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Stable Isotope Ratio Analysis coupled with ICP-MS for geographical discrimination between Italian and foreign saffron

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Abstract

Seventy-six samples of saffron were analysed through inductively coupled plasma - mass spectrometry and stable isotope ratio analysis. The dataset was formed by 67 samples harvested in different areas of Italy, Morocco and Iran and 9 samples purchased in the Italian market. For the first time, 42 elements and five stable isotopes ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{2}H$ and $\delta^{18}O$) were considered to carry out the discrimination of the samples on the basis of their geographical origin. Combined ICP-MS and isotopic composition data turned out to be a useful tool for the geographical discrimination of saffron among predefined cultivation sites.

K, Cr, Mn, Ni, Zn, Rb, Sr, Mo, Cs, Nd, Eu, Pb, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, and $\delta^{2}H$ were identified as the significant variables in geographical discrimination. Moreover, the class models generated for saffron cultivated in two specific areas of Central Italy exhibited 100 % specificity for Moroccan, Iranian and commercial samples and a high specificity (83-84%) for the saffron samples cultivated in other although close Italian sites.

Keywords *saffron, isotopes, inductively coupled plasma - mass spectrometry, geographical classification, class-modelling, authentication.*

Introduction

Saffron is one of the most ancient and valuable spices in human history. It derives from the dried stigmas of *Crocus sativus L*. Since ancient times, Persians used it as an aphrodisiac and Egyptians, Indians, Arabs, Greeks and Romans used it as ingredients in perfumes.[1] Still today, in Oriental countries, saffron is given as a present to wish a long and happy life. The precious spice was originally cultivated in the areas that nowadays include Iran, Turkey and Greece but now also Spain, Italy, France, Switzerland, Morocco, Egypt, Azerbaijan, Pakistan, India, New Zealand, Australia and Japan are producers of saffron.[2]

The compounds that mainly contribute to saffron peculiar characteristics are safranal, a volatile oil responsable of its aroma,[3] picrocin, which gives the spice its bitter taste,[3,4] and crocins (water soluble crocetins esters) which are responsible of saffron characteristic colour.[5] The cultivation, harvest and manufacturing of this spice is entirely manual. Thus, to produce a kilogram of saffron it is necessary to pick up about 150.000 flowers, which entails around 500 hours of work. As many benefits are associated with saffron consumption, the use of this spice for therapeutic purposes has been longly practiced.[6–9] Because of its high cost, its beneficial effects and the great demanding production, saffron has often been subject of adulterations. The frauds consist both in adding extraneous substances to enhance organoleptic properties of saffron and in declaring a different geographical origin than the real one, as saffron quality is closely related to the terroir of production.[10]

Most of the investigations concerning geographical traceability of saffron is based on chromatographic methods,[11–16] or spectroscopic fingerprinting approaches.[17–19] To the best of our knowledge, only few works providing inductively coupled plasma - mass spectrometry (ICP-MS) multi-elemental data of samples of saffron can be found in literature. Jia et al. analysed 19 elements of two samples of saffron coming from two different Chinese regions.[20]

D'Archivio et al. collected ICP-MS data of 62 elements of 27 samples of saffron coming from three different Italian areas (Sardinia, Umbria and L'Aquila).[20,21] ICP-MS data were recently used to authenticate PDO (Protected Designation of Origin) saffron from L'Aquila with respect to commercial and Iranian products.[22]

Furthermore, only two works have been carried out for assessing the provenance of saffron by using stable isotope ratio analysis. IRMS has proven to be indeed a potential tool for the geographical origin and authenticity identification of foodstuffs such as honey, wheat, rice, etc.[23,24] Maggi et al. carried out the geographical discrimination of 28 samples of saffron coming from Greece, Iran, Italy and Spain by using hydrogen, carbon and nitrogen stable isotopes.[10] They provided isotopic values of defatted saffron samples along with chemical composition characteristics of the spice (colour, taste and aroma) to check whether it would be possible to relate saffron properties to its origin using multivariate statistical analysis.[10] Wakefield et al. managed to discriminate between samples of saffron coming from the Chilohorasan province of Iran and the La Mancha province of Spain by using carbon, nitrogen and hydrogen isotopic ratios of the bulk samples in addition to 42 elements.[25]

The present work aims to characterize 76 samples of saffron coming from different Italian regions, Morocco and Iran, and commercial products acquired from the Italian market through EA-IRMS and ICP-MS analysis. Five stable isotopes ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{2}H$ and $\delta^{18}O$) and 42 elements were considered. To the best of our knowledge, except for the work of Wakefield et al.,[25] this is the first investigation combining multi-elemental and isotopic ratio patterns for tracing saffron. The isotopic and the elemental composition was used to attempt a geographical discrimination of the saffron samples through univariate statistics and multivariate classification approaches. Moreover, the potentiality of the combined elemental and isotopic data in saffron authentication was tested for the first time by application of class

modelling.

2. Material and Methods

2.1. Sampling

A set of 76 samples of saffron produced in different years was analysed. The set is made up of 46 Italian samples (harvested in 2016 and 2017), 10 Iranian (harvested in 2016, 2017 and 2018), 12 Moroccan (harvested in 2016, 2018 and 2019) and 9 commercial ones. The Iranian samples were produced in the Khorasan region, while Moroccan ones were produced in Taliouine, in the Taroudant region. Most of the Italian saffron samples came from L'Aquila (16) and Spoleto (21), both in central Italy, while isolate samples are representative of other cultivation sites of northern (Alessandria and Padova), central (Firenze, Pescina and Isernia) and southern part of Italy (Siracusa, Messina, Lucania and Sardinia). The Italian, Iranian and Moroccan saffron samples, in stigma form, were directly acquired from producers or consortia that assured their genuineness and geographical origin, whereas the commercial samples, in powder form, were purchased in the Italian market.

2.2. Chemicals

Trace analysis grade mono and multi-element standard solutions for ICP-MS analysis were provided by Perkin-Elmer (Waltham, Massachusetts, U.S.), Sigma-Aldrich (St. Louis, Missouri, U.S.) and Agilent (Santa Clara, California, U.S.). All the standard solutions were prepared in 8% ultrapure HNO_3 obtained by sub-boiling distillation of super-pure grade HNO_3 in a SUBPUR (Milestone, BG Italy) apparatus. Deionized water (18.2 MW cm) was obtained by a Milli-Q (Millipore Corporation, Billerica, MA, USA) system.

