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Quantification of Crocetin Esters in Saffron (*Crocus sativus* L.) Using Raman Spectroscopy and Chemometrics

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The feasibility of Raman spectroscopy for predicting the content of crocetin esters (crocins), and coloring strength was assessed. 114 samples from Greece, Iran, Italy and Spain were divided into two sets: a calibration set with 49 samples and a validation one with 65 samples. Calibration models for crocetin esters (r 0.97, RMSEC 0.92, RMSEP 0.97, RPD 3.46) and coloring strength (r 0.95, RMSEC 12.2, RMSEP 11.3, RPD 2.59) were built in the spectral region 1700−955 cm^{-1} using partial least-squares (PLS) regression. The calibration models were validated using cross-validation, leaving one sample out (r 0.97, RMSECV 1.09 for crocetin esters and r 0.93, RMSECV 14.5 for coloring strength). The crocetin esters content as determined by liquid chromatography fluctuated between 18.8 and 31.7 mg/100 g saffron. The corresponding values, as calculated using the Raman method, fluctuated between 19.2 and 32.0 mg/100 g saffron. The coloring strength determined by the reference method ranged from 177.0 to 296.7 units, while with the Raman method the values were between 186.8 and 297.6 units. The results, as compared to the reference methods (liquid chromatography and UV−vis spectrophotometry), show that the proposed methodology gives data with acceptable accuracy. The proposed models can be used as a tool for rapid screening of quality in saffron samples.

KEYWORDS: Saffron; Raman spectroscopy; HPLC chromatography; crocins; coloring strength; partial least-squares PLS

INTRODUCTION

Saffron's quality is determined by its taste, aroma and color. Picrocrocin, safranal and crocins are the secondary metabolites that contribute to saffron quality parameters respectively. Picrocrocin, the safranal's glycoside precursor, contributes to the bitterness and is responsible for its taste. Safranal, a monoterpene aldehyde, is the main compound of the essential oil of saffron and gives its distinctive aroma. Crocetin esters with glucose, gentiobiose, neapolitanose or triglucose sugar moieties are water-soluble carotenoids and responsible for saffron's yellowish color. The determination of these compounds is important for establishing the commercial quality criteria and consequently its price.

For world trade, color is the major parameter for saffron's quality. Under the Technical Specifications of the ISO standard ISO/TS 3632:2003 (*1*) this parameter is expressed as coloring strength, which is defined as the absorbance at 440 nm of a 1% aqueous solution in a 1 cm quartz cell ($E^{1\%}_{1cm}$440 nm). According to this value, saffron is classified into three categories. Because this procedure does not give a detailed composition of crocetin esters, thin layer chromatography and liquid chromatography have been applied, instead (*2*−*6*). Although these techniques are very sensitive and accurate, they are expensive and time-consuming and must be supported by specialized staff. In the case that the saffron sector is made up mostly from medium and small size enterprises, affordable and fast techniques are needed. The application of spectroscopy techniques is enforced in order to save time, cost and reagents.

In this context, spectroscopy techniques such Raman and infrared spectroscopy (IR) in combination with suitable chemometric algorithms have been shown to be advantageous for routine basis quality control of foods (*7*−*14*). Raman and infrared spectroscopy are complementary methods that can be applied for a routine control process. They are fast and solvent free techniques. Recently, near-infrared spectroscopy (NIRS) in combination with chemometrics has been shown to be usable for quantifying the main saffron compounds (*15*). However, an NIR spectrum is difficult to be interpreted due to the fact that NIR consists of overtones and combination bands of fundamental transitions. In a Raman spectrum, there are well-resolved bands of fundamental vibrational transitions thus providing more clear structural information. The polyene structure of carotenoids enhances strong signals, so they can easily be detected in complex biological matrices (*12*, *16*). Raman spectroscopy has already

