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Analytical Methods

Evaluation of saffron (*Crocus sativus* L.) adulteration with plant adulterants by ^{1}H NMR metabolite fingerprinting

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ABSTRACT

In the present work, a preliminary study for the detection of adulterated saffron and the identification of the adulterant used by means of ^{1}H NMR and chemometrics is reported. Authentic Greek saffron and four typical plant-derived materials utilised as bulking agents in saffron, i.e., *Crocus sativus* stamens, safflower, turmeric, and gardenia were investigated. A two-step approach, relied on the application of both OPLS-DA and O2PLS-DA models to the ^{1}H NMR data, was adopted to perform authentication and prediction of authentic and adulterated saffron. Taking into account the deficiency of established methodologies to detect saffron adulteration with plant adulterants, the method developed resulted reliable in assessing the type of adulteration and could be viable for dealing with extensive saffron frauds at a minimum level of 20% (w/w).

1. Introduction

Food authenticity is an increasingly important issue for consumers, regulatory agencies, and food industry. Aspects of authentication involve the detection of economically motivated adulteration in food products, usually carried out with less expensive and more readily available substitutes which are difficult to identify by routine analytical methodologies (Cubero-Leon, Peñalver, & Maquet, 2014; Oms-Oliu, Odriozola-Serrano, & Martín-Belloso, 2013).

Among the major candidates for adulteration conducted for economic gain, saffron is one of the most targeted spices (Moore, Spink, & Lipp, 2012); it consists of the dried stigmas of the cultivated species *Crocus sativus* L. Saffron, that has long been used as a colouring and flavouring agent in food, is also known for a wide range of health promoting benefits (Melnyk, Wang, & Marcone, 2010; Winterhalter & Straubinger, 2000). Due to its high price and limited production, saffron has been subjected to various types of adulteration over the centuries. Common fraudulent practices include the addition of inferior plant material with similar appearance to extend the more expensive saffron. This particularly happens when the spice is in powder form or when added to seasonings and other food products as an ingredient (Hagh-Nazari & Keifi, 2007; Torelli, Marieschi, & Bruni, 2014). Within the most frequently reported plant materials to adulterate saffron are cut or dyed *C. sativus* stamens, *Carthamus tinctorius* L. petals (safflower) as well as *Curcuma longa* L. powdered rhizomes (turmeric) (Hagh-Nazari & Keifi, 2007; Ordoudi & Tsimidou, 2004; Saffron in Europe, 2007). Additionally, commercial safflower and turmeric are often mislabeled, using the name "saffron" and the supposed country of origin for misleading consumers (Hagh-Nazari & Keifi, 2007; Sánchez, Maggi, Carmona, & Alonso, 2011). The use of gardenia, the extract obtained from the fruits of *Gardenia jasminoides* Ellis, is another possible and more sophisticated method of adulteration, considering that gardenia and saffron differ merely in the pigments contained (Carmona, Zalacain, Sánchez, Novella, & Alonso, 2006; Ordoudi & Tsimidou, 2004; Sánchez et al., 2011).

Regardless of the practice followed, the detection of commercial frauds in saffron is a challenging task since changes in physical, chemical or organoleptic properties are not always easily identifiable. As a result, the best quality saffron is usually sold in filaments (Melnyk et al., 2010), where the extraneous or foreign matter may be more easily detectable. In the quality assessment of saffron according to the ISO 3632 standards (ISO, 2010, 2011), up to 1% (w/w) of foreign matter is permitted in third-class products. However, microscopic examination is required, which is time-consuming for the screening of large batches of samples. Also, the UV–Vis

