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PTR-TOF-MS and HPLC analysis in the characterization of saffron (*Crocus sativus* L.) from Italy and Iran

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ABSTRACT

Saffron samples from Italy and Iran were analyzed for their content in aroma and bioactive compounds with different analytical techniques. HPLC was used for the identification and quantification of crocins, picrocrocin, safranal and flavonoids content, while the novel proton transfer reaction time-of-flight mass spectrometer was employed for the aroma compounds analysis. Italian saffron turned out to be richer in total crocins and safranal contents. Sample characterization was performed with an unsupervised statistical approach; tests involving different numbers of parameters deriving from the two analytical techniques were performed. The results achieved showed that the best samples classification was obtained by joining the information acquired from both techniques; following such an approach, a sharper separation between Iranian and Italian samples was achieved. Finally, among the variables that most contribute to the description of variability, isophorone, safranal and picrocrocin were identified to be the most significant.

1. Introduction

The dried, red stigmas of *Crocus sativus* L. (saffron) are a very expensive spice known as saffron, which is used as a food flavoring, a coloring agent and as a traditional herbal medicine (Xi & Qian, 2006). The postharvest dehydration process is necessary to convert *C. sativus* stigma into the saffron spice.

Iran is the main saffron producer in the world. A few Iranian eastern and south eastern provinces glean the bulk of modern global production. In 2005, the amount of saffron produced in Iran was 230 tons which constituting 93.7% (82% being exported) of the world saffron production; the second-ranked Greece produced 5.7 tons (5700 kg), while Morocco and Kashmir, tied for third rank, each produced 2.3 tons (2300 kg) (Ghorbani, 2008). It should be underlined that Iranian saffron is less expensive (it costs up to 5 times less than Italian saffron). Saffron quality is determined by its color, taste, and aroma, which depend on many factors such as soil, climate, rainfall, harvest time, and finally postharvest treatments (Bolandi & Ghoddusi, 2006; Carmona et al., 2005; Carmona et al., 2007).

The sites of saffron cultivation are often very small and the product that is obtained is related to the peculiar geographic area, therefore the importance of the characterization of this spice must be pointed out (Alonso, Salinas, Garijo, & Sanchez-Fernandez, 2001; Anastasaki et al., 2009).

The compounds that are responsible of saffron color and taste are crocins, which are glycoside derivatives of crocetin, picrocrocin (mainly responsible for the bitter taste), and safranal (monoterpene aldehyde). Safranal is formed by hydrolysis from picrocrocin during drying and storage (Del Campo et al., 2010; Maggi et al., 2010).

Furthermore, there are compounds that are regarded as pharmacologically active such as crocin derivatives (Li, Lin, Kwan, & Min, 1999; Rios, Recio, Giner, & Manez, 1996) and flavonoids. Many papers deal with analytical aspects to set up methods for the separation and determination of the biological active compounds (Alonso et al., 2001; Li et al., 1999; Pfander & Rychener, 1982; Tarantilis, Polissiou, & Manfait, 1994; Tarantilis, Tsoupras,

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& Polissiou, 1995), and aroma components (Carmon et al., 2006; Carmona et al., 2007; D'Auria, Mauriello, Racioppi, & Rana, 2006; Loskutov, Beninger, Hosfield, & Sink, 2000; Lozano, Delgado, Gomez, Rubio, & Iborra, 2000; Tarantilis & Polissiou, 1997).

The aim of this research is to study both aroma and bioactive compounds in order to improve saffron characterization with the use of techniques such as high-performance liquid chromatography (HPLC) and proton transfer reaction time-of-flight mass spectrometer (PTR-TOF-MS). PTR-TOF-MS is a non-invasive technique which allows the achievement of the whole mass spectra with a time of resolution inferior to 1 s and the detection of high molecular weight molecules with a high resolution power (Cappellin et al., 2010). Therefore, the PTR-TOF-MS technique, combining high sensitivity, good precision, accuracy and very poor fragmentation of the volatile molecules, can be used for fingerprinting purposes as well as for detailed investigation of single compounds. Indeed, this tool has already been used previously to assess the aromatic profile of various agricultural products, such as apple (Soukoulis et al., 2013), hot pepper (Taiti et al., 2014), olives (Masi, Romani, Pandolfi, Heimler, & Mancuso, 2014) and tropical fruits (Taiti et al., 2015).

With the two techniques we compared saffron from Gonabad, Torbat and Ghaen (Razavi Khorasan and south Khorazan regions) in Iran and from Città della Pieve, Cascia and Fiesole (Umbria and Tuscany) in Italy.

2. Materials and methods

2.1. Saffron samples

The analyzed saffron samples were obtained from 6 geographic origins, three in Italy (Fiesole, Cascia, Città della Pieve) and three in Iran (Gonabad, Torbat, Ghaen).

2.2. HPLC analysis

Saffron stigmas (50 mg) were extracted with 10 mL of 70% ethanol, adjusted to pH 2.0 with formic acid for one night and then filtered to eliminate plant residues.

These extracts were analyzed by HPLC/DAD/MS for the determination of saffron components.