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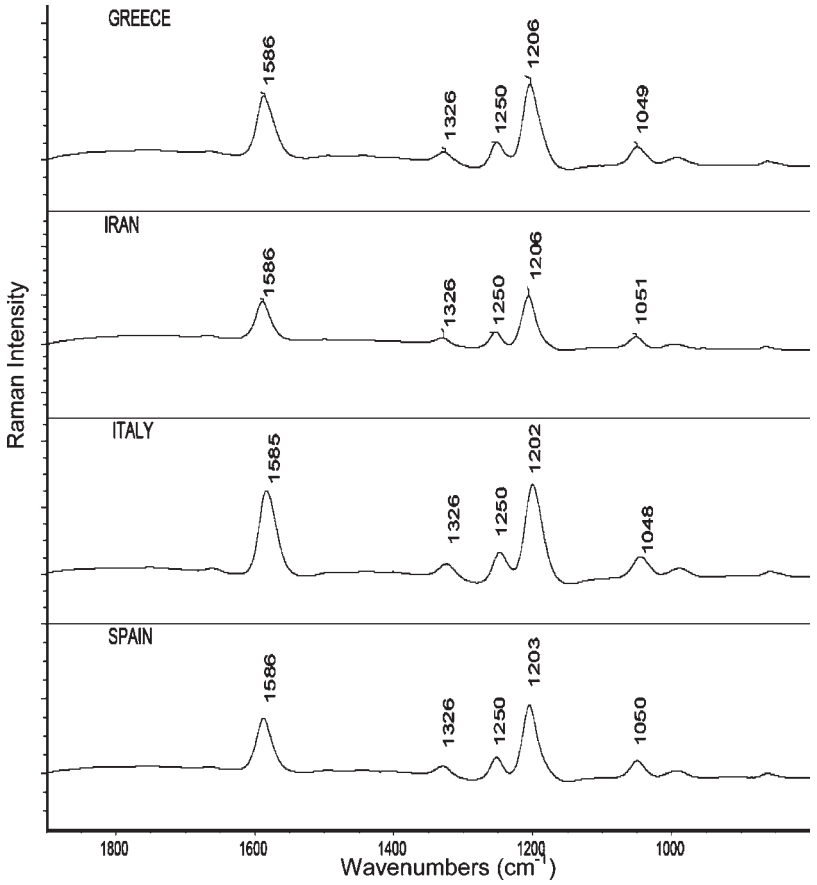

Figure 1. Typical Raman spectra of saffron samples from samples originated from Greece, Iran, Italy and Spain in the spectral region 1900−800 cm^{-1}.

been used for qualitative measurements of carotenoids in saffron (*16*−*18*), but its feasibility for a quantitative approach has not been studied.

The aim of this study is to develop a rapid method for determining the crocetin esters and coloring strength using Raman spectroscopy in combination with partial least-squares (PLS) regression.

MATERIALS AND METHODS

Plant Materials, Chemicals and Reagents. A total of $N = 114$ saffon samples (*Crocus sativus* L.) harvested in the period 2005−2006, in Greece ($N = 28$), Iran ($N = 37$), Italy ($N = 24$) and Spain ($N = 25$), were obtained directly from the producers in order to avoid possible adulteration and to guarantee their origin. The samples were kept at 4 °C in the dark until their analysis. HPLC-grade acetonitrile and formic acid were purchased from Scharlau (Barcelona, Spain). Milli-Q system Millipore (Bedfore, MA) used for the production of ultrahigh-purity water and polytetrafluoroethylene filters (PTFE) (11 mm, 0.45 μm pore) were purchased from Millipore.

Reference Analysis. The determination of coloring strength using a UV−vis spectroscopic method was carried out according to the specification of the ISO/TS 3632-2:2003 (*1*). 500 mg of saffron was weighed in a precision balance (0.001 g). The sample was placed in a volumetric flask, 900 mL of Milli-Q ultrapure water was added and the mixture was stirred by magnetic agitator (1000 rpm) for 1 h kept away from light exposure. Water up to 1 L was then added and homogenized through agitation. An aliquot of 20 mL was transferred to a 200 mL volumetric flask, and water was added. Once homogenized, the solution was filtered through a PTFE filter with a 0.45 μm pore size. Absorbance changes between 190 and 900 nm were recorded in a Perkin-Elmer Lambda 25 (Norwalk, CT) UV−vis spectrophotometer using a quartz cell of 1 cm path length. Coloring strength was calculated according to the following formula:

