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Comparison of IRMS, GC-MS and E-Nose data for the discrimination of saffron samples with different origin, process and age

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ABSTRACT

In this study the use of conventional (Isotope-ratio mass spectrometry-IRMS and solid-phase microextraction gas chromatography mass spectrometric, SPME-GC-MS) and non-conventional analytical techniques (Electronic Nose) to characterize and discriminate origin, drying and age of 35 saffron samples was investigated.

The IRMS technique by the analysis of the stable carbon and nitrogen isotopes has proved to be a reliable method in discriminating the geographical origin of saffron. Taking into account the chemical classes of the detected volatile compounds, the SPME-GC-MS was able to discriminate the different origin, drying and age. An E-Nose was used as alternative and rapid tool to characterize the complex aroma patterns and to exploit authenticity of saffron samples. Overall, the innovative AuNP-peptide based sensors array showed only a good discrimination of their origin.

Results of this study could contribute to select and identify routine quality control methods for quality and authentication of saffron.

1. Introduction

Saffron (*Crocus sativus* L.) stigmas after drying are traditionally used as spice in food preparations as coloring or flavoring agent; recent research has shown also its potential to promote health (Hamzah, Yee, Kadir, & Nayan, 2017; Melnyk, Wang, & Marcone, 2010; Razak, Anwar Hamzah, Yee, Kadir, & Nayan, 2017; Menghini et al., 2018). It is produced in many countries of the world, generally in small-medium size farms and factories. This contributes to the large variability of saffron quality at world level. These factors and the very low product yield lead to a product that is considered one of the most expensive spice in the world and that raise, consequently, high risks of frauds and adulterations and issues regarding its traceability and authenticity. ISO 3632 standards classify saffron in three quality categories based on data obtained by spectrophotometric analysis (ISO 3632–2: 2010 and ISO 3632–1:2011). This approach while useful for quality classification and trade purposes has some limitations. In particular, the spectrophotometric data connected to the absorbance of the key components at specific wavelengths actually are not specific. Moreover, there are no relationships between ISO classification and spectrophotometric results and the presence of compounds of interest for traceability and authenticity along with the possibility to evidence the impact of processing and storage conditions.

The IRMS analysis is one of the most widely used approach for food traceability and authenticity (Zhao et al., 2014). This technique evaluates the stable ratios of the isotopes present in the sample which are related with the geographical origin, the climatic conditions, and the soil pedology and geology of the location from where the products originate (Drivelos & Georgiou, 2012). The IRMS analysis, considering its high accuracy, has found many applications in the field of authentication and discrimination of the geographical origin of food matrices (Bontempo et al., 2019; Camin et al., 2018; Faberi et al., 2018; Perini, Giongo, Grisenti, Bontempo, & Camin, 2018; Semiond et al., 1996). In the case of saffron, Semiond et al. (1996) used the carbon isotope ratio analysis on saffron measuring $\delta^{13}C‰$ values of safranal. They reported the successful discrimination of synthetic and natural safranal, but also the difficulty to point out differences among the various geographical origins.

Saffron is characterized by a number of volatile and aroma-yielding compounds that recently literature has indicated of being ca. 150,

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mainly terpenes, terpene alcohols and their esters (Carmona, Zalacain, Salinas, & Alonso, 2007). Many studies were carried out on food traceability and authenticity of saffron focused on the volatile fraction and its evolution as affected by origin (Carmona et al., 2006; Maggi et al., 2009; Tahri et al., 2015; Karabagias, Koutsoumpou, Liakou, Kontakos, & Kontominas, 2017; D'Archivio, Di Pietro, Maggi, & Rossi, 2018) and storage conditions (Raina, Agarwal, Bhatia, & Gaur, 1996; Kanakis, Daferera, Tarantilis, & Polissiou, 2004; D'Auria, Mauriello, Racioppi, & Rana, 2006), while minor attention was given to the role of the drying process (Raina et al., 1996; D'Auria et al., 2006). Saffron aroma pattern has been mainly analysed by GC techniques, generally coupled to mass spectrometry (Amanpour, Sonmezdag, Kelebek, & Selli, 2015; Maggi et al., 2011a; Sereshti, Heidari, & Samadi, 2014) and recently olfactometry (Amanpour et al., 2015; Culleré, San-Juan, & Cacho., 2011). However, conventional techniques for assessing flavor quality, including sensory analysis and GC–MS, are often too time-consuming and labor-intensive to be used routinely as quality control (QC) methods. The ultimate challenge is to develop fast analytical procedures resulting in classifications of food products that correlate well with sensory panel data.

Among the analytical techniques to characterize the volatiles pattern of food matrices, the electronic nose (E-nose) is among the most innovative ones. These devices mimic the sense of smell and generate a sensor array response to a complete volatile pattern, without separating the aroma compounds and use pattern recognition software for data processing (Arshak, Moore, Lyons, Harris, & Clifford, 2004). These electronic sensing systems could represent a convenient alternative for screening due to their rapidity, simplicity and low cost to classify products with a different chemical "fingerprint".

E-Nose has been applied for the food quality evaluation in several domains of applications in food analysis, for example, food quality monitoring based on seasonal effect, ageing, and geographical origin (Chen et al., 2018; Compagnone et al., 2013, 2015; Roy, Bandyopadhyay, Tudu, & Bhattacharyya, 2018) and also used for the diagnosis of gastrointestinal diseases (Wilson, 2018).

E-Nose based on Metal Oxide Semiconductor (MOS) gas sensors has been shown to be able to discriminate saffron samples from different origins (Carmona et al., 2006; Kiani, Minaei, & Ghasemi-Varnamkhasti, 2016) and also to differentiate non-adulterated and adulterated saffron (Carmona et al., 2006; Heidarbeigi et al., 2015; Kiani, Minaei, & Ghasemi-Varnamkhasti, 2017). In this study we used a quartz crystal microbalance peptide-based E-Nose previously developed in our lab and already successfully applied for detection of different volatiles in food (Compagnone et al., 2013; Del Carlo et al., 2014; Compagnone et al., 2015; Pizzoni, Compagnone, Di Natale, D'Alessandro, & Pittia, 2015).

The aim of this study, thus, was to combine conventional (GC-MS and IRMS) and non-conventional techniques (peptide gas sensors array) to characterize and differentiate saffron samples having different origin, process and age conditions. The IRMS could prove a very reliable method in discriminating the geographical origin of saffron. The peptide gas sensors array could be applied as additional analysis method to other complementary conventional techniques, such as GC-MS, to characterize and discriminate complex aroma patterns. The present study could contribute to select and identify routine saffron quality control and authentication tools.