Authentic standard of safranal was purchased from Sigma–Aldrich (St. Louis, USA), and cinnamic acid, quercitrin and curcumin were purchased from Extrasynthèse S.A. (Lyon, France). All solvents were of HPLC grade purity (BDH Laboratory Supplies, United Kingdom).

Analysis for polyphenols were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector and managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA), and were separated by using a 250 × 4.6 mm i.d. 5 μ Luna C18 column (Phenomenex) operating at 25 °C. UV/Vis spectra were recorded in the 190–600 nm range and the chromatograms were acquired at 280, 330, 350 and 440 nm. The mobile phase was a two-steps linear solvent gradient system, starting from 90% H_2O (adjusted to pH 3.2 by HCOOH) up to 100% CH_3CN during a 40-min period, flow 0.8 mL min^{-1}. Quantification of individual compounds was directly performed by HPLC/DAD using a five-point regression curve ($r^2 = 0.998$) in the range 0–30 mg on the basis of authentic standards. In particular, crocin derivatives were determined at 440 nm using curcumin as reference compound. Flavonols were determined at 350 nm using quercitrin as reference compound, picrocin was determined at 280 nm using cinnamic acid as reference compound and safranal was determined at 308 nm using safranal as reference compound. In all cases, actual concentrations of the derivatives were calculated after applying, were possible, corrections for differences in molecular weight. The identity of polyphenols was ascertained using data from HPLC/DAD analyses by comparison and combination of their retention times and UV/Vis spectra with those of authentic standards and previously reported data (Vignolini et al., 2008).

2.3. PTR-TOF-MS analysis

Volatile organic compounds (VOCs) emitted from all saffron samples were analyzed with a PTR-TOF-MS 8000 (IoniconAnalytik GmbH, Innsbruck, Austria) using H_3O^+ as reagent ion for the proton transfer reaction. The reaction takes place between H_3O^+ ions and all the biogenic or anthropogenic VOCs having a proton affinity higher than that of water (165.2 kcal mol^{-1}). Separation of single ions happens accordingly to their mass to charge (m/z) ratio. Drift applied voltage was set at 600 V, temperature at 110 °C, pressure 2.25 mbar, extraction voltage at the end of the tube Udx = 32 V; this resulted in a field density ratio (E/N) of about 140 Td (E being the electric field strength and N the gas number density; 1 Td = 10^{17} V cm^2).

2.4. Treatment of PTR-TOF-MS samples

Saffron samples, all previously stored in a cool dark room (15 °C), were brought to room temperature (22–23 °C). For each sample (each analyzed in triplicate), about 1 mg of stigmas were transferred to a glass jar (volume = 10 mL, exposed surface = 6 cm^2); the jar lid was fitted with Teflon inlet and outlet tubes which were, respectively, connected to a zero-air generator and to the PTR-TOF-MS system. Samples were then equilibrated at 50 °C in a water bath. The conditions of setup and incubation allowed the formation of a dynamic headspace sampling system with a constant air flow of 0.3 L min^{-1} and a constant humidity, which are critical parameters in for VOCs determination (Mancuso et al., 2015).

The VOCs in the headspace were measured by direct injection into the PTR-TOF drift tube inlet for 120 s. Preliminary measurements on an empty jar were run before every sample measurement and used for background subtraction.

2.5. PTR-TOF-MS spectra analysis

Raw data (count rate of the analytes recorded in number of counts per second, *cps*) were acquired with TofDaq software (Tofwerk AG, Switzerland), using a dead time of 20 ns for the Poisson correction. In order to guarantee high mass accuracy throughout the analysis study, the mass scale was calibrated following the peaks of known components, present in the spectra at any time (NO^+ peak, $m/z = 29.99$, the main isotope of acetone, $C_3H_7O^+$, $m/z = 59.05$, and safranal, $C_{10}H_{15}O^+$, m/z 151.22) (Cappellin et al., 2010). All data from each replicate and background signal were normalized, according to Jardine et al. (2010), by the primary ion signal (cps to *n*cps). For all acquisitions, average spectra considering 50 recorded spectra (corresponding to 50 consecutive seconds of analysis) were obtained and, for a better comparison between samples, data were normalized to sample mass (expressed in grams). Moreover, VOCs were acquired in the range of $m/z = 30–250$ using a high mass accuracy for their identification.

2.6. Statistical data analysis

Principal component analysis (PCA) was used as unsupervised multivariate technique to represent and explore samples and variables correlations. For the PCA analysis, the average spectra of the three replicates of each saffron sample were used. PCA is a mathematical tool used to reduce the variability of complex data set, generating a relative small number of new descriptors

(principal components, PCs) accordingly to the correlation between the original variables. Missing data were estimated by using the mean of the corresponding variables. Pearson's correlation coefficient *r* as a statistical measure of the strength of a linear relationship between paired variables was also calculated. The same coefficient was used to perform hierarchical cluster analysis in order to group samples with similarities and mean linkage clustering was used to draw a dendrogram. The software used to perform statistical analysis was Addinsoft XLSTAT (Ver. 2014.2.04).