$$E^{1\%}_{1\mathrm{cm}}\ 440\ \mathrm{nm} = \frac{A \times 10000}{m(100 - W_{\mathrm{MW}})}$$

where A is the absorbance at 440 nm, m is the mass of the sample after drying expressed in g and W_{MV} is the moisture and volatile content according to the specification of the ISO/TS 3632-2:2003 (*1*).

For the analysis of crocetin esters by HPLC, 500 mg of saffron in 900 mL of Milli-Q ultrapure water was stirred for 1 h at room temperature in the dark. Water up to 1 L was then added and the extract was homogenized through agitation. The extract was filtered through a PTFE filter with a 0.45 μm pore size. Then 1 mL of this solution was placed in an opaque vial. The solution was homogenized, and 20 μL was injected into an Agilent 1100 HPLC chromatograph (Palo Alto, CA) equipped with a 150 mm × 4.6 mm i.d., 5 μm Phenomenex Luna C18 column thermostated at 30 °C. The solvents were water Milli-Q acidified with formic acid 0.25% (A) and acetonitrile (B) using the following gradient: 80% A for 5 min to 20% A in 15 min, at flow rate of 0.8 mL/min. Double online detection was carried out by a diode array spectrophotometer (DAD) and a quadrupole mass spectrometer with electrospray ionization (ESI) (Agilent 1100). The probe of the mass spectrometer was connected to the UV cell outlet. The DAD detector was set at 440 nm. Both auxiliary and the sheath gases were nitrogen with a flow rate of 12 L/min. The drying 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 for LC/MS. The nomenclature for the crocetin ester identified was adopted from Carmona et al. (*6*): *trans*-crocetin di-(D-gentiobiosyl) ester (T4GG), *trans*-crocetin (β-D-gentiobiosyl)-(β-D-glucosyl) ester (T3Gg), *trans*-crocetin di-(β-D-glucosyl) ester (T2gg), *cis*-crocetin di-(β-D-gentiobiosyl) ester (C4GG), *cis*-crocetin (β-D-gentiobiosyl)-(β-D-glucosyl) ester (C3Gg), *trans*-crocetin (β-D-neapolitanosyl)-(β-D-gentiobiosyl) ester (T5nG), *trans*-crocetin (β-D-triglucosyl)-(β-D-gentiobiosyl) ester (T5tG), *trans*-crocetin (β-D-neapolitanosyl)-(β-D-glucosyl) ester (T4 ng), *trans*-crocetin (β-D-gentiobiosyl) ester (T2G), *cis*-crocetin (β-D-neapolitanosyl)-(β-D-glucosyl) ester (C4 ng), *cis*-crocetin(β-D-glucosyl) ester (C1g), *cis*-crocetin (β-D-gentiobiosyl) ester (C2G), *trans*-crocetin(β-D-glucosyl) ester (T1g).

For the quantitative determination of crocetin esters the method based on the extinction coefficient and the related area calculated was