2.3. Samples preparation

As for the stable isotope analysis, the stigmas of saffron were grinded in order to obtain a powder, and directly analysed through the EA-IRMS. For ICP-MS analysis, 20 mg of saffron, previously dried in oven at 103°C for 24 h, were mineralized at 148°C in a closed PTFE vial using 500 µL of HNO_3. The concentrated acid was then evaporated to near dryness and the mineralized sample was dissolved with 4 mL of water. All vessels were conditioned with 5% HNO_3 and rinsed with water before use.

2.4. Stable Isotope analysis

The $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ ratios were measured using an isotope mass spectrometer (IsoPrime, Isoprime Limited, Germany) after total combustion in an elemental analyser (VARIO CUBE, Isoprime Limited, Germany). The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios were measured using an IRMS (Finnigan DELTA XP, Thermo Scientific) coupled with a pyrolyser (Finningan DELTA TC/EA, high temperature conversion elemental analyser, Thermo Scientific). To analyse the samples, the amount introduced in the mentioned instruments was, respectively, 1.5 and 0.2 mg.

According to the IUPAC protocol, the values are denoted in delta in relation to the international V-PDB (Vienna-Pee Dee Belemnite) for $\delta^{13}C$, V-SMOW (Vienna-Standard Mean Ocean Water) for $\delta^{2}H$ and $\delta^{18}O$, V-CDT (Vienna Canyon Diablo Troilite) for $\delta^{34}S$ and Air (atmospheric N_2) for $\delta^{15}N$, according to the following general equation:

$$\delta i\ E = \frac{(i\ RSA - i\ RREF)}{i\ RREF} \qquad (1)$$

where i is the mass number of the heavier isotope of element E, RSA is the respective isotope ratio

of the sample and RREF is the relevant internationally recognised reference material.

The delta values are multiplied by 1000 and expressed in units "per mil" (‰).

The isotopic values were calculated against working in-house standards, which were themselves calibrated against international reference materials: fuel oil NBS-22 with $\delta^{13}C$=−30.03‰, sucrose IAEA-CH-6 with $\delta^{13}C$=−10.45‰ (IAEA-International Atomic Energy Agency, Vienna, Austria), and L-glutamic acid USGS 40 with $\delta^{13}C$=−26.39‰ and $\delta^{15}N$=−4.52‰ (U.S. Geological Survey, Reston, VA, USA) for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ and potassium nitrate IAEA-NO3 ($\delta^{15}N$=+4.7‰) from IAEA for $^{15}N/^{14}N$. Keratins CBS (Caribou Hoof Standard $\delta^{2}H$=−157 ± 2‰ and $\delta^{18}O$=+3.8 ± 0.1‰) and KHS (Kudu Horn Standard, $\delta^{2}H$=−35 ± 1‰ and $\delta^{18}O$=+20.3 ± 0.2‰) from U.S. Geological Survey, were used to obtain $^{18}O/^{16}O$ and $^{2}H/^{1}H$ values. Barium sulphates IAEA-SO-5 ($\delta^{34}S$=+0.5‰) and NBS 127 ($\delta^{34}S$=+20.3‰) from IAEA were used to obtain $^{34}S/^{32}S$ values. The details of the analyses are reported in the literature.[26]

2.5. ICP-MS analysis

Multi-element composition of the saffron samples was determined by means of triplicate ICP-MS analyses carried out in semi-quantitative mode with an Agilent 7500a ICP-MS. A 10 µg/L solution of Li, Y, Ce and Tl was used to set up the instrumental parameters and for daily sensitivity optimisation. $^{140}Ce^{16}O^{+}/^{140}Ce^{+}$ and $^{140}Ce^{2+}/^{140}Ce^{+}$ ratios were monitored to keep the levels of oxides and double charged ions in the plasma below 1% and 2%, respectively. Blank subtraction was applied to correct the solvent and argon-based interferences. A calibration solution containing Ag, Al, Ce, Dy, Li, Mn, Tl, Zn at a concentration of 10 µg/L was analyzed daily to update the element sensitivity factors. Ge and Re were used as internal standards. A multi-element standard solution (Fe, Ca, Na, Mg at 1 mg/L, Sr at 100 µg/L and Ag, Al, As, Ba, Be, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, Tl, V, Zn, U at 10 µg/L) was analyzed after and before each saffron analysis session to check inaccuracy, which resulted to be always below

10%. Blank measurements were performed before and after each analysis by treating the PTFE vial like the saffron samples. Further instrumental details are reported elsewhere,[21] together with the limits of detection (LODs) and recoveries of some representative elements.

2.6. Statistical analysis

The data were statistically evaluated using Statistica For Windows v 13.1 (StatSoft Inc., Tulsa, OK, USA). Statistically significant differences were found using a Kruskal-Wallis test. Differences were considered statistically significant for $p<0.05$.

Preliminary exploration of the experimental data was performed by PCA. PCA[27] allows to represent multivariate information in a low-dimensionality space of mutually orthogonal and uncorrelated principal components (PCs). Transformation of the original data matrix **X** is mathematically described by equation (2)

$$\mathbf{X} = \mathbf{T}\mathbf{P}^{T} + \mathbf{E} \qquad (2)$$

where the columns of matrix **P** (loadings matrix) define the PC directions, the columns of matrix **T** (scores matrix) are the coordinates of the samples in the PC space and the error matrix **E** collects the residuals associated with the approximation of the original data when fewer PCs than the original variables are extracted. A separate or simultaneous graphical projection of the scores and the loadings onto the two- or three-dimensional space of the most significant components allows a straightforward visualisation of the trends within the data samples and the relationships between the original variables and selected PCs, respectively.