3. Results and discussion

3.1. HPLC analysis

The first datum that should be taken into account is the significantly higher ($p < 0.05$) total crocins content in Italian samples with respect to Iranian ones (Table 1). The crocins, a family of red-colored and water-soluble carotenoids, are glycosyl esters of crocetin with different sugar moieties, such as glucose, gentiobiose, neapolitanose or triglucose; they can be present in saffron in *cis* and *trans* isomeric forms. Total crocins content of Italian samples is generally higher than the values reported for Moroccan saffron samples (Lage & Cantrell, 2009). The most abundant compounds were *trans*-crocin 4 and *trans*-crocin 3, with a significant difference ($p < 0.05$) between Italian and Iranian origins. Both compounds accounted for 91% of total crocins content in the case of Italian samples and for 78% in the case of Iranian ones. These two compounds have been already described, in a study on saffron from 10 different countries (Caballero-Ortega, Pereda-Miranda, & Abdullaev, 2007), as the most abundant, even if quantitative data could not be compared since these data were reported as mg/g of stigmas. *Cis*-crocin 4 and *trans*-crocin 2 contents were higher in Iranian than in Italian samples ($p < 0.05$). Concerning safranal, its content was significantly ($p < 0.05$) more abundant in Italian provenances. No picrocrocin and 2,6,6-trimethyl-4-hydroxy-1-car boxaldehyde-1-cyclohexene (HTCC) were found; saffron dehydration process involves the formation of safranal from HTTC and picrocrocin (Boschetti et al., 1999).

As regards flavonoids, three kaempferol derivatives were identified according to previous findings (Carmona et al., 2007; Vignolini et al., 2008), and one compound, also a kaempferol derivative, has not been characterized. Significant differences ($p < 0.05$) have been found only in the case of kaempferol-3-sophor iside-7-glucoside. Among Italian samples both crocins and flavonoids contents were highest in the Fiesole provenance and, in the case of Iranian samples, the Torbat provenance showed the highest crocins content.

3.2. PTR-TOF-MS analysis

Table 2 shows all masses highlighted by PTR-TOF-MS analysis present in the six saffron samples, and their possible identification, taking into account the available fragmentation patterns of pure standards (Diskin, Wang, Smith, & Španěl, 2002; Fujii, Selvin, Sablier, & Iwase, 2001; Lozano, Castellar, Simancas, & Iborra, 1999; Mayr, Märk, Lindinger, Brevard, & Yeretzian, 2003; Wang, Španěl, & Smith, 2004). All masses listed have already been identified in saffron stigmas. An equal number to any possible compound having the same protonated theoretical mass was attributed. In

Table 1
Crocins, picrocrocin, safranal and flavonoids content (mg/g) of stigmas (HPLC data). Different crocin derivatives have been highlighted with roman numerals. Data are mean values of three determinations, standard deviation within brackets (n.d. = not determined; K = kaempferol).