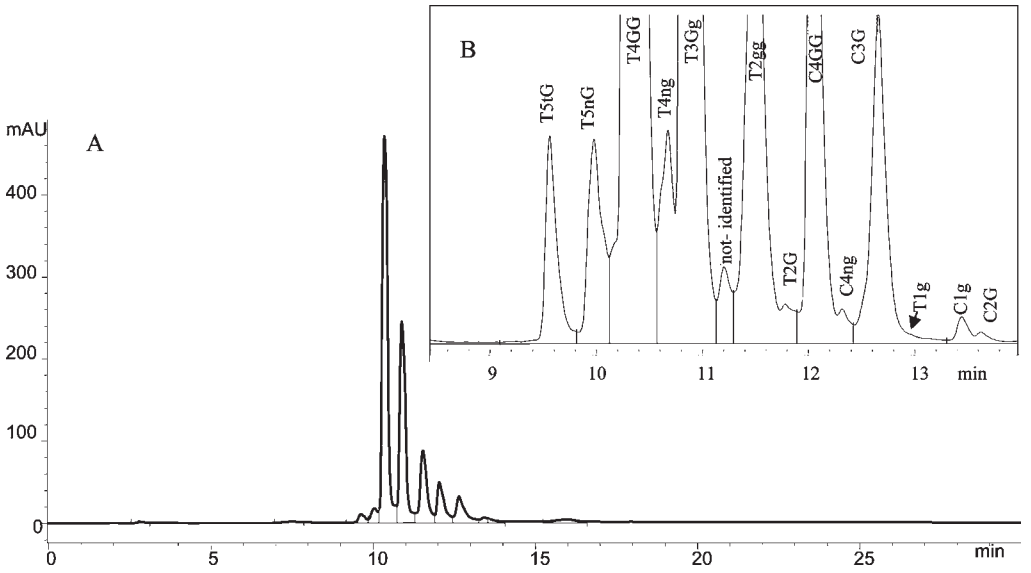

Figure 2. Typical HPLC separation and detection of characteristic crocetin peaks at 440 nm: (**A**) whole view and (**B**) extended view with indication of the main crocetin esters.

used (*19*). So, the concentrations were calculated using the following expression:

$$\text{concentration (mg/100 mg)} = (A \times 100/A_t) \times (\text{mw}_i/\varepsilon) \times E^{1\%}_{1\text{cm}}\ 440\ \text{nm}/10$$

where, for *trans*-crocetin esters, the extinction coefficient (ε) was 89000 M^{-1} cm^{-1} and, for *cis*-crocetin esters, it was 63350 M^{-1} cm^{-1} (*20*), A was the area of the each crocin peak in the chromatogram and A_t was the total area of the crocetin esters, $E^{1\%}_{1\text{cm}}$440 nm was the coloring strength of the samples and mw_i was the molecular weight of the main crocetin esters corresponding to the particular area A.

Raman Spectra. A DeltaNu Advantage 785 near-infrared Raman spectrometer (Laramie, WY) equipped with a 785 nm diode laser for excitation with a maximum output power of 71.6 mW was used to record spectra of saffron cut filaments. The spectral resolution of the instrument is 8 cm^{-1} and the spectral range 2000−200 cm^{-1}. 500 mg of each saffron sample was placed into a 1 mL clear shell vial (VWR International, USA). The vial was placed into a prefixed sampler holder such that the laser was focused into the center of the vial. Each saffron spectrum is an average of two 15 s acquisitions over the spectral range of 2000−200 cm^{-1}. The spectra were collected with the NuSpec software provided by the manufacturer and then treated with the OMNIC (ver. 7.3) software.

Statistics. Raman spectra were imported into TQ Analyst 7.2.0.161 (Thermo Nicolet Corp.). Partial least-squares (PLS) regression was used to correlate Raman spectral data with the chemical reference values for the sum of crocetin esters and the coloring strength. PLS algorithm aims to extract the important information from Raman sprectroscopic data (X-matrix) and the related chemical data (Y-matrix) and to compress it in new independent latent variables (factors).

The PLS models were constructed using a calibration set of 49 samples and a validation set of 65 samples. Leave-one-out cross validation was used for the performance of the established calibration equations and for preventing overfitting of the calibration models (*21*).

Calibration model evaluation was based on correlation coefficient for calibration (r), root-mean-square error of calibration (RMSEC), correlation coefficient for cross validation (r) root-mean-square error of cross-validation (RMSECV). External validation of the calibration models was performed using a set comprising 65 samples not included in the calibration set. The models were used to predict the sum of crocetin esters and the coloring strength of the 65 samples. The predicted values were then compared with the reference values. The root-mean-square error of prediction (RMSEP) and the residual predictive deviation (RPD), defined as the ratio of standard deviation to square error of prediction, were calculated. RPD is a parameter that evaluates the robustness of the model. It has been reported that an RPD value >3 is considered acceptable for screening purposes and RPD >5 is excellent for quality control (*22*). Others divide the continuum differently depending on the material (*22*−*25*). In general, for an RPD value greater than 2.5 it is assumed that the calibration model is adequate to predict a specific parameter (*25*).