2. Materials and methods

2.1. Saffron samples and chemicals

Thirty-five saffron samples of different origin and process were collected during the harvesting period 2012–2016 (Table 1). Samples from Italian regions (n = 26 from Abruzzo, Basilicata, Campania, Lazio, Lombardy and Sardinia) were kindly provided by local farmers and producers. Detailed information on the non-Italian samples was not available, therefore only the country of origin was reported. Non-Italian samples were collected as powders, while the Italian ones were as stigmas and upon arrival underwent to manual grinding in a dry box up to a fine and homogeneous powder. All samples were freeze-dried to remove any residual moisture and then packed in high barrier metalized plastic bags hermetically closed and stored at −18 °C until analysis.

Table 1
List of thirty-five saffron samples with different origin, drying process and year of production. Regions origin labels were provided only for the Italian samples.

Country	Region	Year	Drying treatment	Label
Italy	Abruzzo	2012	Hard	1H
Italy	Abruzzo	2012	Hard	2H
Italy	Abruzzo	2013	Hard	3H
Italy	Abruzzo	2014	Hard	4H
Italy	Abruzzo	2015	Mild	5M
Italy	Abruzzo	2015	Hard	6H
Italy	Abruzzo	2015	Hard	7H
Italy	Abruzzo	2016	Mild	8M
Italy	Abruzzo	2016	Mild	9M
Italy	Abruzzo	2016	Mild	10M
Italy	Abruzzo	2016	Mild	11M
Italy	Abruzzo	2016	Hard	12H
Italy	Abruzzo	2016	Mild	13M
Italy	Basilicata	2016	Mild	14M
Italy	Campania	2016	Mild	15M
Italy	Campania	2014	Mild	16M
Italy	Lazio	2014	Mild	17M
Italy	Lazio	2016	Mild	18M
Italy	Lombardy	2012	Hard	19H
Italy	Lombardy	2013	Hard	20H
Italy	Lombardy	2015	Hard	21H
Italy	Lombardy	2015	Mild	22M
Italy	Lombardy	2016	Hard	23H
Italy	Sardinia	2014	Hard	24H
Italy	Sardinia	2015	Hard	25H
Italy	Sardinia	2016	Hard	26H
Argentina	unknown	2014	Mild	ARG
Greece	unknown	2016	Mild	GR
Iran	unknown	2014	Mild	IR1
Iran	unknown	2016	Mild	IR2
India	unknown	2014	Mild	IND
Lebanon	unknown	2014	Mild	LEB
Spain	unknown	2016	Mild	SP1
Spain	unknown	2016	Mild	SP2
Turkey	unknown	2014	Mild	TUR

All saffron samples were preliminarily classified by age and drying method and intensity that, in turn was based on the applied process temperature. To this aim local farmers and producers were asked to indicate year of production and specific drying conditions of their samples. For commercial samples, some information on process was either provided by producers or based on the generally used drying methods and temperatures used in the country of origin while age was estimated based on the “best consumption date”, taking as reference a 5 years-commercial shelf-life.

Drying was generally carried out in electric oven also by farmers, but other technologies were also used (sun-drying, microwave). For drying intensity, the process temperature allowed to classify samples in two main categories: high temperature drying (“H” samples) processed in electric ovens at temperatures $> 45^{\circ}$ and up to 120 °C, and samples undergone to mild temperature drying (“M”) when process is carried out at temperatures ≤ 45 °C. For the age classification two groups were also defined: fresh (≥ 2015) and aged (< 2015).

It was not possible to analyse all saffron samples by GC-MS for the low amount available and the analyses were carried out on 30 samples (no analysed: 5M; 15M; 23H; 25H and SP2). The E-Nose analyses were achieved on a small set of saffron samples to evaluate the possibility to use this technique based on AuNP-peptide sensors as additional tool.

2.2. Quality evaluation of saffron

Saffron samples were analysed for the colouring, aromatic strength, and bitterness by applying the ISO 3632–1 protocol as modified by Paredi, Raboni, and Mozzarelli (2014) within the COST ACTION Saffronomics and described in Rocchi et al. (2018). Briefly, an aliquot of saffron (10 mg) was dispersed in 2 mL distilled water and subjected to Ultrasound Assisted Extraction (UAE) for 15 min at 25 °C. After centrifugation, the extracts were analysed by a spectrophotometer (PerkinElmer Lambda Bio 20 UV–Visible, US). The absorbance at three fixed wavelengths (flavor strength-picrocrocin: $\lambda_{max} = 257$ nm; aroma strength-safranal $\lambda_{max} = 330$ nm and colour strength-crocins $\lambda_{max} = 440$ nm) of the saffron extract, diluted at 1% w/w in water, was evaluated in a 1 cm pathway quartz cell; MilliQ water (Milliport Corp., Bedford, MA) was used as reference. Moisture and volatiles content (W_{MV}) as a percentage of the initial sample was determined by putting an aliquot of each sample (15 mg) exactly weighted in an oven at 103 °C for 16 h and computed by the following equation (1):

$$W_{MV} = \frac{initial\ weight - final\ weight}{initial\ weight} * 100 \quad (1)$$

Quality indicators were then computed according to the following equation (2):

$$E_{1cm}^{1\%} = \frac{D - 10000}{m*(100 - w_{MV})} \quad (2)$$

where: D is the specific absorbance at the three fixed wavelengths; m is the mass of the sample (in g); W_{MV} is the moisture and volatile content of the sample, expressed as a mass fraction.

Classification of the samples according to ISO 3632 trade specifications was eventually carried out and based on the modified ISO method applied in this study, the class categories were named "ISO-like".

Analyses were carried out in triplicate on different aliquots of each sample.

2.3. Isotope ratio mass spectrometry

The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios were determined according to the elemental analysis EA/IRMS technique as reported in Faberi et al. (2018). Briefly, the analysis was performed by a flash combustion on an elemental analyser EA-Flash 2000 HT (Thermo Fisher, Bremen, Germany), connected to a Delta V Plus (Thermo Fisher, Bremen, Germany) isotope ratio mass spectrometer operating in continuous helium flow mode, via a CONFLO IV interface (Thermo Fisher, Bremen, Germany).