	Compound	Iran			Italy		
		Gonabad	Torbat	Ghaen	Città della Pieve	Cascia	Fiesole
Crocins							
H1	*trans*-Crocin 5	1.63 (0.049)	1.98 (0.098)	1.61 (0.061)	1.13 (0.031)	1.14 (0.054)	1.52 (0.044)
H2	Crocin derivative I	1.05 (0.097)	1.67 (0.108)	1.30 (0.112)	2.22 (0.113)	2.62 (0.080)	2.72 (0.053)
H3	Crocin derivative II	0.66 (0.035)	0.51 (0.059)	0.65 (0.034)	0.45 (0.028)	0.41 (0.048)	0.49 (0.013)
H4	Crocin derivative III	0.92 (0.031)	0.90 (0.026)	1.17 (0.035)	1.16 (0.018)	1.50 (0.062)	1.58 (0.023)
H5	Crocin derivative IV	0.53 (0.062)	0.51 (0.049)	0.52 (0.019)	0.35 (0.031)	0.22 (0.021)	0.51 (0.005)
H6	*trans*-Crocin 4	168.91 (1.356)	238.02 (0.964)	197.80 (0.897)	302.65 (1.974)	343.97 (1.957)	372.49 (0.681)
H7	Crocin derivative I	1.32 (0.009)	2.18 (0.012)	1.69 (0.036)	2.71 (0.022)	2.66 (0.134)	2.75 (0.014)
H8	Crocin derivative II	1.19 (0.035)	2.44 (0.056)	1.30 (0.028)	0.98 (0.052)	1.01 (0.041)	1.03 (0.0392)
H9	*trans*-Crocin 3	61.25 (0.485)	85.36 (0.531)	71.56 (0.68)	109.17 (0.605)	111.94 (0.893)	123.15 (1.0407)
H10	Crocin derivative I	0.40 (0.027)	0.51 (0.021)	0.39 (0.035)	0.66 (0.013)	0.58 (0.040)	0.67 (0.029)
H11	Crocin derivative II	1.71 (0.012)	1.41 (0.010)	1.64 (0.028)	1.37 (0.018)	1.43 (0.141)	0.71 (0.011)
H12	*trans*-Crocin 2′	1.64 (0.091)	3.63 (0.158)	2.53 (0.164)	3.29 (0.146)	2.81 (0.173)	1.23 (0.012)
H13	Crocin derivative I	0.53 (0.035)	0.51 (0.049)	0.52 (0.041)	0.41 (0.033)	0.37 (0.048)	2.38 (0.0437)
H14	Crocin derivative II	0.13 (0.015)	0.26 (0.020)	0.26 (0.012)	0.55 (0.051)	0.71 (0.059)	0.47 (0.012)
H15	Crocin derivative III	n.d.	n.d.	n.d.	n.d.	0.32 (0.042)	0.51 (0.012)
H16	Crocin derivative IV	n.d.	n.d.	n.d.	n.d.	n.d.	0.24 (0.015)
H17	*cis*-Crocin 4	30.42 (1.521)	19.38 (0.851)	26.88 (0.862)	5.73 (0.053)	7.37 (0.390)	12.55 (0.606)
H18	Crocin derivative I	0.79 (0.061)	0.77 (0.072)	0.65 (0.048)	0.41 (0.034)	0.51 (0.058)	0.62 (0.013)
H19	Crocin derivative II	0.53 (0.026)	0.38 (0.027)	0.52 (0.049)	0.30 (0.022)	0.44 (0.057)	0.38 (0.009)
H20	*trans*-Crocin 2	26.00 (0.58)	24.30 (0.69)	24.86 (0.41)	16.12 (0.352)	13.59 (0.172)	21.24 (0.457)
H21	Crocin derivative I	0.40 (0.024)	0.38 (0.018)	0.39 (0.011)	n.d.	n.d.	n.d.
H22	*cis*-Crocin 1	1.58 (0.087)	2.22 (0.091)	1.73 (0.103)	1.06 (0.090)	0.81 (0.077)	1.48 (0.032)
H23	Crocin derivative I	0.53 (0.048)	0.51 (0.039)	0.52 (0.061)	n.d.	n.d.	n.d.
H24	Crocin derivative II	0.40 (0.022)	0.38 (0.035)	0.39 (0.029)	n.d.	n.d.	0.12 (0.011)
H25	Total	**302.51**	**388.23**	**338.87**	**450.73**	**494.42**	**548.84**
H26	Picrocrocin	36.97 (0.031)	67.95 (0.025)	43.82 (0.029)	101.92 (0.783)	127.83 (0.881)	130.35 (1.026)
H27	Safranal	1.26 (0.091)	1.79 (0.126)	1.35 (0.108)	2.41 (0.103)	3.01 (0.298)	2.01 (0.134)
Flavonoids							
H28	K-3-sophoroside-7-glucoside	2.64 (0.015)	2.95 (0.022)	2.99 (0.030)	4.89 (0.026)	4.17 (0.080)	5.18 (0.038)
H29	K derivative	1.05 (0.041)	0.90 (0.029)	1.30 (0.022)	1.07 (0.040)	1.24 (0.038)	1.35 (0.043)
H30	K-3,7,4′-triglucoside	2.51 (0.015)	2.95 (0.016)	2.21 (0.027)	n.d.	n.d.	1.53 (0.039)
H31	K-3-sophoroside	10.02 (0.064)	10.38 (0.061)	8.83 (0.068)	8.39 (0.059)	5.11 (0.063)	9.61 (0.087)
H32	Total	**16.22**	**17.18**	**15.33**	**14.35**	**10.52**	**17.67**

two cases (P11 and P17), different protonated theoretical masses of putative compounds have been associated: in both cases, the system detects only one peak due to the small differences in their *m*/*z*. Other masses that were expected to be found were not actually present in the saffron samples studied. Interestingly, mass identified as 4-hydroxy-2,6,6-trimethyl-1-cyclohexen-1-carboxal dehyde (HTCC), the aglycone precursor of safranal, was not present in any of the samples, indicating quality in the transformation (dehydration) process, thus confirming the data from HPLC. Furthermore, only five fragments have been detected (Tarantilis & Polissiou, 1997), according to the low fragmentation characteristic of the PTR-TOF-MS analysis.

With regard to the content of safranal in the samples, the PTR-TOF-MS approach also underlined great differences between saffron from different geographic origins. Two samples from Italy (Cascia and Città della Pieve) showed to be the richest; two from Iran (Ghaen and Gonabad) had the lowest amount of safranal, while the saffron from Fiesole (Italy) and that from Torbat (Iran) had intermediate amounts (see Fig. 1).

3.3. Principal component analysis on PTR-TOF-MS data

The analysis performed on the PTR-TOF-MS mass spectra of the headspace of the six saffron under study allowed the compilation

Table 2
Masses highlighted by PTR-TOF-MS analysis found in the six saffron samples, their possible identification, theoretical and measured mass. Other masses already described in saffron, but not detected by PTR-TOF-MS analysis in this study are also listed (marked with an asterisk).