Partial Least Square Discriminant Analysis (PLS-DA) was applied to attempt a classification of the saffron samples according to the geographical origin. PLS-DA28 takes origin from partial least squares regression, which allows to link a matrix **X** with a response matrix **Y** overcoming limitations related with ill-conditioned covariance matrix (as in the case of a

number of X variables greater than the number of objects). The regression model is built by iteratively extracting latent variables from X factors and Y responses (also referred as X-scores and Y-score, respectively). The extracted X-scores are used to predict the Y-scores and indirectly the model responses. In classification problems, the model response is categorized by generation of a dummy binary **Y** matrix, in which 1 and 0 indicate respectively the "in-group" and "out-group" samples. After the regression model has been built based on a calibration dataset in which all the classes in the problem are well represented, the calibration or external data samples are classified according to the computed or predicted outputs, but, since the PLS-DA responses are continuous and not binary, a threshold must be defined to assign the objects. The simplest approach, here used, is to assign the sample to the category for which is highest the Y predicted value.

Soft independent modelling of class analogy (SIMCA), which is the first and one of the most used class modelling methods, assumes a PCA model for each category in the problem to describe its typical variability.[29] Once a PCA model has been built by extracting information from the objects belonging to the modelled class, the acceptance or rejection criteria for each sample is based on an F-test on the ratio between the squared distance of the tested object from the principal component model and rsd^2, where rsd (class residual standard deviation) is the mean distance of the objects used to generate the model. A leave-one-out cross-validation scheme was adopted to select the number of PCs representing the classes in SIMCA and the kind of data scaling.

Multivariate statistical analyses were run in Matlab (The Mathworks, Natick, MA; version 2015b), using in-house routines.

3. Results and discussion

3.1. Isotopic Composition

The dataset was divided into 4 major groups: Italian (IT), Moroccan (MO), Iranian (IR) and commercial (CS) samples. Moreover, samples coming from L'Aquila and harvested both in 2016 (AQ2016) and in 2017 (AQ2017), samples coming from Spoleto and harvested both in 2016 (SP2016) and in 2017 (SP2017) and the Other Italian samples (OI), not belonging to any of the previous groups, were considered as IT subgroups. All the mentioned groups were taken into account for the statistical analysis and their values are reported in the supplementary materials (Table 1S). The isotopic mean values for all the samples of saffron, together with the box plots displaying the differences in the δ^2H, δ^{18}O, δ^{13}C, δ^{15}N and δ^{34}S among the groups, are reported in Figure 1. Kruskal-Wallis test was performed to verify whether it was possible to discriminate among the four groups CS, IT, IR and MO and among the Italian groups of samples AQ2016, AQ2017, SP2016, SP2017 and OI. The results are reported in Figure 1 (1-6).

3.1.1 δ^2H, δ^{18}O and δ^{13}C

As shown in Figure 1 (1 and 2), a strict correlation between the parameters δ^2H and δ^{18}O of saffron and their geographical origin could be found, with a good discrimination between Italian and Iranian samples and only partial between Italian and Moroccan ones. The relatively low δ^2H and δ^{18}O values of the Italian samples could reflect the higher latitude of the Italian sampling points (41°54'39"24 North, 12°28'54"48 East) with respect to the Iranian (Khorasan region: 36° 19' 16" North, 59° 31' 58" East) and Moroccan (Taliouine: 34° 48' 36" North, 4° 0' 0" West) ones (Figure 1 (1 and 2)). Indeed it is well known that δ^2H and δ^{18}O of water are strictly related parameters,[30] that depend on variables such as latitude, altitude, closeness to the sea.[31] For plants the correlation is still valid but not that strict, indeed the only source of hydrogen is the water absorbed through the roots, while oxygen also derives from O_2 and CO_2 absorbed through the stomata.[32] This could explain the lack of correlation

between δ^2H and $\delta^{18}O$ for the Moroccan samples (Figure 1 (1-2)). The same scarce correlation has already been noticed in previous studies as for garlic from the Italian region Sicily.[33]

While in Italy the saffron crops are normally not irrigated,[34] in Iran and Morocco the farmers irrigate during flowering (in October) and during the reproductive period (in March-April) only one or two times per month.[35,36] The correlation between the δ^2H and $\delta^{18}O$ values and the isotopic signature of rainfall, which remains the primary source of water origin, was therefore evaluated. In the absence of direct measurement of the δ^2H and $\delta^{18}O$ of rainwater, we used water isotope data from the WaterIsotope database administered by Gabriel Bowen. The data available in the database http://wateriso.utah.edu are the monthly weighted average precipitation values for sites all over the world. As for the δ^2H, the correlation between the values of saffron and the ones of the precipitations are really well correlated (R=0.94), as this parameter is only related to the water provided to the plant. On the other hand, for $\delta^{18}O$ the correlation is not that good (R=0.68). This further confirms that oxygen has other sources of variability except from water.

As for Italian AQ and SP samples, for which we have two different years of harvest (2016 and 2017), it is possible to notice the influence of the year on the oxygen and hydrogen isotopic ratios (Figure 1 (3)). Kruskall-Wallis test showed that the oxygen isotopic ratio permits to discriminate between L'Aquila and Spoleto samples harvested in 2016 and 2017 (Figure 1 (3)). Samples of saffron harvested in the two zones of AQ and SP have lower values for 2016 compared to 2017. Moreover, in 2017 the total precipitations have been 4 times less abundant than in 2016 (-216.3 mm in 2017 compared to -45.8 mm in 2016, with reference to the mean value of the period 2007-2016).[37] In particular, the maximum precipitation decrease was registered during October 2017, just before the harvest period, which took place in

November. A decrease in the amount of the precipitation is associated with an increase of the hydrogen and oxygen isotopic ratios.[38]

The $\delta^{13}C$ mean value for the Italian samples is -27.8±1.1 ‰ (among them, SP $\delta^{13}C$ mean value is -28.0±1.0 ‰, while AQ one is -27.0±0.8 ‰), a typical value for a C3 plant like saffron.[39] The $\delta^{13}C$ mean values of the Moroccan and Iranian samples, as well as the commercial ones, are significantly higher. The less negative values of the samples of saffron are compatibles with the arider conditions of Iran and Morocco, with respect to Italy, only partially muted by the irrigation practice adopted in these countries.[35,36] Plants generally show higher $\delta^{13}C$ values when high temperatures, low air humidity and a high ground-water deficit lead to narrower stomatal apertures in the leaves of the plants.[40] The same behaviour has already been noticed in a previous study as on goji berries.[41] Indeed, both $\delta^{2}H$ and $\delta^{18}O$ and $\delta^{13}C$ in plants depend on factors of influence in the same way.[42] Kruskall-Wallis test showed that carbon isotopic ratio permits to discriminate between Italian saffron and the rest of the dataset (Figure 1 (4)).