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spectrophotometric method proposed by ISO 3632-2 (ISO, 2010) may not detect saffron contamination with amounts of up to 20% (w/w) of safflower or turmeric, as it was recently reported (Sabatino et al., 2011). For the detection of plant adulterants in saffron, several chromatographic (Alonso, Salinas, & Garijo, 1998; Haghighi, Feizy, & Hemati Kakhki, 2007; Lozano, Castellar, Simancas, & Iborra, 1999; Sabatino et al., 2011; Sampathu, Shivashankar, Lewis, & Wood, 1984) and molecular (Babaei, Talebi, & Bahar, 2014; Javanmardi, Bagheri, Moshtaghi, Sharifi, & Hemati Kakhki, 2011; Ma, Zhu, Li, Dong, & Tsim, 2001; Marieschi, Torelli, & Bruni, 2012; Torelli et al., 2014) methods have been employed so far with encouraging results. The use of DNA markers has allowed the detection of low amounts (up to 1%) of several bulking materials including safflower and turmeric (Javanmardi et al., 2011; Marieschi et al., 2012). Nevertheless, there is still an ongoing demand for the development of faster, simple and robust screening methods suited for identifying saffron adulteration, especially at levels that make practical economic sense.

Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique largely applied for its rapidity and reproducibility, having the potential for high-throughput analyses with minimal sample pretreatment (Longobardi et al., 2013; Mannina, Sobolev, & Viel, 2012). NMR based metabolite fingerprinting may identify the subtle differences that often exist between authentic and fraudulent products. As a matter of fact, this metabolomic approach has been recently explored to discriminate authentic Iranian saffron from commercial samples; the results indicated relative amounts of picrocrocin and the sum of different crocetin glycosides as the characteristic metabolites for authentic saffron (Yilmaz, Nyberg, Mølgaard, Asili, & Jaroszewski, 2010). The complexity of NMR data in food metabolomics studies is clearly the primary impetus for the coupling of NMR spectroscopy with multivariate statistical methods, capable of gathering samples with similar features (Consonni & Cagliani, 2010; Tomassini, Capuani, Delfini, & Miccheli, 2013). Among them, supervised methods that may enhance classification performance, such as orthogonal projection to latent structures – discriminant analysis (OPLS-DA) and its bidirectional modifications (O2PLS-DA) (Bylesjö et al., 2006), have shown great potential to determine the authenticity of various foodstuffs, mainly on the basis of their geographical or botanical origin (Consonni, Cagliani, & Cogliati, 2012a,b, 2013; Consonni, Cagliani, Stocchero, & Porretta, 2009; Consonni, Cagliani, Stocchero, & Porretta, 2010; Fotakis et al., 2013).

The present work describes a preliminary study for the detection of adulterated saffron and the identification of the adulterant used by means of ^{1}H NMR and chemometrics. The two-step approach proposed herein relied on the application of both OPLS-DA and O2PLS-DA models to the ^{1}H NMR data. Taking into account the deficiency of established methodologies to detect saffron adulteration with plant adulterants, the method developed could be viable for dealing with extensive saffron frauds at a minimum level of 20% (w/w). The efforts focused on four typical plant-derived materials utilised as bulking agents in saffron, i.e., *C. sativus* stamens, safflower, turmeric and gardenia.

2. Materials and methods

2.1. Samples

Ten samples of Greek *C. sativus* dried stigmas of commercial grade, harvested in 2012, were supplied by Kozani Saffron Producers Cooperative (Cooperative De Saffran). The Greek saffron samples selected were either organic ($n = 6$) or conventionally produced ($n = 4$), to extend variability among them. Prior to ^{1}H NMR analysis, their quality and authenticity had been checked according to the ISO 3632 parameters and HPLC analysis at the Laboratory of Chemistry, Agricultural University of Athens. All saffron samples belonged to the commercial category I. Samples of turmeric (branded as "Like safran"), safflower (branded as "Turkish saffron") and *C. sativus* stamens (branded as "Safran") were purchased from local markets. *G. jasminoides* fruit extract (single herb extract, Zhi Zi) was acquired from Plum Flower Brand (Anguo, China).