	Compound	Protonated formula	Protonated mass (*M*/*Z*)		Refs.
			Theor.	Meas.	
P1	Acetic acid	$C_2H_5O_2^+$	61.05	61.03	4,5
P2	2(5H)-furanone	$C_4H_5O_2^+$	85.07	85.03	2,5
P3	3-Methylbutanal	$C_5H_{11}O^+$	87.13	87.08	2,5
P3	2-Methylbutanal	$C_5H_{11}O^+$	87.13	87.08	2,5
P4	1-Pentanol	$C_5H_{13}O^+$	89.15	89.10	2,5
P5	Phenol	C_6H7O^+	95.06	95.05	6
P6	Hexanal	$C_6H_{13}O^+$	101.16	101.10	2,5
P7	Benzaldehyde	$C_7H_7O^+$	107.12	107.05	2
P8	1,1,3-Trimethylcyclopentadiene	$C_8H_{13}^+$	109.18	109.10	2
P9	2,5-Dimethyl-2,4-hexadiene	$C_8H_{15}^+$	111.20	111.11	2,5
*	Heptanal	$C_7H_{15}O^+$	115.19		2,5
P10	1,3,5-Trimethylbenzene	$C_9H_{13}^+$	121.19	121.10	2,5
P11	2-Phenylethanol	$C_8H_{11}O^+$	123.16	123.08	2,3,5
P11	1-t-Butylcyclopentadiene	$C_9H_{15}^+$	123.21	123.12	2,5
P12	6-Methyl-3,5-heptadien-2-one	$C_8H_{13}O^+$	125.18	125.10	2,5
P13	2-Hydroxy-5-cyclohexen-1,4-dione	$C_6H_7O_3^+$	127.11	127.04	3
P14	6-Methyl-5-hepten-2-one	$C_8H_{15}O^+$	127.19	127.11	2,5
P15	Octanal	$C_8H_{17}O^+$	129.21	129.13	2,5
P16	β-Phellandrene	$C_{10}H_{17}O^+$	137.24	137.13	2,5
P17	5,5-Dimethyl-2-cyclohexen-1,4-dione	$C_8H_{11}O_2^+$	139.16	139.08	2,5
P17	3,5,5-Trimethyl-3-cyclohexen-1-one	$C_9H_{15}O^+$	139.21	139.11	3,5
P17	3,5,5-Trimethyl-2-cyclohexen-1-one (isophorone)	$C_9H_{15}O^+$	139.21	139.11	1,3–5
P17	3,5,5-Trimethylcyclohex-3-en-1-one	$C_9H_{15}O^+$	139.21	139.11	2,5
P17	3,5,5-Trimethylcyclohexenone	$C_9H_{15}O^+$	139.21	139.11	5
P17	3,7-Dimethyl-1,6-octadiene	$C_{10}H_{19}^+$	139.21	139.15	3
*	Nonanal	$C_9H_{19}O^+$	143.24		2,5
P18	4-(1-Methylethyl)-benzaldehyde	$C_{10}H_{13}O^+$	149.20	149.10	2,5
P18	2,4,5-Trimethylbenzaldehyde	$C_{10}H_{13}O^+$	149.20	149.10	2,5
P19	2,6,6-Trimethyl-1,4-cyclohexadien-1-carboxaldehyde	$C_{10}H_{15}O^+$	151.22	151.11	3,5
P19	2,6,6-Trimethyl-1,3-cyclohexadien-1-carboxaldehyde (safranal)	$C_{10}H_{15}O^+$	151.22	151.11	2–5
P19	4-Methylene-3,5,5-trimethyl-2-cyclohexen-1-one	$C_{10}H_{15}O^+$	151.22	151.11	3
P19	4,6,6-Trimethyldicyclo-[3.1.1]hept-3-en-2-one	$C_{10}H_{15}O^+$	151.22	151.11	3
P20	2-Hydroxy-2,6,6-trimethyl-2-cyclohexen1,4-dione	$C_9H_{13}O_2^+$	153.19	153.09	1–4
P20	2-Hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one	$C_9H_{13}O_2^+$	153.19	153.09	1–4
P20	3,5,5-Trimethylcyclohex-2-en-1,4-dione	$C_9H_{13}O_2^+$	153.19	153.09	5
P21	3,7-Dimethyl-1,6-octadien-3-ol (linalool)	$C_{10}H_{19}O^+$	155.15	155.14	3
P22	2,2-Dimethyl-4-oxocyclohexan-1-carboxaldehyde	$C_9H_{15}O_2^+$	155.21	155.11	3
P22	2,6,6-Trimethylcyclohexan-1,4-dione	$C_9H_{15}O_2^+$	155.21	155.11	1–3
P22	4-Hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one	$C_9H_{15}O_2^+$	155.21	155.11	4
P22	2,6,6-Trimethyl-1,4-cyclohexanedione	$C_9H_{15}O_2^+$	155.21	155.11	1
P22	3,5,5-Trimethylcyclohexan-1,4-dione (2-hydroxyisophorone)	$C_9H_{15}O_2^+$	155.21	155.11	5
P23	3,3,4,5-Tetramethylcyclohexan-1-one	$C_{10}H_{19}O^+$	155.25	155.14	3
P24	2,6,6-Trimethyl-3-oxo-1-cyclohexen-1-carboxaldehyde	$C_{10}H_{15}O_2^+$	167.22	167.10	3
*	4-Hydroxy-2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde (HTCC)	$C_{10}H_{17}O_2^+$	169.27		1
P25	4-Hydroxy-2,6,6-trimethyl-3-oxo-cyclohexan-1-carboxaldehyde	$C_{10}H_{13}O_3^+$	181.20	181.09	1,3
*	4-Hydroxy-2,6,6-trimethyl-3-oxo-1,4-cyclohexadien-1-carboxaldehyde	$C_{10}H_{17}O_3^+$	185.27		3
P26	4-(2,6,6,-Trimethyl-1-cyclohexen-1-yl)-3-buten-2-one	$C_{13}H_{21}O^+$	193.30	193.16	3
P26	β-Ionone	$C_{13}H_{21}O^+$	193.30	193.16	2,5
P27	4-(2,2,6,-Trimethyl-cyclohexan-1-yl)-3-buten-2-one	$C_{13}H_{27}O^+$	195.31	195.17	3
P27	Dihydro-β-ionone	$C_{13}H_{27}O^+$	195.31	195.17	2,5
P27	(E)-6,10-Dimethyl-5,9-undecadien-2-one	$C_{13}H_{27}O^+$	195.31	195.17	2,5
P28	Dihydro-β-ionol	$C_{13}H_{25}O^+$	197.33	197.19	2,5
*	2,6-di-(1,1-Dimethylethyl)-phenol	$C_{14}H_{27}O^+$	207.14		2,5
*	2,4,4-Trimethyl-3-(3-oxo-1-butenyl)-2-cyclohexen-1-ol	$C_{13}H_{21}O_2^+$	209.15		3