RESULTS AND DISCUSSION

Reference Analysis: Analysis of Crocetin Esters by HPLC and Coloring Strength by UV−Vis Spectrophotometry. Figure 1 shows a typical chromatogram with the identified crocetin esters. Their content, as determined by HPLC, fluctuated between 16.9 and 32.6 mg/100 g saffron. The first six crocetin esters account for more than 96% of the total esters area of the HPLC chromatogram. The T4GG and the T3Gg are the major crocetin esters in saffron, followed by C4GG, C3Gg and T2gg. The distribution of the crocetin esters among the four countries is different (**Figure 2**), indicating that their content could serve to distinguish the origin of saffron.

The coloring strength as determined with the UV−vis method fluctuated between 164.5 and 299.0 units. The majority of the samples, 106 samples of the total, fulfilled the ISO specifications for category I; only 7 samples belong to category II and none to category III. All Italian and Spanish samples belong to category I, one sample originated from Greece and 6 Iranian samples were classified in category II.

Saffron Raman Spectra Assessment. Figure 3 shows the Raman spectra of representative saffron samples from Greece, Iran, Italy and Spain in the spectral region between 1900 and 800 cm^{-1}. There are some differences in the intensities of the peaks, but the profile is the same. The Raman effect is so strong that only bands due to carotenoids appear in the visible excited resonance Raman spectra of carotenoid containing biological materials (*7*). The peak at 1586 cm^{-1} corresponds to C=C stretching (*16*, *18*, *26*−*28*). The region between 1400 and 1100 cm^{-1} is assigned to C−C stretching and C−H in-plane bending modes (*27*). The peaks at 1202, 1203, and 1206 cm^{-1} correspond to C−C stretching (*16*, *18*, *27*, *28*). The medium peaks at 1250 cm^{-1} and 1326 cm^{-1} are due to C−C stretching and C−H in-plane bending modes and to CH deformation, respectively (*16*, *27*, *28*). The peak at 1050 cm^{-1} is due to $-CH_3$ in-plane rocking (*16*, *18*, *27*).

Comparing the profile of saffron samples obtained in previous work (*16*), there is a shift in band positions and intensities. Raman