3.1.2 $\delta^{15}N$ and $\delta^{34}S$

$\delta^{15}N$ in cultivated plants is affected mainly by the fertilization process used. Synthetic fertilizers, produced from atmospheric nitrogen via the Haber process, have $\delta^{15}N$ values ranging between -4 and +4 ‰. On the other hand, organic fertilizers are characterized by values ranging between +0.6 and +36.7‰.[43,44] In Iran, the use of synthetic fertilizers is now applied in a systematic way during all the production steps of saffron,[45] and this is confirmed by the narrow range of variability of $\delta^{15}N$ whose value is around +2‰. On the other hand, in Italy the use of synthetic fertilizers is alternated or replaced with organic ones. This entails a wide range of $\delta^{15}N$ isotopic variability related to the different input made by the different types of fertilizer. On the other hand, for Moroccan samples, the nitrogen isotopic ratio

reflects the use of wholly organic fertilizers. Indeed, Moroccan farmers have found that manure is enough for the crops and there is thus no need to use chemical fertilizers.[35] Kruskall-Wallis test showed that nitrogen isotopic ratio permits to discriminate between Moroccan saffron and the rest of the dataset, which may indicate a different class of fertilizer used during the cultivation process (Figure 1 (5)).

The $\delta^{34}S$ is influenced by different factors, such as the abundance of sulphides in soil, the plants aerobic and anaerobic growth, the local bedrocks,[46] the active microbial process in the soil, the fertilization procedures and the active deposition.[47] Kruskall-Wallis test showed that sulphur isotopic ratio permits to discriminate between Italian saffron and the rest of the dataset (Figure 1 (6)). Due to the huge variety of the influence factors, it is hard to carry out considerations about the $\delta^{34}S$ values.

3.1.3 Commercial samples

The isotopic composition $\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ of the commercial samples, whose geographical origin is unknown, seems to indicate a high correspondence with that of Iranian samples. The Kruskall-Wallis test did not show significant differences, both for $\delta^{2}H$ and $\delta^{18}O$, between Iranian and commercial samples. Nevertheless, the latter showed tendentially higher $\delta^{2}H$ values, probably due to different years of harvest.

The low $\delta^{15}N$ seems to exclude the possible Moroccan origin of the commercial samples, while the $\delta^{13}C$ and $\delta^{34}S$ fall perfectly into the range of variability of an Iranian sample. This is not a surprise. As well know, about 90% of world production of saffron is carried out in Iran,[48] due to the lower cost of the raw material and the industrial scale of this crop.

3.2. ICP-MS analysis

Multi-elemental analysis by ICP-MS allowed to determine 65 elements in saffron. Nevertheless, neither the elements presenting concentrations just above the corresponding limits of detection (LOD) in a non-negligible number of samples nor those undetected in at least one saffron sample were considered in successive data elaboration. The only exception was Ni, despite it was systematically undetectable in all the 12 saffron samples coming from Morocco. The observed concentrations in the remaining samples, ranging between 0.3 and 12 µg/g, were instead largely above the LOD (0.05 ng/g). Thus, Ni seems to be an excellent geographical marker for the identification of Moroccan saffron. To retain this important piece of information in further statistical analysis, Ni concentration of Moroccan samples was identified with its LOD value. Finally, 42 elements were considered to characterise the saffron composition. The mean concentrations of the selected elements along with the standards deviations in the saffron samples, grouped according to the geographical origin, are collected in Table 1.

3.3 Multivariate statistical analysis

The ICP-MS and isotopic ratio data matrices were separately handled by PCA for exploratory purposes. A logarithmic transformation was applied to the concentrations (ng/g) of the elements detected by ICP-MS, which are very different in magnitude, to make them comparable and to restrict the effect of the possible outliers. Moreover, PCA was conducted in both cases on the auto-scaled variables to exalt the role of low-variance quantities. Figure 2 displays the saffron samples projected in the subspace of the first three PCs extracted from the ICP-MS multi elemental data whereas Figure 3 shows the variable loadings.

A good separation of the Italian saffron samples and the spices cultivated in Morocco (MO) and Iran (IR) along with the commercial ones (CS) can be observed in the PC1-PC2 plane. Even IR and MO samples are discriminated, whereas the CS samples are partially

superimposed to the MO ones. A better separation between CS and MO samples occurs along PC3 that seems also to describe the discrimination, although incomplete, between Italian saffron samples produced in L'Aquila (AQ) and those of Spoleto (SP). As expected, the other Italian saffron samples coming from various regions are superimposed to both AQ and SP samples. The trend of loadings (Figure 3) suggests that almost all the elements contribute positively to PC1. The apparent increase in the overall mineral content described by PC1, apart from the differences among specific groups of samples (for instance CS and MO with respect to the Italian saffron), seems also to take into account the internal variability of the samples cultivated in a same geographical area. This is quite evident for the MO and IR saffron which give rise to asymmetric clusters elongated in the PC1 direction. Fewer elements are instead the significant variables in defining PC2 and PC3, these components carrying more specific information on the saffron geographical origin. Cr, Cd and Re, with positive loadings, and K, with a negative loading, are in particular the most relevant in PC2, whereas Ni, Zn and Sr, with positive loadings, and Rb, with a negative loading, are the most influent in PC3.

Simultaneous projection of the saffron samples and variable loadings (biplot) on the PC1-PC3 subspace extracted from the isotopic ratio data is shown in Figure 4. A separation, although less clear than that provided by the ICP-MS data, between Italian and non-Italian saffron samples can be observed. The MO saffron samples are in particular well isolated in the first quadrant of the PC1-PC2 plane, whereas the MO and CS samples are mainly located in the fourth quadrant. PC3, meanwhile, describes the separation although incomplete between SP and AQ samples. As suggested by preliminary univariate statistical analysis (Section 3.1), a differentiation of the Italian saffron samples according to the harvest year, which involves not only the two well represented production territories (AQ and SP), but also the isolate samples coming from the other Italian areas, can be noticed along PC1 and partly

along PC3.