1 = Alonso et al. (2001); 2 = Pfander and Rychener (1982); 3 = Tarantilis et al. (1994); 4 = Lozano et al. (2000); 5 = Jardine et al. (2010); 6 = Pujimulyani, Raharjo, Marsono, and Santoso (2013).

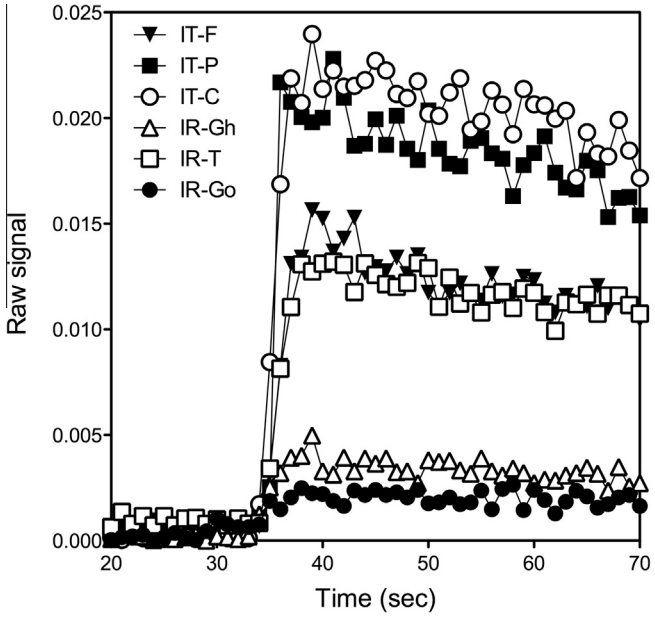

Fig. 1. Signal intensity (*n*cps) of the putative mass safranal (mass P19) in the 6 saffron samples.

of a table of 28 mass peaks (masses with intensity equal to zero *n*cps in all the samples were ignored).

The first two components obtained with PCA explained more than 75% of the total variability and the deriving two-dimensional scatter plot provided the main separation of saffron from Cascia (IT-C) and Città della Pieve (IT-P), showing them to be clearly separated from each other and from the rest of the samples (Fig. 2, *top left*). Saffron from Iran and the Italian one from Fiesole shared almost the same region of the plot, mainly in the negative range of both PCs; nevertheless, each group of replicates of the same geographic origin appeared quite distinct, especially those from Fiesole that occupied also the positive range of the PC1.

Further interesting information could be achieved from the analysis of the contribution of each original variable to the new ones generated with the PCA (Table 3, case "A"). For example, the mass tentatively identified as isophorone (P17) was one with the bigger role in the definition of the first component, that described 46.21% of total variability. This occurrence has already pointed out in saffron from Tuscan Maremma (Macchia, Ceccarini, Molfetta, Cioni, & Flamini, 2013). Isophorone is characterized by floral notes. This is in contrast with safranal, that confers to saffron its spicy characteristic aromatic notes (Maggi et al., 2009). Isophorone was abundant in saffron from Città della Pieve (IT-P), whose position in the bi-factors plot is in the positive range of PC1 (Fig. 2, *top left*). Concerning the second principal component (PC2), the compounds that mainly participate to its definition are