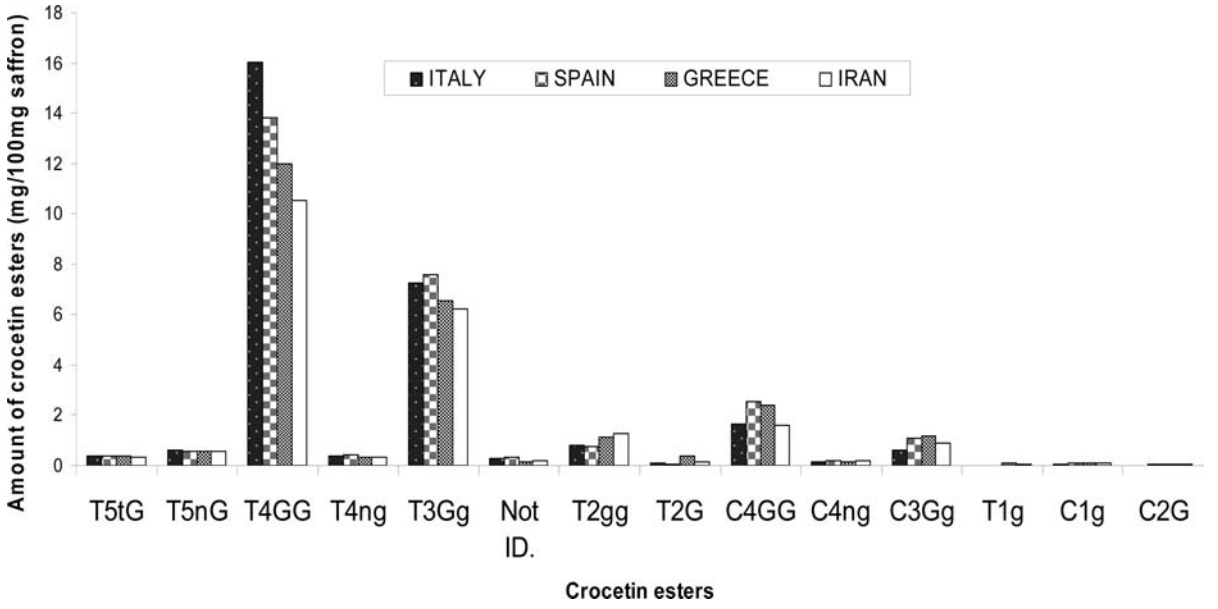

Figure 3. Profile of crocetin esters in samples from four countries. Not ID = not identified.

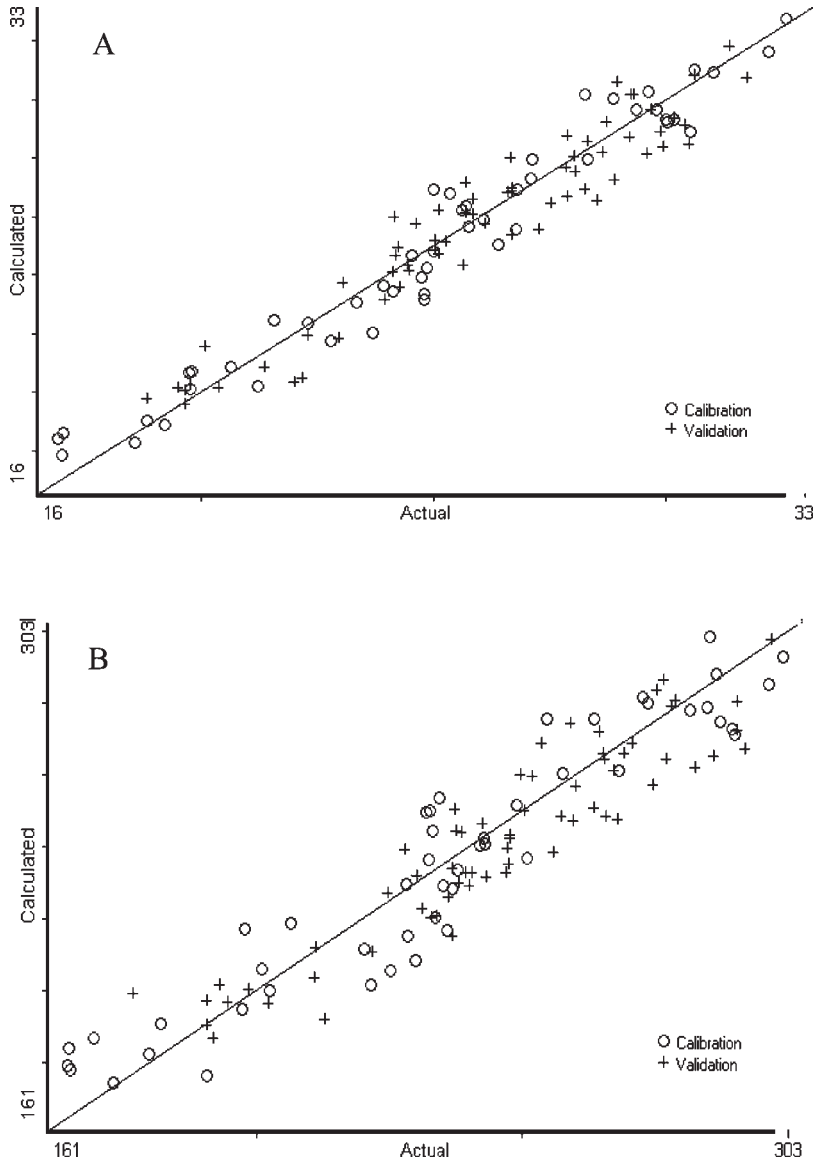

Figure 4. PLS model calibration for crocetin esters (**A**) and coloring strength (**B**).