The harvest year by contrast does not seem to affect the mineral composition of the samples here analysed, which is consistent with the results of a previous study concerning AQ saffron.[22] Classification of saffron on the geographical basis was attempted by PLS-DA after combination of ICP-MS and isotopic ratio datasets into a single data matrix (low-level data fusion). Four distinct classes according to the cultivation territory were considered: AQ, SP, IR and MO. CS samples and the spices coming from other Italian sites were instead excluded from this kind of analysis, since geographical origin of the first group was unknown and the latter was too heterogeneous to be considered as a distinct class.

The available objects were divided into a calibration and an external data set by Duplex Kennard-Stone algorithm,[49,50] which ensures a comparable representativeness of both subsets. Finally, the PLS-DA model was generated with 39 objects (10 AQ, 14 SP, 8 MO and 7 IR) and tested on 19 external saffron samples (5 AQ, 7 SP, 4 MO and 3 IR). Based on the model performance in leave-one-out cross-validation, autoscaling was selected as the optimal data pre-treatment and the final model was built with five latent variables. Descriptive and predictive performances of PLS-DA classification are displayed in Table 2 reporting the rate (%) of correct classifications, and Figure 5, showing the PLS-DA computed and predicted responses.

The inspection of these data reveals that all the calibration samples were correctly classified, whereas only one AQ and one SP sample were misclassified in prediction. Looking at Figure 5 in more detail, it can be observed that the erroneously classified external SP saffron sample was accepted by the AQ class, although the Y response of this sample for the class AQ (0.64) was just greater that for the class SP (0.52). Moreover, the misclassified AQ sample was assigned to the MO class, but the associated Y response for this class (0.44) was noticeably

lower than those of the genuine MO saffron samples (close to 1). In summary, information carried out by the combined ICP-MS and the isotopic ratio data provided a very good discrimination of the saffron samples on geographical basis. Moreover, even SP and AQ saffron samples coming from close territories of Central Italy, despite both cultivated in two different years, are almost quantitatively discriminated from each other. It follows that the systematic differences associated with the geographical origin can overcome the observed effect of the harvest year on the isotopic composition of the samples.

The importance of the predictors in PLS-DA classification can be quantified by VIP (Variable Importance in Projection) scores;[51] the variables presenting VIP values greater than 1 can be considered as relevant. The VIP indices associated with the PLS-DA model here developed are displayed in Figure 6. It can be observed that all the isotopic ratios, except $\delta^{18}O$ which has a VIP value just below the unity, are significant in the geographical classification of the saffron samples along with a subset of ICP-MS variables including K, Cr, Mn, Ni, Zn, Rb, Sr, Mo, Cs, Nd, Eu and Pb. It is worth noting that Ni has the highest VIP index among all the variables, which reflects the above-mentioned high power of this element in the discrimination of the MO samples with respect to the others.

PLS-DA, as well as other classification strategies, is a suitable method to classify samples among predefined categories. Nevertheless, when the classification model is called to make predictions, even the samples belonging to none of the predefined classes will be assigned to one of them, which makes conventional classification unsuitable for authentication purposes. To evaluate whether a sample complies or not with a given specification (the geographical origin, for instance) a class modelling approach should be applied.[52,53] Once a class model has been established by investigation of a suitable number of target samples class modelling performance can be evaluated by sensitivity and specificity, representing the fraction of

genuine samples correctly accepted and the fraction of non-genuine samples correctly refused by a given class model, respectively.

In this work, two independent class models, for AQ and for SP, were generated by SIMCA, one of the most common class-modelling methods. The remaining saffron samples, including the commercial ones and those cultivated in different Italian territories excluded from previous PLS-DA classification, were all used to test the specificity of the two class models. SIMCA class modelling was performed on the subset of the variables presenting VIP>1 in PLS-DA, namely K, Cr, Mn, Ni, Zn, Rb, Sr, Mo, Cs, Nd, Eu, Pb, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, and $\delta^{2}H$. A leave-one-out cross-validation scheme was adopted to select the number of significant PCs representing the two modelled classes and the variable scaling. Finally, one and three PCs extracted from the autoscaled variables were adopted to build the AQ and SP class models, respectively. Sensitivity and specificity of the two SIMCA class models are reported in Table 2.

Class modelling performance is also graphically shown in the so-called Coomans' plot,[53] displayed in Figure 7. In this diagram, each data sample is represented by its distance to the models developed for SP and AQ, and the lines parallel to the axes represent the acceptance/rejection thresholds for these categories, whereas the lower right and upper left rectangles define the related space classes. The objects falling within the lower left and upper right squares are instead those accepted or rejected by both models, respectively. As to sensitivity, it can be observed that the two class models accept all the genuine samples in calibration while only one external sample is refused by each class model. Specificity of the class models built for AQ and SP saffron is also very high, since 91.8 and 94.4% of non-genuine samples, respectively, were correctly rejected. Looking at the data in more detail, all the IR, MO and CS samples are rightly refused by both class models, whereas the few miss-

classified samples come all from Italy. Four SP and one IT sample are in particular wrongly classified in the AQ class, while two AQ and two IT samples are erroneously assigned to the SP class. The overall specificity of AQ and SP classes for Italian saffron samples cultivated outside the related territories is however acceptable (83 and 84%, respectively).

These results revealed that the variables relevant in the PLS-DA classification model are also useful for the authentication of saffron cultivated in specific territories of Central Italy, including PDO saffron of L'Aquila. It must be stressed that the class models for AQ and SP were built by exploring variability coming from samples cultivated in two different years. The non-negligible effect of the harvesting year on the isotopic composition is expected to enlarge the class spaces being in part responsible for a loss of specificity for the saffron samples cultivated in close areas.