Aliquots of 1 mg of saffron powder were exactly weighted into a tin capsule, inserted in the autosampler for solids and introduced in the reactor for combustion of the elemental analyser. All organic matter was oxidized under synchronized helium flow and oxygen pulse flow (3 s) through combustion flash which occurs in a quartz reactor at 920 °C. Packaged redox catalysts Cr_2O_3 and $(Co_3O_4)Ag$ are contained in the reactor. The combustion gas (CO_2, N_2, NO_x, and H_2O) was passed through a layer of metallic Cu allowing elimination of the O_2 excess and reduction of nitrogen oxides to N_2 while water was removed by a trap filled with anhydrous $Mg(ClO_4)_2$. N_2 and CO_2 are then separated on a gas chromatographic column Pora-PLOT Q, L = 5 m, thermostated at the temperature of 45 °C, and sent through the ConFlo IV to the IRMS, to determine the isotopic composition.

As reference, CO_2 and N_2 gases were injected in the continuous He flow gas pure standard pulses. The carbon isotope ratio ($^{13}C/^{12}C$) was calculated from the ionic currents *m/z* 44 ($^{12}C^{16}O^{16}O$), *m/z* 45 ($^{13}C^{16}O^{16}O$), and *m/z* 46 ($^{12}C^{16}O^{18}O$ or $^{13}C^{16}O^{17}O$), being the latter used by the software to make a small correction $\delta^{13}C$, according to Craig (1957), due to the contribution of ^{17}O produced by CO_2. The nitrogen isotope ratio $^{15}N/^{14}N$ was determined from the ionic currents *m/z* 28 ($^{14}N^{14}N$), *m/z* 29 ($^{14}N^{15}N$), and *m/z* 30 ($^{15}N^{15}N$). Two different reference materials have been used in each sequence of analysis, one for the calibration of the instrument and one for the quality control of the analysis. To this aim a skimmed milk protein powder by ultra-filtration and whey protein powder (Fonterra Italy S.p.A., Cernusco Lombardone, CL, Italy) whose reference values of $\delta^{13}C$ and $\delta^{15}N$ have been previously standardized against certified reference materials (IAEA-NBS-22, IAEA-CH-6, USGS-40 for $\delta^{13}C$ and IAEA-NO-3, USGS-40 for $\delta^{15}N$) are used. Isodat™ 3.0 software (Thermo Fischer Scientific, Bremen, Germany) management tool calculated the values of $\delta^{13}C$ in the samples by comparison of the signal ratios of the masses 45 and 44 corrected according to Craig (1957) with reference CO_2 gas. $\delta^{15}N$ was calculated similarly using the ratio of the masses 29 and 28. Stable isotope ratios are calculated the conventional δ – notation, according to Brand, Coplen, Vogl, Rosner, & Prohaska, 2014:

$$\delta^i E = \frac{^iRSA - {}^iRREF}{^iRREF}$$

where E is the element considered; i is the mass number of the heavier isotope of element E (for example, ^{13}C); RSA is the respective isotope ratio of a sample; RREF is the relevant internationally recognized reference material.

Delta values are expressed in "per mil" units (%_0). The delta obtained for the samples was processed in order to verify the repeatability limit of the MR used as "reference material" (MR1); only for values below 0.3% δ units (usually accepted for this analysis), the values of $\delta^{13}C$ and $\delta^{15}N$ are averaged. The difference between the resulting value and the "true value" (by setting) is, then, calculated and all the other data of $\delta^{13}C$ and $\delta^{15}N$ of the series, including MR used as "control material" (MR2), and corrected for the difference. Samples are analysed in duplicate and the $\delta^{13}C$ and $\delta^{15}N$ average of the measurement checked for the error and corrected as reported earlier.

2.4. SPME-GC–MS

A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber 50/30 μm (Supelco, Bellefonte, PA, USA) was used to extract headspace volatiles from saffron. Prior to use, the fiber was conditioned following the manufacturer's recommendations. The samples (10 mg of saffron in 2 mL of distilled water) were placed in a 20 mL crimp cap vial equipped with PTFE/silicone septum. The vial was maintained at 40 °C for 30 min under stirring to favour the partitioning of the volatiles, then the DVB/CAR/PDMS fiber was exposed to the headspace at 40 °C for 5 min. The absorbed volatile compounds on the SPME fiber were thermally desorbed for 2 min in the closed GC inlet at 230 °C. The injections were performed in splitless mode (2 min) and with a split flow of 50 mL/min, as helium was used to transport gas at a constant flow of 1 mL/min, the transfer line temperature was maintained at 230 °C. The temperature of the EI source was set at 250 °C, the potential was set to the standard value of 70 eV, all measurements were performed in full scan range *m/z* 15–450.

The initial temperature of the oven was set at 40 °C for the first 4 min; the temperature was then, increased 5 °C/min until 170 °C and there kept for 1 min, and increased again to 230 °C and maintained at this temperature for 10 min in order to eliminate impurities and interfering compounds. The program run was 45 min. Test samples were prepared daily prior to SPME–GC-MS analysis. Each sample was run in duplicate (n = 2).

SPME-GC-MS analysis was carried out by gas chromatographic Trace GC Ultra (Thermo Scientific) interfaced with a mass spectrometry DSQ II of Thermo Scientific fitted with CP-WAX capillary column (30m × 0.25 mm ID x 0.25 μm film). Compounds identification was carried out using the NIST library.

2.5. Peptide gas sensors array set-up (E-Nose)

The peptide gas sensors array consisted of four peptides covalently bound to gold nanoparticles (AuNPs) (AuNP-IHRIC, AuNP-WHVSC, AuNP-KSDSC, and AuNP-LAWHC). The peptides were purchased from Espikem (Florence, Italy, purity > 85%).

These peptides were deposed onto the 20 MHz quartz crystal microbalance sensors (QCM), bought from KVG GmbH (Germany). The QCM sensors modification was achieved by drop casting 5 μL of the AuNP-peptide suspension on each side of the crystal and let dry for few minutes as reported in Compagnone et al. (2013).

The piezoelectric measurements were carried out using an Enose-UTV from Sensor group, University of Rome Tor Vergata (Italy).

Nitrogen was selected as gas carrier for the experiments and fluxed at 4 L/h (flowmeter from DK 800R KROHNE, Germany) through the glass bottle connected to the measuring chamber containing sensor array via three-way stop-cocks. Before measurement, the saffron samples (50 mg) were placed in an aluminum basket and located inside a glass bottle. Measurements of the glass bottle headspace were carried out after 10 min at 40 °C. The measurement started opening the stop-cocks and, then, flowing the glass bottle headspace into the sensor chamber. This range of time was selected as the optimum time required to reach a steady state by the saffron volatile compounds in the headspace sample's volume based on preliminary experiments (*data not shown*). The frequency shift (ΔF), taken as analytical signal was recorded. Δf for each sensor was defined as the difference of frequency values between the beginning and the end of the measurement (8 min). After each measurement, a complete recovery of the signal was achieved under N_2 flow in about 6 min.