Table 1. Crocetin Esters Content and Coloring Strength as Determined by HPLC, UV−Vis and Raman Methods for the 49 Samples Used for Calibration

		crocetin esters content (mg/100 mg saffron on dry basis)		coloring strength (units)	
sample origin		HPLC	Raman	UV−vis	Raman
Greece		22.3−26.8	22.4−27.0	220.3−250.9	211.7−253.7
$N = 8$	mean ± SD	25 ± 1	25 ± 1	237 ± 9	236 ± 13
Iran		16.9−27.1	17.8−27.3	164.5−241.9	174.5−250.0
$N = 16$	mean ± SD	21 ± 3	21 ± 3	199 ± 29	201 ± 25
Italy		21.2−32.6	20.2−32.8	197.7−289.4	200.0−298.3
$N = 12$	mean ± SD	27 ± 4	27 ± 4	253 ± 37	251 ± 36
Spain		24.8−31.0	23.2−31.1	232.0−299.0	216.9−292.6
$N = 13$	mean ± SD	28 ± 2	28 ± 3	261 ± 25	261 ± 25
RMSEC		0.92		12.2	
RMSECV		1.09		14.5	
RMSEP		0.98		11.3	

Table 2. Crocetin Esters Content and Coloring Strength as Determined by HPLC, UV-Vis and Raman Methods for the 65 Samples Used for Validation

		crocetin esters content (mg/100 mg saffron on dry basis)		coloring strength (units)	
sample origin		HPLC	Raman	UV−vis	Raman
Greece		18.8−30.2	19.8−29.4	177.0−269.1	191.8−266.1
$N = 20$	mean ± SD	26 ± 3	26 ± 3	241 ± 22	236 ± 22
Iran		19.5−27.9	19.6−28.0	190.7−265.6	186.8−259.7
$N = 21$	mean ± SD	23 ± 3	24 ± 3	225 ± 23	222 ± 21
Italy		21.4−31.7	20.9−31.8	202.3−296.7	196.2−297.6
$N = 12$	mean ± SD	29 ± 3	29 ± 3	276 ± 25	266 ± 25
Spain		25.6−29.7	24.3−30.6	236.0−277.9	226.0−279.3
$N = 12$	mean ± SD	28 ± 1	28 ± 2	261 ± 12	261 ± 16

spectra are obtained with different instruments each time. Choquette et al. (*29*) report that the variations in the measured relative peak intensities and in the wavenumbers of Raman spectra recorded with different instruments are mainly due to the differences in their wavelength-dependent optical transmission and their detector quantum efficiency.

Prediction Models. Methods that are capable for routine basis quality control must work directly on the saffron stigma. For this purpose, partial least-squares (PLS) regression was used. The method tries to correlate Raman sprectroscopic data (X-matrix) with the related chemical data (Y-matrix) and to build a calibration model enabling the prediction of the content of crocetin esters and coloring strength (y) from a measured spectrum (x).

An important aspect of multivariate calibration is to create a model that is accutate and robust. Region selection can improve the performance of the prediction models. If many uncessarary spectral regions are including, the model is too complex and tends to overfit the data. On the other hand, if the spectral region is too tight, important information is ignored and the model leads to being distorted (*21*).

Four different spectral regions were examined in the spectral region 1700−800 cm^{-1}. In fact the spectral regions between 2000 and 1700 cm^{-1} and between 800 and 200 cm^{-1} were excluded because they do not contain any bands. The model in the spectral region 1700−955 cm^{-1} had the best correlation coefficient, a low RMSEC, a low RMSECV and a low RMSEP, but also small differences between RMSEC and RMSECV, as well as between RMSEC and RMSEP.