Conclusions

The Stable Isotope Ratio Analysis showed the capability of $\delta^{13}C$ and $\delta^{34}S$ to discriminate between Italian samples and the rest of the dataset, while $\delta^{15}N$ permitted to individuate Moroccan samples among all the others. On the other hand, $\delta^{18}O$ and $\delta^{2}H$ allowed to discriminate between samples of Italian saffron harvested in different years (2016 and 2017). Ni, among the 42 elements determined by ICP-MS, can be considered as a powerful geographical marker for Moroccan saffron, being not detected in none of the 12 samples analysed in this work. Multi-elemental ICP-MS patterns combined with isotopic ratios provided a good geographical classification of Moroccan, Iranian saffron and Italian saffron cultivated in two distinct, although close, sites of Central Italy, L'Aquila and Spoleto. K, Cr, Mn, Ni, Zn, Rb, Sr, Mo, Cs, Nd, Eu, Pb, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, and $\delta^{2}H$ were identified as the significant variables in geographical discrimination. The same descriptors were used to build a class model for both L'Aquila and Spoleto saffron. The two modelled classes presented a

100 % specificity for Moroccan, Iranian and commercial saffron samples and a high specificity (83-84%) for those coming from different Italian areas. In summary, the combined ICP-MS and isotopic composition data, apart from providing a good geographical discrimination of saffron coming from predefined cultivation sites, are also powerful tools for the authentication of high-quality and certified saffron cultivated in specific territories of Central Italy.

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Table 1. List of the monitored elements, limits of detection (LOD) and observed mean contents with related standard errors (SE) in saffron samples from L'Aquila (AQ), Spoleto (SP), other Italian areas (OI), Morocco (MO) and Iran (IR), and commercial saffron (CS); n is the number of the investigated samples for each group.

element		mean content (ng/g)± SE					
	LOD (ng/g)	AQ (n=15)	SP(n=21)	OI(n=9)	CS(n=9)	MO(n=12)	IR(n=10)
Li	0.3	38±7	68±12	27±6	394±34	188±45	114±60
Be	0.1	1.9±0.4	2.7±0.5	0.9±0.2	10±1	7.5±1.6	2.8±1.3
B	8.5	17±1[a]	14.8±0.6[a]	13.1±1.5[a]	21.9±0.7[a]	15.9±0.9[a]	17.1±0.7[a]
Na	39	38±9[a]	34±3[a]	25±2[a]	343±168[a]	87±19[a]	76±10[a]
Mg	30	1.27±0.06[b]	1.19±0.03[b]	1.13±0.07[b]	1.44±0.04[b]	1.76±0.08[b]	1.9±0.2[b]
Al	2.3	27±6[a]	44±8[a]	16±2[a]	215±18[a]	249±47[a]	147±79[a]
K	1.1[a]	8.7±0.4[b]	8.6±0.3[b]	8.2±0.5[b]	9.2±0.5[b]	11.3±0.2[b]	12.2±0.5[b]
Ca	0.05	311±29[a]	300±35[a]	218±10[a]	565±36[a]	568±121[a]	482±110[a]
Sc	0.6	22±2	20±2	19±3	44±3	63±8	75±21
Ti	1.0	1.59±0.15[a]	1.52±0.08[a]	1.34±0.14[a]	4.2±0.5[a]	4.7±0.9[a]	4.0±0.9[a]
V	0.16	48±11	77±13	30±5	483±49	332±66	334±222
Cr	0.45	700±90	1.31±0.12[a]	1.8±0.4[a]	3.1±1.1[a]	574±112	1.1±0.6[a]
Mn	11	13.1±0.4[a]	13.0±0.4[a]	13.2±0.6[a]	21.0±0.7[a]	18±1[a]	23±3[a]
Fe	11	53±7[a]	75±10[a]	54±4[a]	193±16[a]	160±27[a]	157±76[a]
Co	2.5	54±12	66±6	47±5	202±20	120±15	171±75
Ni	0.06	827±142	1.19±0.09[a]	1.26±0.15[a]	3.1±0.7[a]	<LOD	2.8±0.9[a]
Cu	6.5	8.8±0.9[a]	8.2±0.4[a]	6.7±0.3[a]	10.0±1.7[a]	6.2±0.3[a]	6.0±0.2[a]
Zn	2.9	16±1[a]	22.7±0.5[a]	21.7±0.3[a]	22±2[a]	18.2±0.7[a]	20.5±0.6[a]
Ga	0.27	45±5	51±5	41±12	159±13	210±38	115±28
As	1.9	11±2	13±2	12±3	95±10	51±9	63±17
Rb	0.16	11.3±1.7[a]	3.9±0.5[a]	6.5±1.7[a]	5.1±1.5[a]	4.2±0.5[a]	2.6±0.2[a]
Sr	0.09	1.0±0.3[a]	1.9±0.3[a]	7±6[a]	15±2[a]	1.5±0.2[a]	5.7±1.1[a]
Y	0.7	15±4	34±7	8.5±1.3	115±11	74±15	49±29
Zr	0.16	21±2	16±2	22±9	47±9	87±28	64±34
Nb	0.15	3.1±0.5	2.8±0.4	2.2±0.3	8.8±1.6	11±3	5±3
Mo	0.38	634±65	639±108	937±165	337±58	411±59	200±10
Ag	0.07	28±16	11±2	11±4	17±2	35±15	12±5
Cd	0.02	75±13	181±111	59±8	31±4	15±3	19±3
Cs	0.09	13±2	8±1	8±2	40±5	19±3	12±4
Ba	0.23	1.17±0.15[a]	1.37±0.16[a]	1.5±0.5[a]	3.0±0.4[a]	2.7±0.5[a]	1.7±0.3[a]
La	0.1	30±7	36±7	35±14	130±17	159±37	61±28
Ce	0.7	64±12	92±17	65±20	272±37	316±65	129±58
Pr	0.02	4.8±1.2	8.2±1.7	3.8±0.8	32±4	35±8	14±7
Nd	0.7	59±12	69±7	42±4	222±30	140±34	56±26
Sm	0.13	5±1	7.8±1.5	3.2±0.7	28±3	28±7	13±6
Eu	0.12	31±12	725±277	531±332	63±12	7.6±1.3	9.8±1.5
Dy	0.7	2.2±0.5	4.2±0.9	1.3±0.3	17±2	15±3	8±4
Ho	0.03	0.4±0.1	0.75±0.15	0.25±0.05	3.3±0.4	2.9±0.6	1.6±0.9

Er	0.02	7±4	3.1±0.5	1.8±25	16±5	8.1±1.6	8±4
Hf	0.05	0.44±0.12	0.84±0.09	3.3±0.5	5±2	3.3±0.4	4.3±1.2
Re	0.04	41±18	33±11	3.3±0.1	123±45	9±6	4.2±1.7
Pb	1.7	420±90	210±40	200±30	859±198	342±117	109±25

[a]μg/g;[b]mg/g

Table 2. Rate (%) of correct classifications in PLS-DA. Sensitivity (% of compliant samples correctly accepted) and specificity (% of non-compliant samples correctly refused) in SIMCA class modelling of AQ and SP saffron.