2.2. Preparation of commercial and spiked samples

All of the plant-derived materials, namely "saffron samples" and "plant adulterants", were finely ground in a mortar. To simulate conditions of commercial samples, artificial counterfeit mixtures containing saffron and 20% (w/w) of plant adulterant were prepared. Overall, 10 mixtures were used for each adulterant and thus five classes were defined, including the authentic saffron samples. Those fifty samples (10 mg) along with the pure plant adulterants used for reference were extracted with 600 μL DMSO-d_6 by stirring (vortex) for 3 min at room temperature. After 10 min, they were submitted to centrifugation at 12,100 rcf for 10 min and then 500 μL aliquots of the supernatant were transferred into 5 mm NMR tube for analysis. DMSO solvent was used because of its capability to dissolve both hydrophobic and hydrophilic compounds, leading to NMR signals with sharp line width.

2.3. NMR analysis

^{1}H NMR spectra were recorded on a Bruker AVANCE 600 spectrometer (Bruker Biospin GmbH, Rheinstetten, Karlsruhe, Germany), operating at 14.09 T and equipped with a 5-mm inverse probe with a *z*-gradient. All monodimensional spectra were acquired at 300 K with a spectral width of 10,000 Hz over 32 K data points. Residual water suppression was achieved by applying a presaturation scheme with low power radiofrequency irradiation for 1.2 s. Spectra were processed using TOPSPIN software (Bruker BioSpin GmbH, version 3.0, Rheinstetten, Karlsruhe, Germany) by applying an exponential function for resolution enhancement with a line broadening of 0.5 Hz before Fourier transformation; phase and baseline were manually corrected. Spectra were aligned on the residual solvent signal at 2.50 ppm. The NMR spectra were reduced to integrated regions (buckets) of equal width of 0.04 ppm each in the range of 0.40–10.50 ppm, excluding solvent and water regions from 2.47 to 2.52 ppm and from 3.31 to 3.34 ppm, respectively. Buckets were scaled with respect to the total spectrum intensity, thus taking into account the different composition of samples (ACD/NMR v. 11.0, ACD Labs, Toronto, Canada).

2.4. Multivariate data analysis

Principal component analysis (PCA), orthogonal projections to latent structures-discriminant analysis (OPLS-DA) and bidirectional OPLS-DA (O2PLS-DA) were performed with Pareto scaling. PCA was applied to represent the sample distribution in the multivariate space. Supervised OPLS-DA and O2PLS-DA were used in order to reduce the model complexity by removing the systematic variations in the *X* matrix that were not related to *Y* response (structured noise) maximising the separation among samples. When the dimension of the joint correlated space is one, a useful visualisation tool, such as the *S*-plot, could be adopted (Wiklund et al., 2008). The non randomness of all classification models was checked by performing the permutation test, in which a total of 200 models were calculated by randomising the order of *Y* variable in the corresponding PLS-DA (partial least squares-discriminant analysis) models. Multivariate data analysis was performed with

