-gentibiosyl) ester (T4), representing almost the twice the *trans*-crocetin (β-D-glucosyl)-(β-D-gentibiosyl) (T3) and between 13.65 and 26.55 times the other three (T2, C4, and C3) (**Table 4**) (*25*). *trans*- and *cis*-crocetin glycoside configurations are clearly identified as shown by different spectroscopy behaviors. In relation to their trans homonyms, *cis*-crocetin presents an additional absorption band around 324 nm in their UV−vis spectrum, and their maximum

Table 2. Retention Time, Mass Fragmentation Pattern in the Positive and Negative Ion Modes, and UV Maximum Band of Saffron Crocins and Picrocrocin[a]

		mass fragmentation pattern		
	RT (min)	positive ion mode	negative ion mode	UV λ_{max}
P	6.64	353 (100), 185 (35)	375 (100)	250
T4	12.51	999 (100), 675 (10), 513 (25)	975 (78), 651 (54), 533 (100)	420, 460
T3	13.36	837 (100), 675 (7), 329 (15)	813 (20), 651 (100), 489 (15)	440, 468
T2	14.35	675 (100), 513 (85), 386 (84)	651 (100), 487 (41), 287 (25)	435, 461
C4	14.84	999 (100), 511 (30), 386 (11)	975 (75), 651 (100), 533 (30)	330,435, 462
C3	16.21	837 (100), 675 (10), 543 (85)	837 (20), 651 (100), 565 (23)	330,435, 460

[a] P, 4-(β-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde; T4, C4, *trans*- and *cis*-crocetin di-(β-D-gentibiosyl) ester, respectively; T3, C3, *trans*- and *cis*-crocetin (β-D-glucosyl)-(β-D-gentibiosyl) ester, respectively; T2, *trans*-crocetin di-(β-D-glucosyl) ester.

Table 3. Principal Component Statistics of NIRS Calibration and Validation for the Different Parameters Measured According to ISO/TS 3632 (2003)[a]

	iso measurements			
characteristic	moisture	$E^{1\%}_{1cm}$ (440 nm)	$E^{1\%}_{1cm}$ (330 nm)	$E^{1\%}_{1cm}$ (250 nm)
no. of analysis	222	222	222	221
outliers	19	15	15	18
no. of inde- independent standards	33	26	26	22
no. of principal components	12	10	9	13
SEC	0.96	19.94	3.30	6.26
SEP	0.99	20.40	3.40	6.48
R^2	0.93	0.91	0.72	0.90
mean value	9.75	209.60	31.92	82.27
% variance	87.07	82.68	52.16	80.37

[a] SEC, standard error of calibration; SEP, standard error of prediction.

Table 4. Principal Component Statistics of NIRS Calibration and Validation for the Crocin and Picrocrocin Content Determined by HPLC[a]

	HPLC measurements					
characteristic	T4	T3	T2	C4	C3	P
no. of analysis	222	222	211	205	189	220
outliers	23	24	31	43	54	27
no. of inde- pendent standards	25	25	28	22	27	23
no. of principal components	11	37	9	12	5	15
SEC	1101.00	573.80	109.00	192.20	99.67	452.40
SEP	1143.03	688.60	112.10	204.31	102.10	472.81
R^2	0.93	0.94	0.85	0.84	0.76	0.92
mean value	8130.00	4529.00	419.30	595.20	306.10	2043.00
% variance	85.56	88.78	72.73	70.57	57.14	83.90

[a] T4, C4, *trans*- or *cis*-crocetin di-(β-D-gentibiosyl) ester; T3, C3, *trans*- or *cis*-crocetin (β-D-glucosyl)-(β-D-gentibiosyl) ester; T2, *trans*-crocetin di-(β-D-glucosyl) ester; P, 4-(β-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde; SEC, standard error of calibration; SEP, standard error of prediction.

absorption at 440 nm presents a hypsochromic effect of some 5 nm (*3*, *8*, *10*).

Chemical Composition Predicted by NIRS. Common problems found in near-infrared reflectance such as noise signals or interferences between the scatter and the sample particle size were not observed when saffron spectra were recorded. This could be due to the homogenization of the sample procedure, where all samples pass through a 0.5 mesh, not requiring any pretreatment spectral data. The NIRS fingerprint of saffron was compared to that of a pure crocetin glycosides standard, as