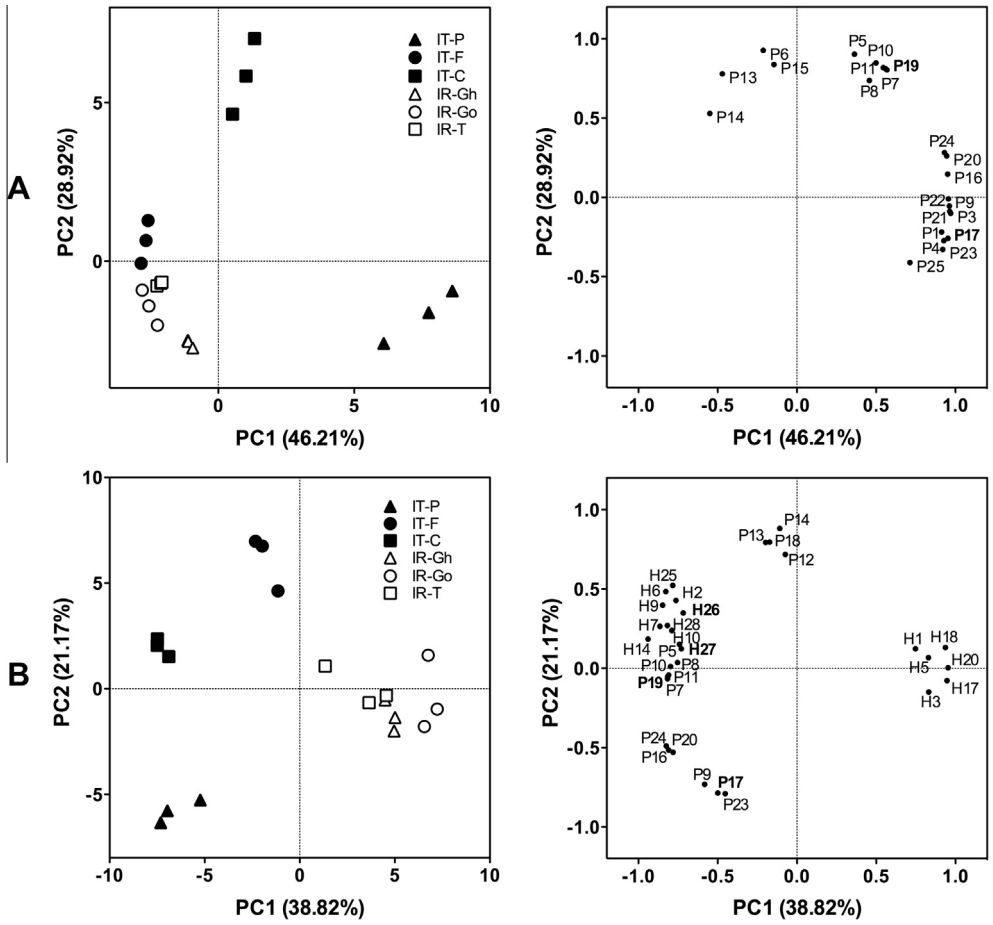

Fig. 2. (*Left*) Biplots showing the projection of the samples in the two-dimensional space (IT-C: Italy, Cascia; IT-P: Italy, Città della Pieve; IT-F: Italy, Pieve; IR-Gh: Iran, Ghaen; IR-T: Iran, Torbat; IR-Go: Iran, Gonabad). (*Right*) Correlation biplots where only variables that most contributed to the definition of variability are shown. The analysis was applied to 28 parameters (PTR-TOF-MS analysis, case "A") and to 60 parameters (28 from PTR-TOF-MS analysis and 32 from HPLC analysis, case "B"). Mass labels are listed in Tables 1 and 2; P, obtained with PTR-TOF-MS analyses; H, obtained with HPLC analyses; in bold character the most interesting variables are underlined.

mostly present in Cascia samples (IT-C), that are in the positive range of this PC. The compound tentatively identified as safranal was one of these. The compound 2(H5)-furanone (P2), known as a useful compound for fingerprinting Iranian saffron (Jalali-Heravi, Parastar, & Ebrahimi-Najafabadi, 2010; Maggi et al., 2009), did not have a significant role in the definition of the samples variability.

As underlined in the correlation plot (Fig. 2, *top right*), that shows a projection of the initial variables in the PC space, most of the variables were positioned in the positive range of PC1 and PC2, showing significant correlations ($p < 0.05$). For example, the compound tentatively identified as safranal (P19) showed to be strongly correlated with compounds identified as phenol (P5, $r = 0.971$), benzaldehyde (P7, $r = 0.999$), 1,3,5-trimethylbenzene (P10, $r = 0.994$), and 1-t-butylcyclopentadiene (P11, $r = 1.000$). The other interesting compound in saffron studies, isophorone, here identified as P17, was positively and significatively correlated with the following compounds: P1 ($r = 0.946$), P3 ($r = 0.961$), P4 ($r = 0.969$), P9 ($r = 0.903$), P21 ($r = 0.947$), P22 ($r = 0.940$) and P23 ($r = 0.976$). Negative correlations, when present, were not significant.

To conclude, PCA on PTR-TOF-MS parameters showed that saffron from Fiesole (IT-F) appeared to share more analogies with saffron aromatic profile from Iran than with those from the same country, and that isophorone and safranal were the most useful compounds to describe samples variability.

Table 3
Variables that most contribute to the description of samples variability as underlined by principal component analysis (PCA). The analysis was applied to 28 parameters (PTR-TOF-MS analysis, case "A") and to 60 parameters (28 from PTR-TOF-MS analysis and 32 from HPLC analysis, case "B"). Here we select all the variables with highest influence for the first two components (PC1 and PC2), namely with squared cosine bigger than 0.5.