2.6. Statistical analysis

Statistical analysis was performed using box and whisker plot by means XLSTAT 2016 and PLS-DA by means of MatLab 2011 (Mathworks, Natick, MA, USA) integrated with a classification toolbox for MATLAB obtained from Milano Chemometrics and QSAR Research Group (version 3.0). Data have been autoscaled (zero mean and unitary variance). The numerical evaluation of the models achieved was validated by 'venetian blinds' cross-validation with number of cv groups equal to 2.

3. Results and discussion

3.1. Saffron quality parameters

The quality parameters values of flavour, aroma and colour strength for the different saffron samples under investigation and the corresponding ISO-like class are summarised in Table 2.

These parameters are determined by spectrophotometric analysis of the saffron extracts at the wavelengths corresponding to the max absorbance of three main quality compounds, namely picocrocin, safranal and crocins. However, as reported in other studies (García-Rodríguez, Lopez-Còrcoles, Alonso, Pappas, Polissiou, & Tarantilis, 2017; García-Rodríguez et al., 2014), this method does not give an accurate measurement of picrocrocin and safranal content due to the interferences in the measurements caused by the crocin isomers that also absorb at their same wavelengths. Indeed, the ISO 3632 method does not allow to classify saffron based on aroma strength value related to safranal while it is based mainly on the flavour and colour strength values.

Aroma, flavour, and colour strength values of the saffron samples varied significantly while three samples resulted non-classified due to the very low absorbance values and, among them, the ARG saffron sample did not show any absorption at the selected wavelengths, which could be a possible index of adulteration. Overall, eighteen samples were classified in the first (higher) quality category, eight in the second, six in the third (lower). Independently on age, Italian samples showed a higher colouring strength ($E_{1\%}^{1cm} > 121$), related to the crocins content, followed by the saffron samples from other countries (Rocchi et al., 2018).

Table 2
Quality parameters and ISO-like classification of saffron samples. The coefficient of variation (CV) is < 0.3% for all samples.

	$E_{1cm}^{1\%}$			
Sample	257 nm	330 nm	440 nm	ISO-like
	Flavour strength	*Aroma strength*	*Colouring strength*	Category
1H	64	31	121	III
2H	77	34	156	III
3H	77	34	155	II
4H	86	23	193	II
5M	82	22	190	II
6H	118	34	293	I
7H	94	39	241	I
8M	88	23	223	I
9M	72	25	166	III
10M	89	35	236	I
11M	93	25	223	I
12H	100	28	233	I
13M	78	31	193	II
14M	99	36	211	I
15M	101	27	244	I
16M	82	23	200	I
17M	98	42	207	I
18M	100	39	238	I
19H	68	32	138	III
20H	120	54	216	I
21H	101	22	180	II
22M	85	27	208	I
23H	132	28	270	I
24H	88	32	204	I
25H	118	37	268	I
26H	123	24	276	I
ARG	/	/	/	n.c.
GR	111	38	288	I
IND	40	22	80	n.c.
IR1	72	37	165	III
IR2	89	40	197	II
LEB	59	31	120	III
SP1	72	36	181	II
SP2	84	45	181	II
TUR	31	26	15	n.c.

*n.c. = non-classified.

3.2. IRMS results

The IRMS analysis was used to provide the traceability of saffron samples before the analysis of the volatile compounds fraction. Isotopes of carbon and nitrogen, often added to a system in enriched form, are powerful tools to trace relationships in a food chain and to assess physiological metabolic processes (Peterson & Fry, 1987).

Table S1 reports the δ isotopic composition of $\delta^{13}C$ and $\delta^{15}N$ of 35 saffron samples collected from Italian (different harvesting year) and non-Italian regions. The values of $\delta^{13}C$ for saffron of the Italian regions were in the range of −27.37 to −31.52, while saffron from other countries had values of $\delta^{13}C$ between −25.19 and −27.10 (*see Supplementary Material,* Table S1 *the δ isotopic composition of* $\delta^{13}C$ *and* $\delta^{15}N$ *of the 35 saffron samples*). The range of values for $\delta^{15}N$ was from 0.55 to 5.14 for Italian samples and for non-Italian from 2.39 to 6.83. The range of values of the δ isotopic composition of $\delta^{13}C$ and $\delta^{15}N$ of the Italian samples resulted wider than the values reported by Maggi, Carmona, Kelly, Marigheto, and Alonso (2011b), that analysed saffron samples produced in the same year (2006) in Italy, Greece, Spain and Iran. Therefore, this variability could be due to the different year of production of the Italian samples from 2012 to 2016.

All data were firstly analysed by using a univariate procedure to

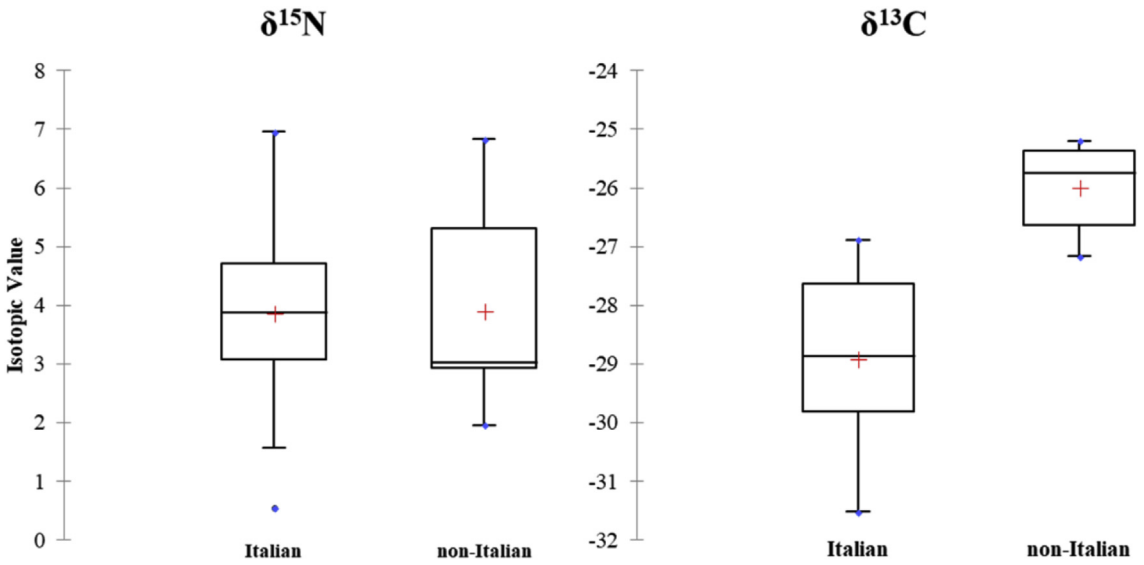

Fig. 1. Box and whisker plot of δ ^{15}N and δ ^{13}C of the Italian and non-Italian saffron samples.

discriminate Italian and non-Italian samples and in Fig. 1 the corresponding results as box and whisker plot are shown.