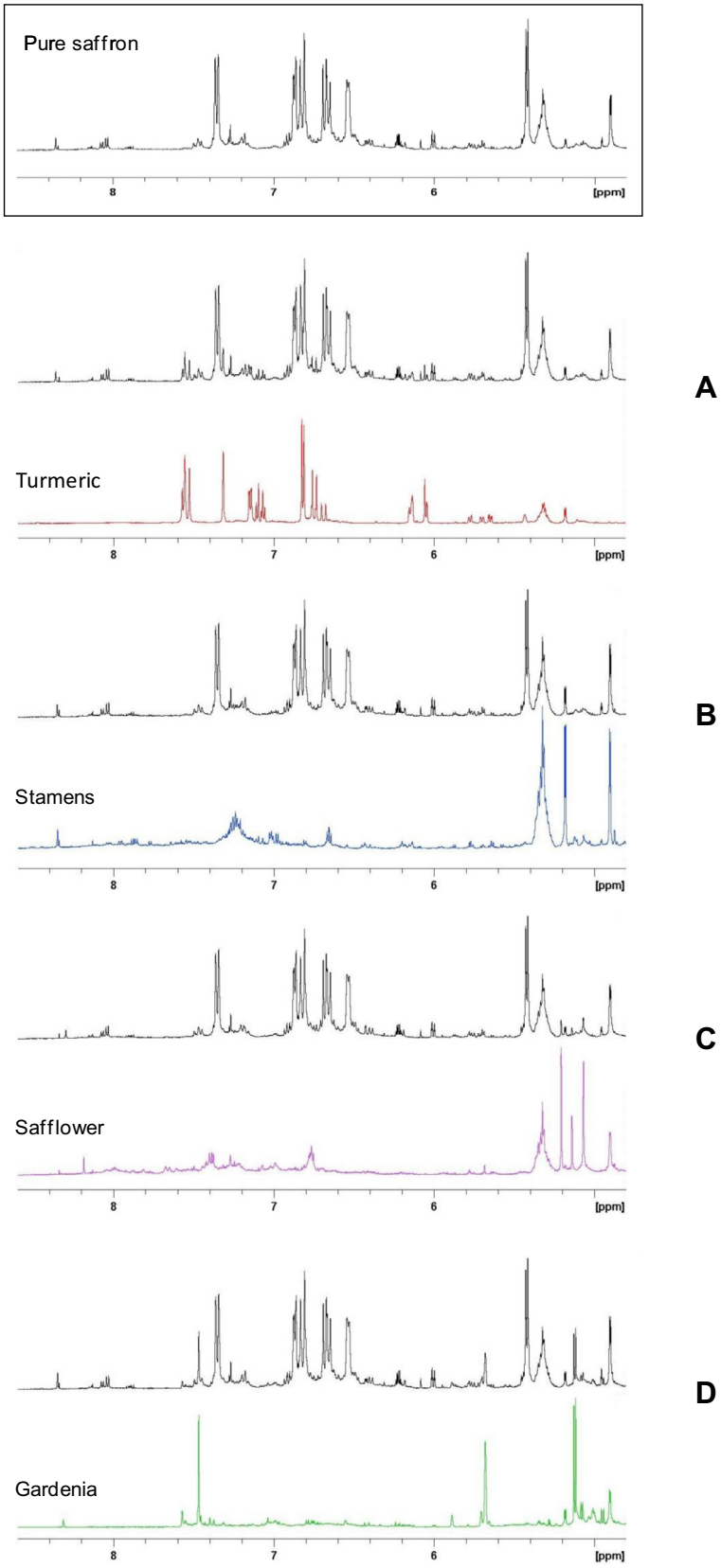

Fig. 1. Selected regions of ^{1}H NMR spectra acquired from DMSO-d_6 extracts. Squared top spectrum is characteristic of pure saffron. Spectra of pure plant material (turmeric, *C. sativus* stamens, safflower, and gardenia *jasminoides* fruit extract) are reported in panels A, B, C, and D, respectively in bottom traces, while spiked saffron with 20% (w/w) concentration of plants adulterants are reported in panels A, B, C and D in top traces.