Figure 4A shows the PLS model calibration for the determination of crocetin esters content in saffron by Raman spectroscopy in the spectral region between 1700 and 955 cm^{-1}. **Table 1** demonstrates the crocetin esters content of the 49 saffron samples used for the calibration of the model. The model developed has correlation coefficient r 0.98, RMSEC 0.92 and RMSEP 0.98. The correlation coefficient r for cross-validation was 0.97 and RMSECV 1.09, indicating that the accuracy of the method was good. The number of PLS factors was six. These six factors describe 98% of the spectral variability of the model. The two first factors explain 82% of the variance, indicating that they include the most relevant information. The low differences between RMSEC and RMSEP reveal the robustness of the model. Using the RPD criteria, it was concluded that spectral region selection (1700−955 cm^{-1}) can give a good prediction model since the RPD values are 3.46.

The crocetin esters content for the 65 samples as determined with the HPLC method fluctuated between 18.8 and 31.7 mg/100 g saffron (**Table 2**). The corresponding values, as calculated using the Raman method, fluctuated between 19.6 and 31.8 mg/100 g saffron. The relative standand deviation (RSDV) ranged from 0.1 to 7.7%. The closely corresponding values for crocetin esters in each of the four origin's saffron samples, as determined by HPLC and Raman spectroscopy, are presented in **Table 2**.

Sanchez et al. (*19*) have reported better RMSEC (0.469 vs 0.92) and RMSEP (0.339 vs 0.98) values for the PLS model for the determination of crocetin esters using UV−vis spectroscopy. The authors applied PLS calibration models to the sum of crocetin esters and to individual esters. These models managed to give better correlations with saffron composition than by the UV−vis spectrometric method determined according to ISO specifications. However, the above models do not discharge us from sample preparation, use of sovent (water) and potential exposure to light and high temperature.

In the case of the proposed Raman method, the challenge is the direct adequate prediction in intact saffron samples for routine basis analysis with no interference of solvents, temperature and other exogenous conditions. Under this perspective, the above results confirm that the Raman method can be used as a fast screening method for quality control.

In terms of quality control, the ISO standard 3632 (*1*) is used for the determination of quality parameters of saffron. The absorbance at 440 nm of the aqueous extract of saffron has been correlated with the crocetin esters content. Since Raman

spectroscopy gave a good prediction model for the sum of crocetin esters as they were determined by the HPLC method, the next step is to build a PLS model for the coloring strength determined by the UV−vis method.

For PLS model construction, the same samples were used for calibration ($N = 49$) and validation ($N = 65$) and the spectral region (1700−955 cm^{-1}), as well. The coloring strength of the 49 saffron samples used for calibration is demonstrated in **Table 1**. The reference values ranged from 164.5 to 299.0 units. Seven samples belong to category II, while the rest belong to category I according to ISO 3632 specifications. The predicted ones fluctuated between 174.5 and 298.3 units. In **Table 2**, the corresponding values for the validation set as determined by UV−vis spectophotometry and the Raman method are presented.

Four PLS factors accounting for 96% of the variability in the data set were used for building the PLS model. **Figure 4B** shows the scatter plot of measured versus predicted values for coloring strength. The model has correlation coefficient r 0.95, RMSEC 12.2 and RMSEP 11.3. The values demonstrate also that the model is robust. The cross validation procedure gave r 0.93 and RMSECV 14.5. The RPD value is 2.59, interpreting that the model has the possibility to predict the coloring strength accurately in saffron samples. Better results were achieved in this study compared to the results reported for the coloring strength prediction model with NIR spectroscopy (*15*). This indicates that Raman spectroscopy is more adequate than NIR spectroscopy for the study of saffron carotenoids.

In conclusion, a novel methodological approach to the quantitative determination of a sum of crocetin esters in saffron samples and their coloring strength based on Raman spectroscopy was presented. Raman spectroscopy is a promising techique for routine basis quality control of saffron as it is a rapid method for measuring coloring components in saffron. It can be used as an alternative method to time-consuming and complicated methods for monitoring the quality of the most expensive spice in the world.

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