It could be noticed that the use of the δ^{15}N isotopic composition led to no statistical differences between the two saffron classes whilst the Italian and non-Italian saffron samples were differentiated by the δ^{13}C composition but not at a statistically level with an overlap of the Italian highest and non-Italian lowest observations. A multivariate statistical procedure by means of Partial Least Squares Discriminant Analysis (PLS-DA) was then applied to the same IRMS dataset to obtain a good separation among classes, i.e., geographical origin, using as variables the δ^{15}N and δ^{13}C isotopic values. The corresponding score and loading plots of PLS-DA model are reported in Fig. 2.

The model was evaluated using the following parameters: the explained variance, the Root Mean Squared Error in Calibration (RMSEC) and the Root Mean Squared Error Cross Validation (RMSECV), which are presented in Table 3. The discrimination of two classes (Italian and non-Italian) was mostly due to the first latent variable having an explained variance of 50.94%. The second latent variable (49.06%) contributed only to the spread of the results within the class. The loadings, that are represented by the δ^{15}N and δ^{13}C variables, displayed a different behaviour; they were located in an opposite part of the plot and contributed to the discrimination of saffron origin to a different extent.

As expected, the variable δ^{13}C contributed largely to the

Table 3
PLS-DA statistical data obtained of the IRMS and E-Nose results. Root Mean Squared Error in Calibration (RMSEC) and Root Mean Squared Error Cross Validation (RMSECV).

	IRMS			E-Nose		
	Origin	**Storage**	**Process**	**Origin**	**Storage**	**Process**
samples	35	35	35	27	27	27
variables	2	2	2	4	4	4
classes	2	2	2	2	2	2
component in model	2	2	2	2	1	1
explained variance %	100%	57%	100%	93%	90%	90%
RMSEC	0.09	0.44	0.43	0.24	0.34	0.32
RMSECV	0.07	0.44	0.58	0.19	0.38	0.40

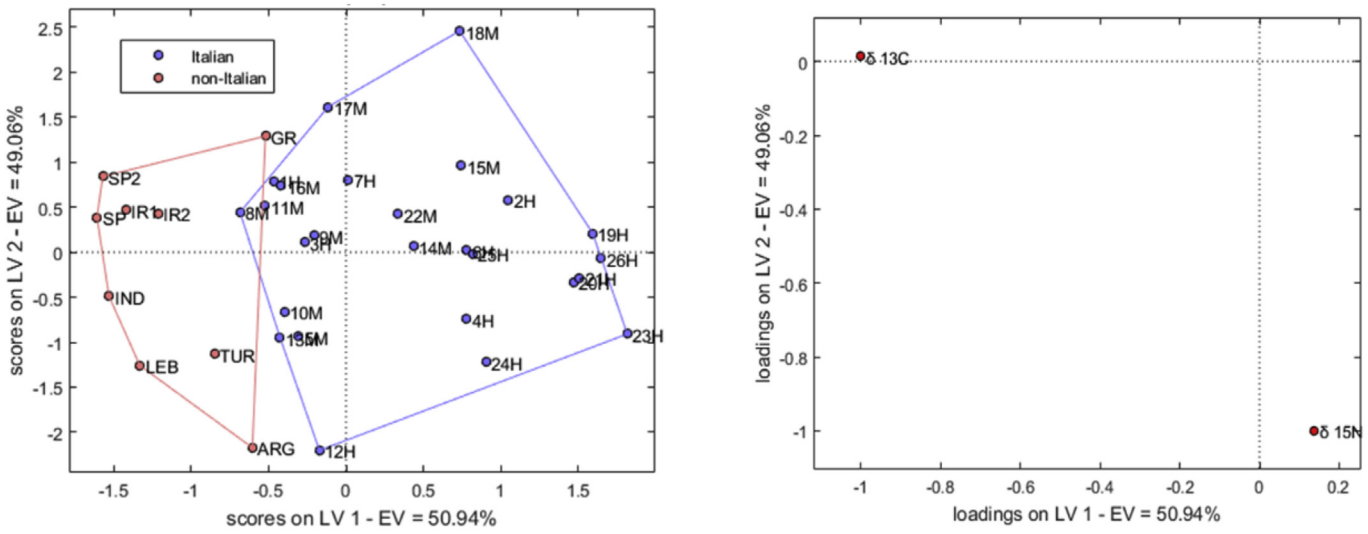

Fig. 2. Score and loading plot of PLS-DA analysis of the IRMS data of the 35 saffron samples. Data have been linearly normalized and autoscaled (zero mean and unitary variance). The two saffron classes are marked with different colors: blue, for Italian saffron samples and red, for the non-Italian saffron samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

discrimination of the non-Italian saffron samples with −0.9 value on the first latent variable. The $\delta^{15}N$ with a value of 0.2 on the first latent variable partly contributed to the separation of Italian samples (Fig. 2). The statistical summary results of the PLS-DA algorithm are reported in Table 3, showing a very good discrimination between the two classes with an explained variance of 100% and low classification errors in both calibration and cross validation (12% RMSEC and 10% RMSECV). The saffron samples misclassified in CV were one sample from Italian (8M) and one from non-Italian class (GR).

IRMS results were also analysed by PLS-DA using age or drying intensity criteria, but high classification errors were obtained (Table 3). Within the Italian samples a tight discrimination origin of samples was applied to discriminate those from different regions. However, despite the different geographical and climatic differences of the Italian regions (Lombardy-North Italy; Lazio, Campania, Abruzzo-Central Italy; Basilicata-South Italy) both $\delta^{13}C$ and $\delta^{15}N$ results did not allow their discrimination as a high classification error between measured and predicted classes (26% RMSEC and 34% RMSECV) was observed.