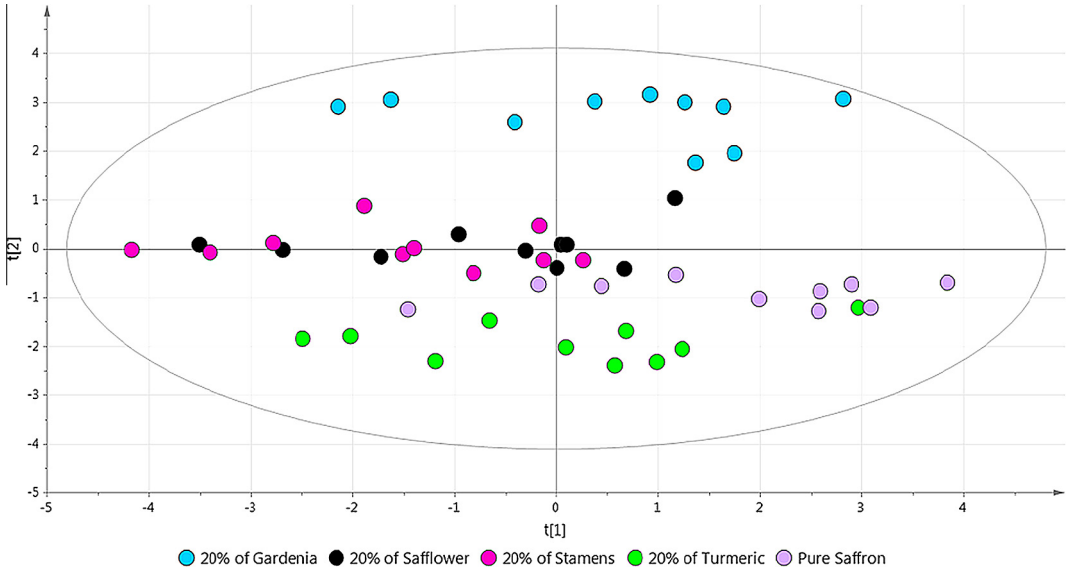

Fig. 2. PCA score plot performed considering 10 pure Greek saffron samples (purple circles) and the same samples spiked at 20% (w/w) concentration with *Gardenia jasminoides* fruit extract (light blue circles), safflower (black circles), *C. sativus* stamens (pink circles), and turmeric (green circles) for a total of 40 samples. PC1 = 36.8%, and PC2 = 26.9%. R^2X = 99.5%, and Q^2 = 96.2%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

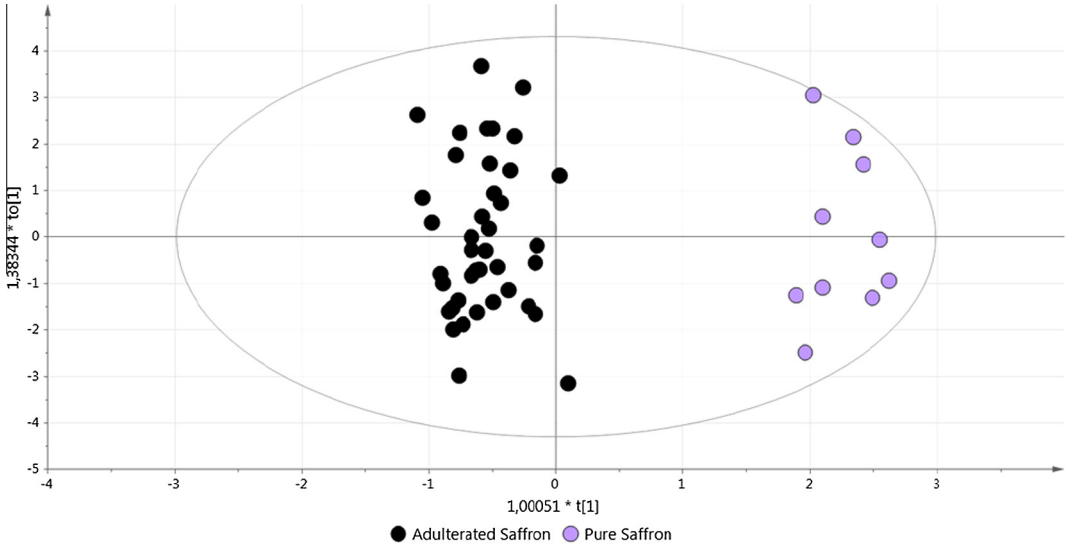

Fig. 3. OPLS-DA score plot performed by considering all saffron samples analysed divided in two classes: pure (purple circles) and adulterated (black circles) saffron. R^2X = 82.4%, R^2Y = 94.5% and Q^2 = 92.3%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the SIMCA-P+ 13 software (Umetrics, Umea, Sweden). T2 and distance to the model (DModX) tests were applied to verify the presence of outliers and to evaluate whether samples fall within the model applicability domain.

2.5. Training and test set selection

To investigate the predictive capability of the models, training and test sets were extracted from the 50 samples containing the 4 classes of adulterated saffron with plant adulterants and the class of pure saffron samples. Seven out of ten samples for each class were randomly selected to build the training set, while the three remaining samples were used for the test set. In total, training and test set consisted of 35 and 15 samples, respectively.