PLS-DA							
calibration				prediction			
AQ	**SP**	**IR**	**MO**	**AQ**	**SP**	**IR**	**MO**
100.0	100.0	100.0	100.0	80.0	85.7	100.0	100.0

SIMCA							
	sensitivity			specificity			
	calibration	**prediction**	**total**	**for IT[a]**	**for CS**	**for IR**	**for MO**
AQ	100.0	80.0	91.8	83.3	100.0	100.0	100.0
SP	100.0	85.7	94.4	84.0	100.0	100.0	100.0

[a] Saffron sample cultivated in Italy except those of the modelled class

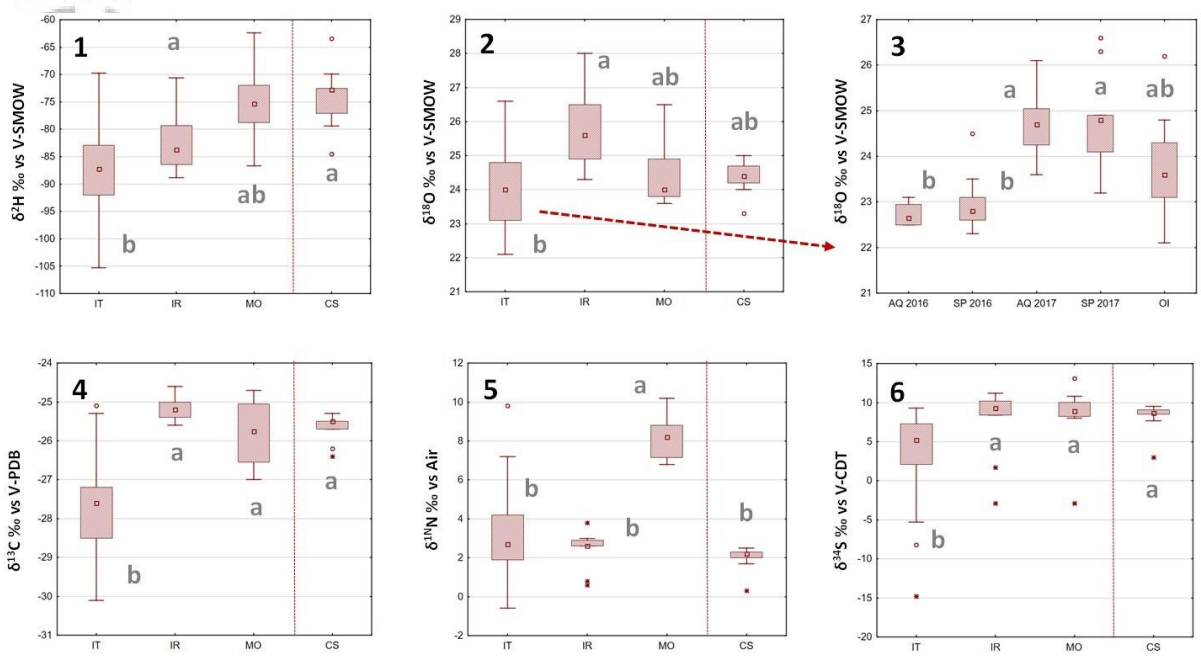

Figure 1. Box plot of 1) $\delta^{2}H$, 2) $\delta^{18}O$ for the group of samples IT, IR, MO and CS 3) $\delta^{18}O$ for the Italian groups of samples AQ2016, AQ2017, SP2016, SP2017 and OI 4) $\delta^{13}C$, 5) $\delta^{15}N$ and 6) $\delta^{34}S$ for the group of samples IT, IR, MO and CS.

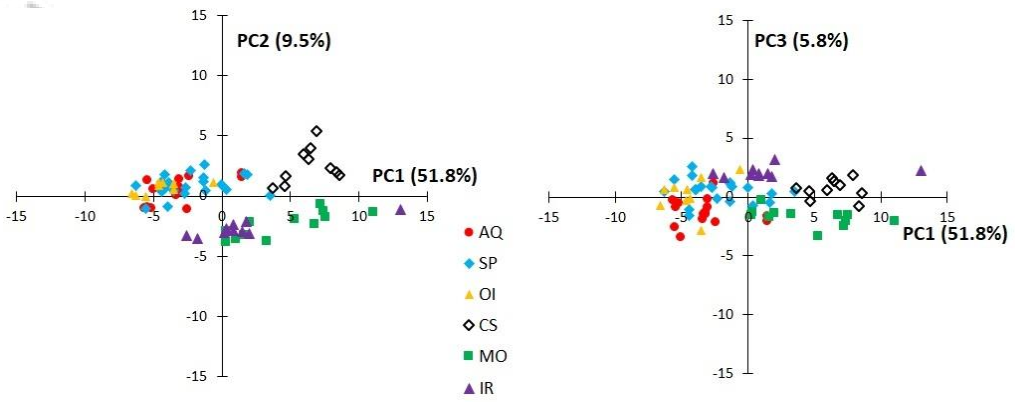

Figure 2. Saffron samples projected onto the subspace of the first three PCs extracted from the ICP-MS multi-elemental data.