Compound	Case "A"		Case "B"	
	PC1 (%)	PC2 (%)	PC1 (%)	PC2 (%)
P1	6.46			
P3	7.29			
P4	6.66			
P5		10.08	2.29	
P6		10.64		
P7		8.13	2.86	
P8		6.73	2.44	
P9	7.08			4.22
P10		8.88	2.74	
P11		8.28	2.82	
P12				4.06
P13		7.50		4.97
P14				6.13
P15		8.70		
P16	7.00		2.63	
P17	7.02			4.88
P18				4.97
P19		7.98	2.86	
P20	6.91		2.82	
P21	7.19		1.88	
P22	7.15		2.04	
P23	6.56			4.92
P24	6.72		2.92	
P25	3.93			
H1			2.39	
H2			2.50	
H3			2.97	
H5			2.96	
H6			2.94	
H7			3.21	
H9			3.08	
H10			2.68	
H14			3.79	
H17			3.85	
H18			3.77	
H20			3.90	
H25			2.64	
H26			2.21	
H27			2.36	
H28			2.87	

3.4. Principal component analysis on PTR-TOF-MS data and HPLC data

PCA performed on the 60 variables showed in Tables 1 and 2 (obtained with PTR-TOF-MS and HPLC analysis, respectively) explained about 60% of total variability by mean of the first two PCs. The two-dimensional scatter plot (Fig. 2, *bottom left*) underlined a clear separation of the three Italian saffron samples in regard to each other and to the saffron from Iran. The main separation was achieved between the samples from Italy; in details, the three saffron shared the negative range of the PC1, with saffron from Città della Pieve (IT-P) and that from Cascia (IT-C) and Fiesole (IT-F) occupying respectively the negative and the positive range of the PC2. Iranian samples were positioned in the positive range of PC1, quite close one to each other.

Most of the original variables contributed to the first PC, that described 38.82% of variability; among those obtained with HPLC analysis, it is worth noting the contribution of picrocrocin (H26) and safranal (H27). Interestingly, also the compound detected using PTR-TOF-MS technique and identified as safranal (P19) had a significant role in the definition of the first component. On the other hand, the compound identified as isophorone (P17) was determinant for the definition of PC2.

The correlation plot (Fig. 2, *bottom right*) confirmed the correlation underlined in case "A". No significant positive correlations were found between variables deriving from the two different analytical approaches, while most of the variables obtained with PTR-TOF-MS analysis showed negative correlation with many HPLC parameters. Furthermore, safranal content (H27) was not significantly correlated with other HPLC parameters, except for the case of picrocrocin (H26, $r = 0.887$). The latter, instead, showed positive correlation with *trans*-crocin 4 (H6, $r = 0.814$), and *trans*-crocin 3 (H9, $r = 0.830$).

Pooling all dataset, derived from PTR-TOF-MS analysis and that obtained with HPLC analysis, the difference of saffron from Iran in respect to the Italian ones was explained; more information on the samples from Fiesole (Italy), that was here better distinct from the rest of the Iranian samples, was achieved. Furthermore, key compounds detected with PTR-TOF-MS analysis (identified as safranal and isophorone) confirmed to be useful for the characterization of samples; similar consideration could be done concerning safranal and picrocrocin data obtained with HPLC analysis.

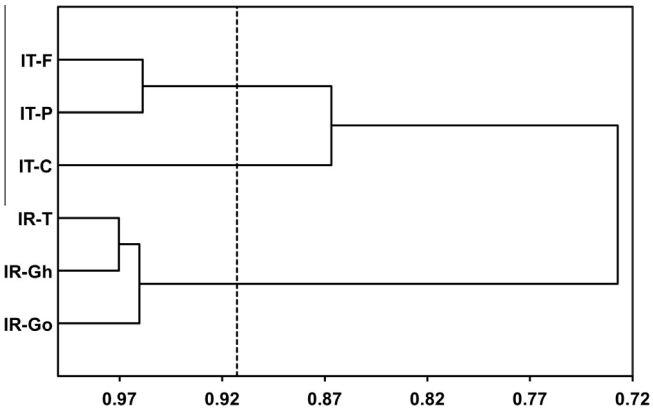

Fig. 3. Hierarchical cluster analysis dendrogram obtained analysing 60 variables (from HPLC and PTR-TOF-MS investigation); dotted line indicates the automatic truncation (IT-C: Italy, Cascia; IT-P: Italy, Città della Pieve; IT-F: Italy, Pieve; IR-Gh: Iran, Ghaen; IR-T: Iran, Torbat; IR-Go: Iran, Gonabad).

The whole dataset was finally used to build the dendrogram obtained with the Pearson's correlation matrix (Fig. 3). Three main groups (as underlined by the dotted line which represents the automatic truncation) could be identified. The group of saffron from Iran was homogeneous and well distinguished by the rest of the samples that clustered in two different groups, with saffron from Cascia clearly distinct from the rest of the Italian samples.

4. Conclusion

HPLC and PTR-TOF-MS analyses were performed to characterize saffron samples of different geographical origin. A higher content of total crocins (especially *trans*-crocin 4 and *trans*-crocin 3) is highlighted in saffron from Italy. HPLC analysis shows also Italian saffron to be richer in safranal (especially Cascia and Città della Pieve); this result is confirmed analyzing the intensity of the raw signal generated by PTR-TOF-MS approach.

The origin of the samples cannot be established using only the fast PTR-MS method, while the use of PCA applied to the whole dataset, including data from HPLC analysis, underlined the existence of groups, helping to better distinguish the geographical origin of each sample and pointing out safranal and isophorone to be the most informative compounds, together with picrocrocin.

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