3. Results and discussion

Adulteration of saffron could be easily evaluated for each plant adulterant by comparing ^{1}H NMR spectra of authentic and spiked saffron. Typical signals concerning the different plant-derived materials used as bulking agents were present along the entire spectral region. Fig. 1 reports the aromatic and anomeric regions of ^{1}H NMR spectra for pure plant adulterants and the corresponding spiked saffron for the sake of clarity. In panel A (bottom trace), the spectrum of turmeric extract is reported; the typical signals of curcuminoid moiety could be identified at 7.541, 6.751 ppm for H1,7 and H2,6, respectively, at 6.059 ppm for H4, and signals at 7.318, 7.147, and 6.819 ppm for the aromatic protons. These assignments and the corresponding carbon signals (140.26,

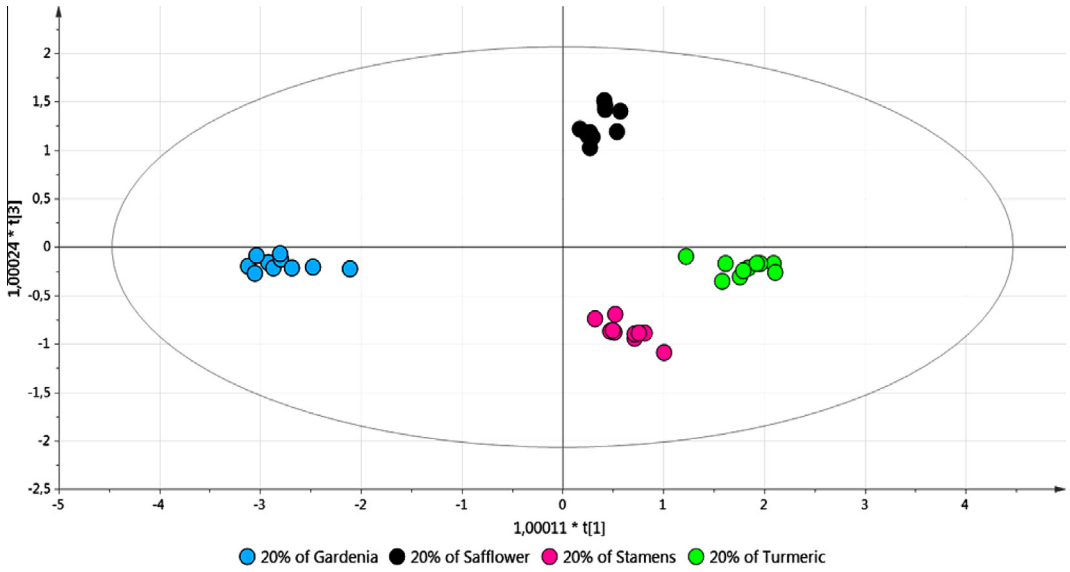

Fig. 4. O2PLS-DA score plot (PC1 versus PC3) performed by considering only adulterated saffron divided into 4 classes according to the type of plant adulterant: saffron adulterated with 20% (w/w) concentration with *Gardenia jasminoides* fruit extract, safflower, *C. sativus* stamens and turmeric are presented with light blue, black, pink, and green circles, respectively. $R^2X = 95.2\%$, $R^2Y = 97.6\%$ and $Q^2 = 96\%$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

120.75, 100.55, 110.93, 122.86, and 115.42 ppm) resulted in full agreement with previously reported data (Saladini et al., 2009). Curcuminoid signals could be easily recognised in the saffron adulterated with turmeric extract (top trace, panel A) by comparison with the pure saffron spectrum. Analogously, panel B evidenced the increase of a doublet at 5.181 ppm most likely referred to a saccharidic moiety, largely present in stamens extracts; Panel C showed the increase of signal at 5.205, 5.138, and 5.066 ppm and finally, panel D evidenced the increase of doublets at 7.569 and 7.466, a broad signal at 5.679 ppm, and doublets at 5.121 ppm most likely due to a saccharide moiety.