3.3. SPME-GC-MS analysis

The SPME-GC-MS analysis was aimed to characterise the VOCs fraction in the headspace of saffron samples with different origin, age and drying process. Fresh stigmas are virtually odourless and the characteristic aroma of saffron is generated during drying by enzymatic and/or thermal degradation of picrocrocin (Cadwalleder, 2001).

The saffron VOCs are generally originated by degradation of safranal and lipophilic carotenoids; they can be divided into two groups (Maggi et al., 2010), depending on their chemical structure and/or precursors: (a) C9–C10 compounds structurally and (b) C13-norisoprenoids. In Table 4 the list of the volatile compounds identified in headspace of the saffron samples is reported along with the average concentration, expressed as % of the total GC-MS area, of the saffron samples and the min-max range (*see Supplementary Material,* Table S2 *for the concentration, as relative % GC-MS area of the detected volatile compounds for each sample*).

Under our experimental conditions, overall 36 compounds were identified belonging to different chemical classes, namely, aldehydes, ketones, alcohols and hydrocarbons. In general, safranal (2,6,6-trimethyl-1,4-cyclohexadiene-1-carboxaldehyde) is the main volatile compound of saffron according to the literature (Carmona et al., 2007; Maggi et al., 2009), whose presence is complemented in significant lower content by other volatile compounds that overall contribute to define the characteristic aroma of the spice. 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde is present in all analysed samples in the range from 22.80 to 96.75%; the lower values corresponded to ARG and TUR samples. In particular, ARG sample had a highest content of 3,5,5-trimethyl-3-cyclohexen-1-one (β-isophorone) and 3,5,5-trimethyl-2-cyclopenten-1-one; instead TUR sample had a higher content also of β-isophorone and 1,3,4-trimethyl-3-cyclohexene-1-carboxaldehyde than other saffron samples. No differences on the amount of safranal were noticed on the different applied drying conditions as instead reported by Carmona et al. (2006), that stated an increasing of safranal concentration depending on the temperature applied during drying. Three other compounds 3,5,5-trimethyl-2-cyclohexen-1-one, 3,5,5-trimethyl-2-cyclopenten-1-one and 1,3,4-trimethyl-3-cyclohexene-1-carboxaldehyde together with safranal constituted more than 90% of the total volatile fraction. Isophorone derivatives are considered as key aroma compounds in saffron (Condurso, Cincotta, Tripodi, & Verzera, 2017) and, in our samples, 2,6,6-trimethyl-2-cyclohexene-1,4-dione (ketoisophorone or 4-oxoisophorone), 3,5,5-trimethyl-2-cyclohexene-1-one (α-isophorone), 3,5,5-trimethyl-3-cyclohexen-1-one (β-isophorone) and 3,5,5-trimethyl-2-cyclohexen-1-ol (isophorol) were identified. Some ionones (β-ionone, dihydro-β-ionone and dihydro-β-ionol) were also identified indicating that stigmas are matured as reported by Condurso et al. (2017).

The high values obtained for the standard deviation (SD%) highlight the high variation of the volatile compounds in the saffron samples analysed, in particular, 3,5,5-trimethyl-2-cyclopenten-1-one, 1,3,4-trimethyl-3-cyclohexene-1-carboxaldehyde and hexanal presented a high variation among samples (Table 4). Not all the detected volatile compounds were present in all samples, they were absent and/or below the limit of detection of the analysis. The relative percentage area of the detected volatile compounds for each sample was reported in Table S2 (*see Supplementary Material,* Table S2). To evaluate the efficiency of the discrimination among origin, age and process, a supervised multivariate discriminant analysis was applied by using the results of the VOCs fraction grouped in six different ways: all the detected VOCs, only the main ones, and other 4 groups including the aldehydes, the ketones, the alcohols and the hydrocarbons (*see Supplementary Material,* Table S3 *for the labels of saffron detected VOCs by GC-MS)*.

The dataset was represented by the 30 samples described in Table 1. The following supervised classes used to build the models were the following: origin (Italian or non-Italian), age (aged: year of

Table 4
Statistical analysis of the SPME-GC-MS results of the main VOCs detected in the saffron samples. GC-MS data are reported as relative percentage on the total area. D = detected VOC in the sample; ND = below the limit of detection of the GC-MS analysis.

	D	ND	GC-MS area (% on total)			
			Max	Min	Average	SD%
3,5,5-trimethyl-2-cyclopenten-1-one	17	13	29.21	0.02	1.79	396
2,2,5,5-tetramethyl-3-cyclopenten-1-one	15	15	0.24	0.03	0.13	42
3-Buten-2-one 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)	16	14	0.45	0.02	0.08	133
3,5,5-trimethyl-2-cyclohexen-1-ol	15	15	0.65	0.06	0.21	80
1,3,4-trimethyl-3-cyclohexene-1-carboxaldehyde	15	15	11.49	0.03	1.21	239
2,5-dimethyl-benzaldehyde	17	13	0.49	0.07	0.23	60
4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-Butanone	16	14	0.24	0.01	0.11	59
2,6-dimethyl-2,5-heptadien-4-one	15	15	0.95	0.25	0.52	47
2-methyl-5-(1-methylethyl)-2,5-cyclohexadiene-1,4-dione	17	13	0.41	0.03	0.13	66
3,5,5-trimethyl-3-cyclohexen-1-one	24	6	3.48	0.01	0.78	139
2,5,5-trimethyl-1-hexen-3-yne	22	8	1.02	0.07	0.40	65
2,4,5-trimethyl-benzaldehyde	26	4	0.46	0.06	0.16	55
Hexanal	25	5	8.49	0.01	0.42	404
3,3,6,6-tetramethyl-1,4-cyclohexadiene	26	4	3.93	0.26	1.37	82
2,6,6-trimethyl-1,4-cyclohexadiene-1-carboxaldehyde	28	2	6.71	1.34	3.11	53
3,5,5-trimethyl-2-cyclohexen-1-one	28	2	39.50	0.26	4.30	189
2,6,6-trimethyl-2-cyclohexene-1,4-dione	28	2	3.03	0.06	0.67	109
2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde	30	0	96.75	22.80	87.51	16

Table 5
PLS-DA statistical data obtained by using GC-MS dataset. Samples classified based on A) origin B) age C) process. The GC volatile compounds detected in the headspace were divided into six different groups: all, major compounds, alcohols, aldehydes, hydrocarbons and ketones. Root Mean Squared Error in Calibration (RMSEC) and Root Mean Squared Error Cross Validation (RMSECV).