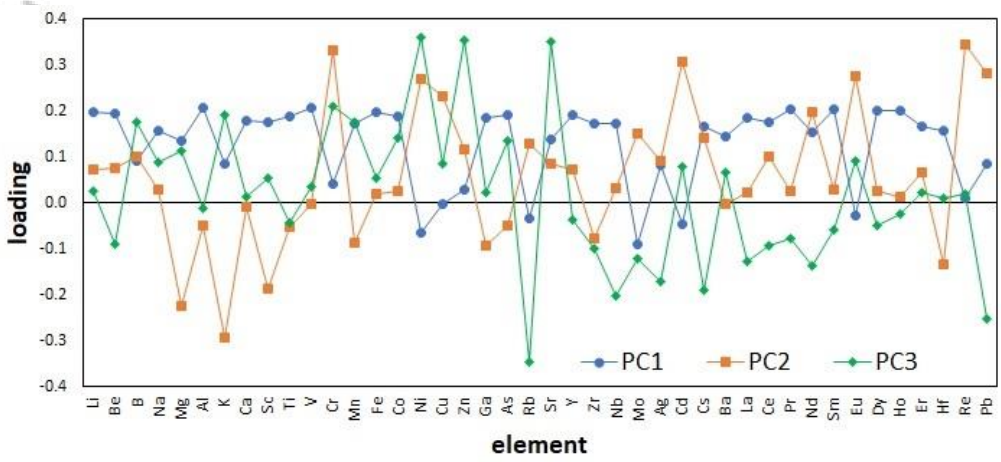

Figure 3. Variable loadings of PCs extracted from the ICP-MS data.

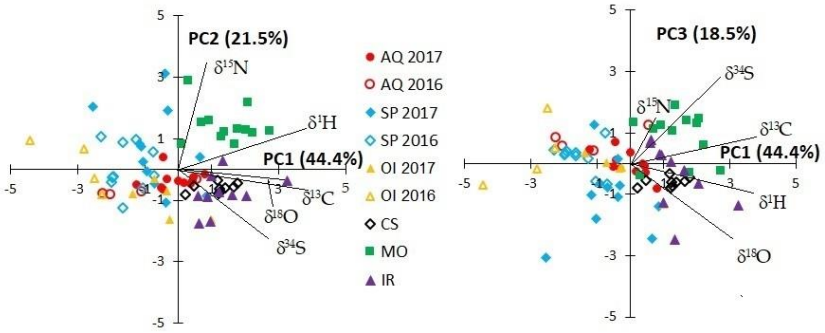

Figure 4. Projection of saffron samples and variable loadings on the subspace of the first three PCs extracted from isotopic ratio data.

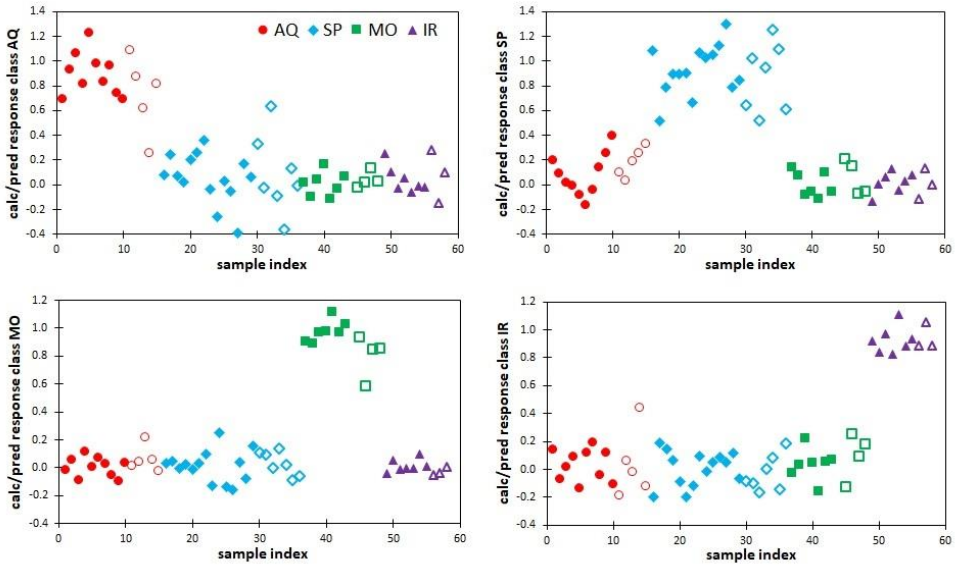

Figure 5. Computed and predicted responses of the PLS-DA classification model. Empty symbols identify the external saffron samples.

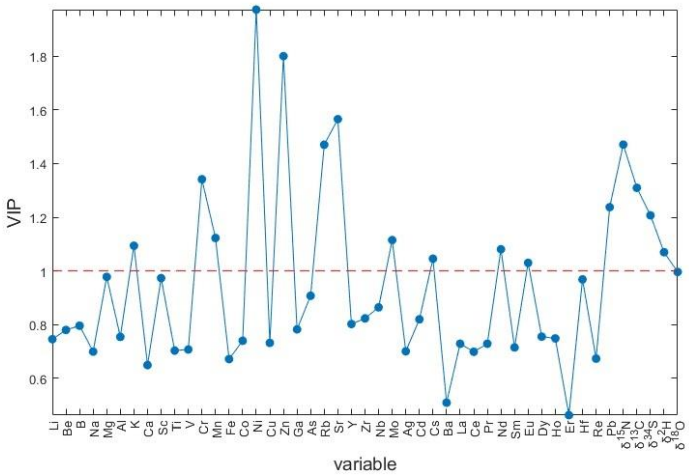

Figure 6. Importance of variable (VIP indices) in PLS-DA geographical classification of saffron samples.

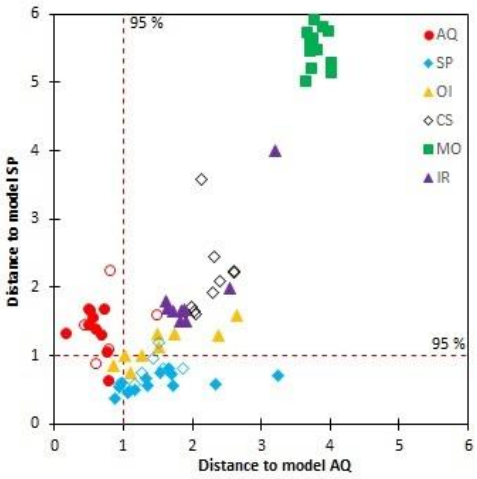

Figure 7. Coomans' plot representing the two SIMCA models of classes AQ and SP.