Full ^{1}H NMR spectra were considered for statistical analysis. PCA was initially performed on all samples to evaluate possible differentiation according to the purity and type of plant adulterant used. The first two PCs explained 63.7% of the total variance; the corresponding score plot (Fig. 2) revealed a poor separation for the majority of samples. Only saffron samples adulterated with 20% (w/w) gardenia extract resulted sufficiently differentiated, followed by saffron samples containing turmeric as bulking agent. A two-step approach with supervised classification models was performed to improve the differentiation of samples; pure and adulterated saffron were discriminated at first, while all artificial mixtures containing 20% (w/w) plant adulterants were successively evaluated.

The OPLS-DA model performed by considering two classes (authentic Greek and adulterated saffron), resulting in one predictive and three orthogonal components ($R^2X = 82.4\%$, $R^2Y = 94.5\%$, $Q^2 = 92.3\%$), is presented in Fig. 3, demonstrating a clear discrimination between the two classes of samples. The corresponding *S*-plot (data not shown) evidenced a higher content of picrocrocin (buckets at 1.12, 1.16, 2.08, 4.28, and 10.04 ppm) and crocins (buckets at 1.96, 4.16, 5.40, 6.52, 6.64, 6.84, and 7.32 ppm) in authentic Greek saffron with respect to saffron adulterated with the bulking agents, which generally presented higher levels of fatty acids (buckets at 1.20 and 1.24 ppm) and buckets including specific plant adulterant signals. Our results were in agreement with previously published data (Yilmaz et al., 2010), reporting picrocrocin and glycosyl esters of crocetin as the most important markers for distinguishing authentic Iranian saffron from commercial saffron purchased in different countries. It should be noted that ^{1}H NMR metabolite fingerprints revealed no marked differences between organic and conventional saffron samples, indicating potential uniformity of Greek saffron.

Successively an O2PLS-DA model was performed by considering all artificial mixtures containing 20% (w/w) plant adulterants. This model resulted in three predictive and three orthogonal components ($R^2X = 95.2\%$, $R^2Y = 97.6\%$, $Q^2 = 96\%$). By scoring the first and the third latent variables (Fig. 4), a clear classification of the adulterated saffron samples according to the plant adulterant used could be obtained.

The reliable capability in categorising unknown saffron samples as pure or adulterated is based on the possibility to obtain a stable and reliable model from supervised OPLS-DA. This critical aspect was checked by selecting training and test sets constituted by 35 and 15 samples, respectively, both including authentic and adulterated saffron. The new two-class OPLS-DA model performed on training set resulted in one predictive and two orthogonal components. The overall goodness of fit were $R^2X = 72.5\%$ and $R^2Y = 93.8\%$, with the overall cross validation coefficient of $Q^2 = 88.2\%$. On the basis of T2 and DModX tests, the created model resulted suitable for the prediction of authentic or adulterated saffron test set samples. The classification list represented in Table 1 highlighted the model performance in prediction capability; no adulterated sample from any of the four classes was assigned as pure saffron and all samples were correctly classified, by using a classification threshold of 0.6. Only the twelve adulterated test set samples were successively re-projected in the O2PLS-DA model, built on the 28 adulterated saffron samples comprising the training set, resulting in three predictive and two orthogonal components ($R^2X = 93\%$, $R^2Y = 96.7\%$, $Q^2 = 93.4\%$). T2 and DModX tests evidenced that the model created was suitable for the prediction of adulteration type for test set samples. The classification list shown in Table 2 presented all adulterated saffron samples correctly categorised.