	GC	GC	GC	GC	GC	GC
	total	*main VOCs*	*alcohols*	*aldehydes*	*hydrocarbons*	*ketones*
A) Origin						
Samples	30	30	30	30	30	30
Variables	36	18	11	12	9	11
Classes	2	2	2	2	2	2
component in model	3	9	10	9	2	6
explained variance (%)	40	93	100	92	94	86
RMSEC	0.07	0.13	0.2	0.13	0.27	0.15
RMSECV	0.2	0.25	0.38	0.17	0.25	0.29
B) Age						
Samples	30	30	30	30	30	30
Variables	36	18	11	12	9	11
Classes	2	2	2	2	2	2
component in model	1	9	2	5	2	7
explained variance (%)	17	93	30	67	39	90
RMSEC	0.19	0.07	0.16	0.16	0.16	0.1
RMSECV	0.22	0.16	0.32	0.29	0.19	0.2
C) Process						
Samples	30	30	30	30	30	30
Variables	36	18	11	12	9	11
Classes	2	2	2	2	2	2
component in model	1	98	3	5	1	7
explained variance (%)	17	98	39	67	29	90
RMSEC	0.25	0.05	0.3	0.19	0.32	0.1
RMSECV	0.45	0.4	0.33	0.2	0.63	0.22

production < 2015 or fresh: year of production ≥2015); drying intensity (high temperature: > 45 °C or medium-low temperature ≤45 °C). Based on this classification scheme, eighteen PLS-DA models were built. Data were linearly normalized and then autoscaled (zero mean and unitary variance) before analysis. Table 5 shows the calculated statistical parameters of the results obtained by the eighteen PLS-DA models.

A very good discrimination of the origin was obtained using only aldehydes as variables having classification errors of 0.13 and 0.17, respectively RMSEC and RMSECV, with 92% of explained variance. The contribution of the aldehydes to the sample discrimination could be evaluated considering the position of the loadings with respect to the scores in the first two latent variables space (*see Supplementary Material,* Fig. S1a). The 1,3,4-trimethyl-3-cyclohexene-1-carboxaldehyde and octanal contributed significantly to the separation of non-Italian and Italian samples. Remarkably, the octanal greatly contributed in spreading the GR saffron sample from the non-Italian class. The majority of the Italian saffron samples were influenced by 2,4,5-trimethyl-benzaldehyde, 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde and its isomer along with 2,6,6-trimethyl-2-cyclohexene-1-carboxaldehyde (cyclocitral), 4-(1-methylethyl)-benzaldehyde. Italian samples were lightly scattered on the second latent variable by the contribution of 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde, propanal, 2-ethylidene-6-methyl-3,5-heptadienal and hexanal. Some of these detected volatile compounds have been previously reported in other studies on the saffron aroma composition (Cadwalleder, 2001; D'Auria et al., 2006; Condurso et al., 2017; Karabagias et al., 2017).

The 2,4,5-trimethyl-benzaldehyde compound, also reported by D'Auria et al. (2006), contributes in the discrimination of the Italian samples to the non-Italian ones; it is present with a higher content in IND and IR1 samples. Karabagias et al. (2017) demonstrated the contribution of the latter volatile compound on the total volatile fraction of saffron from Greece, Iran, Spain and Morocco.

When the process was taken as criterion of classification, the aldehydes were the only variables having the RMSECV of about 0.2 (*see Supplementary Material,* Fig. S1b). In this case, 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde was the most important variable in discriminating the hard treatment from the mild one. Results of the classification on process indicated the possibility to consider some saffron volatile compounds (1,3,4-trimethyl-3-cyclohexene-1-carboxaldehyde; octanal and 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) belonging from aldehyde group as indicators of origin and drastic drying conditions. When the "age" of production of the saffron samples was accounted for, the data elaboration showed a low prediction errors for main VOCs and ketones groups (RMSECV between 0.16 and 0.2) with an explained variance of ca. 90%. It was possible to use the main volatile compounds, in particular 2-methyl-5-(1-methylethyl)-2,5-cyclohexadiene-1,4-dione and 1,3,4-trimethyl-3-cyclohexene-1-carboxaldehyde compounds, to discriminate the older storage samples. In the fresh samples, the important variables were 3,3,6,6-tetramethyl-1,4-cyclohexadiene, hexanal and 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (safranal isomer), while 2,6,6-trimethyl-2-cyclohexene-1,4-dione, known as 4-ketoisophorone, contributed to the spreading of the fresh samples class (*see Supplementary Material,* Fig. S1c). For the aged samples, 1,3,4-trimethyl-3-cyclohexene-1-carboxaldehyde, 3,5,5-trimethyl-2-cyclohexen-1-ol (isophorol), 2,6,6-trimethyl-2-cyclohexene-1,4-dione and 3,5,5-trimethyl-2-cyclopenten-1-one were the most important variables. Remarkably, the 3,5,5-trimethyl-3-cyclohexen-1-one (β-isophorone) greatly contributed in spreading the 18M saffron sample; it is reported to significantly decrease its content from the first year compared to the other years of storage (Maggi et al., 2010). This compound is important in the generation of other volatile compounds during storage as reported by Carmona et al. (2006). It is important to note that the class of the main volatile compounds is characterized by a high number of ketone compounds.

3.4. E-nose analysis

A preliminary optimisation of the E-Nose analysis parameters was carried out to find the best condition for the measurement. To have reproducible measurement conditions (i.e. different Relative Humidity) an initial step of air removal from the sample headspace through the flowing of nitrogen in the sample aluminium basket (pre-opening) was set.

Each AuNP-peptide sensor of the array presents a different adsorption kinetic and thus exhibits a specific response in terms of the ΔF Max intensity (maximum difference in frequency achieved) as well as of the frequency referred to the signal at the end of the measurement. Robustness of the measurement was tested varying the following parameters: temperature in the 25–40 °C range, amount of sample (10–100 mg) and flow rate of carrier gas (3–4 L/h). The inter-day variability of the sensor response was taken over 6 days using the same batch of selected samples; the CV % were in the 2–13% range for most of the sensors (*see Supplementary Material,* Table S4 *for the ΔF response of peptides modified sensors arrays (QCMs) for selected saffron sample*).

The E-Nose data obtained from measurements under optimised and standardised conditions were processed with PLS-DA algorithm in order to discriminate saffron samples based on origin, drying and age. The analyses were carried out only on a set of selected saffron samples to evaluate the possibility to use this innovative E-Nose system as additional tool and complementary to conventional analytical techniques for saffron authenticity.