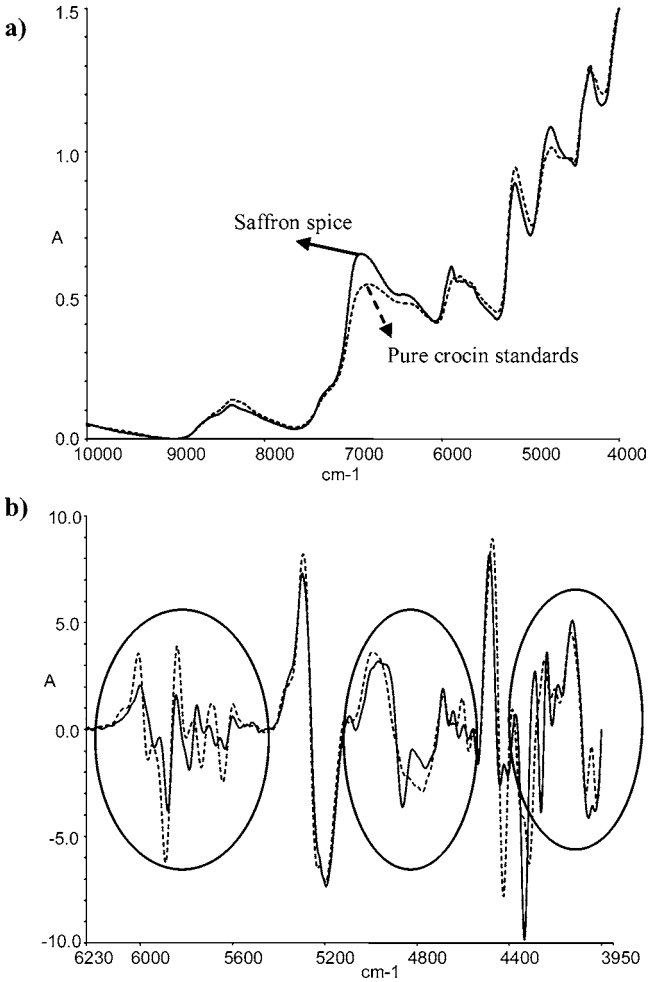

Figure 1. Near-infrared fingerprint spectra of (**a**) saffron spice and a pure standard of crocetin glycosides and (**b**) their respective second-derivative spectra and the differences observed.

illustrated in **Figure 1a**. Differences between them were only slight, and calculation of the second-derivative spectra revealed some characteristic differences in three regions (**Figure 1b**). The latter means that calibrations are expected to be more sensitive within those ranges. Principal component regression (PCR) was used to analyze the NIR spectra; the statistics of calibrations and validation for the different samples when compared with the UV−vis measurements are reported in **Table 3**. The number of principal components varied from 9 to 13, although it has to be pointed out that the two principal components accounted for 98 ± 0.5% of the total variability. The validation carried out was an independent procedure, and the number of independent standards used for each specific

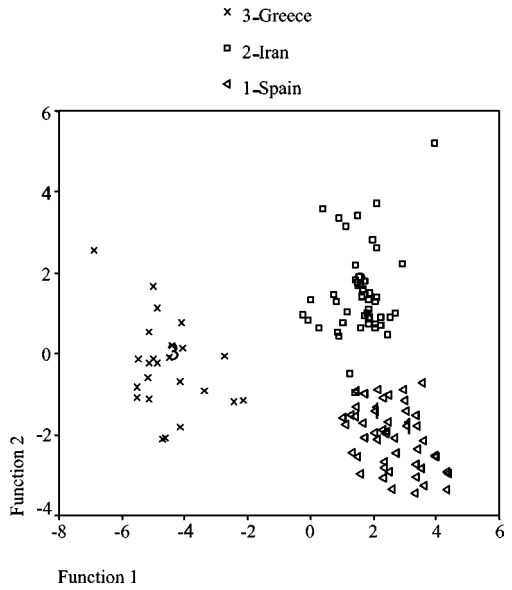

Figure 2. Discriminant plot of the different saffron samples when geographical origin was used as differentiating variable.

property is shown in **Table 3**. The low differences found between the standard error of calibration (SEC) and the standard error of prediction (SEP) reveal the robustness of the equation which best fit for each of the parameters. The low R^2 value obtained for the $E^{1\%}_{1cm}$ (330 nm) parameter is understandable as the determination was carried out in an aqueous extract, but safranal and other volatile compounds are poorly water-soluble. Also, at the wavelength chosen for this determination there are other compounds such as *cis*-crocetin glycosides that also absorb (*8*). The other R^2 value ranged from 0.90 to 0.93. The value for the moisture content should be noted. This is an important parameter because it is fraudulent, although very lucrative, to sell water at saffron price. The maximum limit established by ISO/TS 3632 (*2*) is 12%, a value far from the average of 9.75% obtained.

When the crocetin glycosides content and picrocrocin analysis carried out by HPLC with the NIRS data are compared (**Table 4**), the number of principal components varied in this case from 5 to 37, although with the first two principal components, it was possible to account for 97 ± 0.5% of the total variability. In this case, the R^2 values for crocetin glycosides determination range from 0.76 to 0.94; the lower values of T2, C4, and C3 may be due to the difficulty to integrate such crocetin glycosides area. Slightly higher differences were found between the SEC and the SEP when HPLC analysis was carried out, especially for T3. Good correlations are obtained when bitterness is evaluated by UV−vis spectrometry and individual analysis of picrocrocin by HPLC ($R^2 = 0.92$).

In both cases (UV−vis and HPLC), the capacity of the technique chosen to measure saffron color was demonstrated, as a pool of compounds by UV−vis or one by one by HPLC.

Geographical Origin Discrimination. The potential of NIRS for discriminating saffron geographical origin was also assayed. The diagnosis of the three family tests (Iran, Greece, and Spain) showed a critical probability level of 0.01%. The interclass distances between different countries reveal that Iranian samples ($D_{Iran-Greece} = 180.16$; $D_{Iran-Spain} = 319.22$) are the most different, whereas Greek and Spanish samples ($D_{Greece-Spain} = 21.64$) are more similar, as they have lower interclass distances. Percent recognition for each group of samples was 100% for Iranian, ∼95% for Greek, and ∼88% for Spanish ones.

If the chemical composition data were used, saffron samples were clearly separated by two canonic discriminating functions, when geographical origin was used as differentiating variable (**Figure 2**). The first function explained 83.8% of the variance and the second one 12.2%, the *trans*-crocin 4 area percentage (at 440 and 330 nm), together with the coloring strength of the variables, contributing most to the differentiation.

In summary, NIRS seems to be a powerful tool for application to saffron quality control and origin characterization. Time-savings and low labor and running costs are important merits that should not be overlooked because of the considerable capital cost of the instrumentation. Therefore, the above-mentioned procedure is highly recommended for consideration in the next ISO/TS 3632 (2003) amendment.

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