In order to check the non randomness of the classification models, the permutation test was performed in the corresponding PLS-DA model for each of the OPLS-DA and O2PLS-DA models. The decreased values of both parameters R^2 and Q^2 (R^2 regression line and vertical axis intersection point of the Q^2 resulted in near zero

Table 1
Classification list for the 15 test set saffron samples (3 authentic and 12 spiked with the four different plant adulterants) re-projected onto the two-class OPLS-DA model (authentic and adulterated saffron) performed by considering a training set constituted by 35 samples (7 authentic and 28 spiked with the four different plant adulterants). Letters T, Sf, St, and G stand for turmeric, safflower, *C. sativus* stamens, and *G. jasminoides* fruit extract, respectively. Each test set sample was classified by means of a classification score (*Y* predicted) indicative of its representativeness. In bold the values exceeding the threshold of 0.6, chosen for the correct classification, are reported.

Type of sample	*Y* predicted adulterated saffron	*Y* predicted pure saffron
Adulterated saffron – Sf	**1.04**	−0.04
Adulterated saffron – Sf	**0.98**	0.02
Adulterated saffron – Sf	**1.07**	−0.07
Adulterated saffron – G	**0.91**	0.09
Adulterated saffron – G	**1.02**	−0.02
Adulterated saffron – G	**0.99**	0.01
Adulterated saffron – St	**1.01**	−0.01
Adulterated saffron – St	**1.16**	−0.16
Adulterated saffron – St	**0.88**	0.12
Adulterated saffron – T	**0.82**	0.18
Adulterated saffron – T	**1**	0
Adulterated saffron – T	**0.82**	0.18
Pure saffron	−0.06	**1.06**
Pure saffron	−0.04	**1.04**
Pure saffron	0.15	**0.85**

Table 2
Classification list for the 12 test set saffron samples (3 spiked saffron samples for each plant adulterant) re-projected onto the O2PLS-DA model (adulterated saffron) performed by considering a training set constituted by 28 samples (7 spiked saffron samples for each plant adulterant). Each test set sample was classified by means of a classification score (*Y* predicted) indicative of its representativeness. In bold the values exceeding the threshold of 0.6, chosen for the correct classification, are reported.

Type of adulteration	*Y* predicted safflower (Sf)	*Y* predicted gardenia (G)	*Y* predicted stamens (St)	*Y* predicted turmeric (T)
Sf	**1.05**	−0.04	−0.13	0.12
Sf	**0.94**	0.02	−0.08	0.12
Sf	**0.95**	−0.04	0.07	0.02
G	0.01	**1.04**	0.02	−0.06
G	0.03	**1.07**	0.05	−0.15
G	0.02	**0.95**	0.13	−0.1
St	0	−0.02	**0.99**	0.03
St	0.01	0	**1.03**	−0.04
St	0.02	0.13	**0.73**	0.12
T	0.03	−0.04	0	**1.01**
T	−0.01	−0.02	−0.15	**1.18**
T	0.01	−0.03	0.13	**0.89**

and negative values, respectively) confirmed the validity of the models.

In the present study, the capability of distinguishing authentic against adulterated saffron containing other plant material by untargeted NMR fingerprinting and chemometrics was evaluated for the first time. The approach demonstrated herein led to detect adulteration of pure Greek saffron with four frequently utilised plant-derived materials in two steps. The first OPLS-DA model successfully differentiated adulterated from authentic saffron, owing to specific secondary metabolites representing markers for saffron authenticity, while the O2PLS-DA model identified the type of plant adulterant occurring in the samples, when found adulterated. The good predictive capability of both models, as was verified by using a test set, strongly supported the validity of the protocol proposed. The suggested approach is very low demanding in terms of required amount of saffron, sample preparation and is endowed with high reproducibility and fast execution. Thus, it may be used for screening large commercial batches of Greek saffron, and it could be adaptable for the analysis of samples of different grade or diverse geographical origin after further study.

In conclusion, NMR metabolite fingerprinting proves to be efficient for determining and identifying fraudulent additions of bulking agents to saffron, considering the difficulties in detecting saffron fraud according to the ISO 3632 standard methods, especially when plant adulterants are involved and the spice is commercialised in powder form. The obtained results confirmed the combined use of ^{1}H NMR spectroscopy and multivariate data analysis as a valid and powerful tool to investigate quality and authenticity of food products.

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