For this PLS-DA, the three classification criteria were also origin,

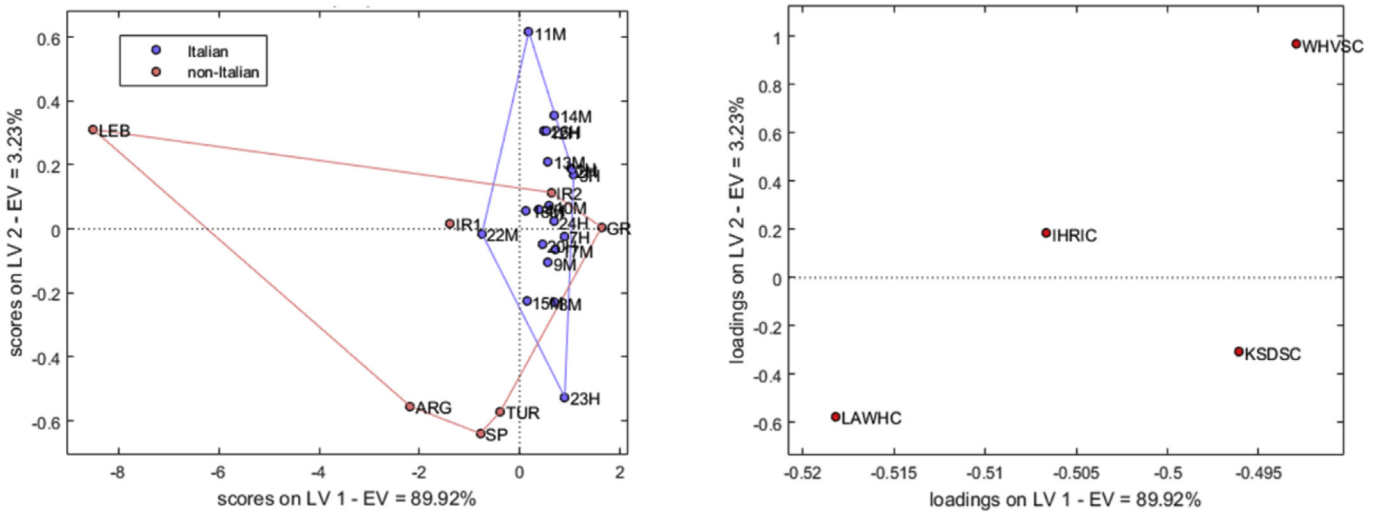

Fig. 3. Score and loading plot of PLS-DA model built to classify 27 saffron samples using E-Nose data. The two saffron classes are marked with different colour: blue for Italian samples; red for the non-Italian saffron samples. Data have been linearly normalized and autoscaled (zero mean and unitary variance). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

age and process and the classes were the same applied in the GC multivariate analysis. Fig. 3 reports the PLS-DA graphical representation of the origin classification criterion, respectively for the first two latent variables representing the full-explained variance captured by the regression model.

All variables showed a similar behavior in the discrimination on the first latent variable (89.92% of the explained variance) contributing to the discrimination of the two origin classes. In particular, LAWHC had a strong influence in the separation of the LEB sample from the others within the non-Italian class. The second latent variable (3.23% of the explained variance) increased the spread within both classes, on this latent variable the peptides WHVSC and IHRIC had an opposite position compared to LAWHC and KSDSC. The saffron samples from Iran and Greece resulted similar to the Italian ones with no a clear discrimination.

The statistical summary results of the PLS-DA analysis carried out with the AuNP-peptide based sensors array are reported in Table 3. The sensors array showed a good discrimination for saffron samples only based on origin with RMSECV of ca. 0.81of the samples correctly assigned in cross validation (CV), while the results did not allow to discriminate them based on age and drying process.

The use of electronic nose to evaluate the quality authentication of saffron samples has been already experienced. Some authors report the use of Metal Oxide Semiconductor (MOS) sensors (Heidarbeigi et al., 2015; Kanakis et al., 2004; Kiani et al., 2016) and demonstrated the possibility to discriminate the volatile profiles of saffron samples produced in different countries and also the non-adulterated and adulterated samples.

The GC-MS (only the class of the “main volatile” compounds) and E-Nose data were also compared by using Pearson correlation. Results (*see Supplementary Material,* Table S5) showed no clear correlation between the main detected volatile compounds and gas sensors, but the E-Nose sensors Au peptides IHRIC, WHVSC, KSDSC and LAWHC were closely related to each other that means they detected almost the same aroma profile. These gas sensors could detect not only the main saffron volatile compound, but also the total contribution of aldehydes.

It should be pointed out that the measurement using the whole array of sensors represents the fingerprint profile of volatiles present in the headspace of the sample and, thus any single volatile compound gives its contribution on each sensor depending on the selectivity and sensitivity of the sensing material deposited on the sensor. Thus, considerations on the performance of the set of sensors *vs.* each single volatile compound added are not straightforward.

4. Conclusion

Conventional (IRMS and SPME-GC-MS) and non-conventional (E-Nose) analytical techniques were applied to evaluate the feasibility to discriminate saffron samples based on geographical origin, storage and drying conditions. Data results were processed by means of Partial Least Squares Discriminant Analysis.

All techniques employed in this study were able to classify saffron based on origin of stigmas from *Crocus sativus* L., cultivated in Italy or other countries. The IRMS, with the analysis of the stable isotopes of carbon and nitrogen, has proved to be a very reliable method in discriminating the geographical origin of saffron. As regards the volatile compounds pattern, the SPME-GC-MS technique revealed that, within all detected volatile compounds, aldehydes pattern could be used to classify the origin. As alternative way, also the peptide gas sensors array was applied as additional complementary analysis method to characterise complex aroma patterns, and it could be use as tool to recognize the traceability of saffron samples. No clear correlation between the detected volatile compounds and gas sensors was found. Each gas sensor had very similar response, influenced not only by the main volatile compound, safranal, but also by the total contribution of aldehydes. Instead, more difficult has been to find some potential indicators to be used to mark the drying treatment and storage. Only the SPME-GC-MS analysis can be used with these purposes. When the process was taken as criterion of classification, some saffron volatile compounds belonging from aldehyde group showed good values of classification, while the main volatile compounds discriminated the saffron age.

In conclusion, the present study contributes to routine food quality control, for the characterization and discrimination of saffron samples with different origin, process and age conditions using conventional and non-conventional techniques. It can be interesting to develop new gas sensors to be used as E-Nose system in order to collect other minor volatile compounds of saffron aroma and investigate their contribution on the discrimination.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2019.106736.

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