Food Chemistry 264 (2018) 241–249

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Crocins pattern in saffron detected by UHPLC-MS/MS as marker of quality, process and traceability

R. Rocchi, M. Mascini, M. Sergi*,[1], D. Compagnone, D. Mastrocola, P. Pittia*,[1]

Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, 64100 Teramo, Italy

ARTICLE INFO

Keywords:
Saffron
Crocins
UHPLC-MS/MS
Process marker
Multivariate analysis

ABSTRACT

This study was carried out to develop an UHPLC-MS/MS analytical procedure, to determinate all isomers and isoforms of crocins of 42 saffron samples, with different origin, age and dried using different process conditions.

A preliminary experimental design was applied to optimize the extraction of crocins; UHPLC-MS/MS conditions were set to obtain the best analytical performances in terms of sensitivity and selectivity. The optimised conditions allowed to determine ten crocins; their amount in samples was significantly different and affected by process, age and origin. Drying conditions influenced the crocins pattern and this was particularly evidenced in the more recently produced samples, with a clear separation between mild and high thermally treated samples. Principal Component Analysis of all crocins data allowed to discriminate samples based on origin (Italy *vs.* other countries) and age.

Results confirm the feasibility of the use of crocins pattern as marker of quality and traceability of saffron.

1. Introduction

Saffron is a precious spice known as “red gold” obtained from the dried stigmas of the *Crocus sativus* L., a plant of the Iridaceae family. Its importance is related to the peculiar quality and sensory properties, in particular aroma, colour, and taste, that are associated to the presence of three main compounds, safranal, crocins and picrocrocin (Maggi et al., 2010; Tsimidou & Tarantilis, 2017) (Fig. S1). Besides colouring and flavouring properties, saffron has been recognized as able to exert various and diverse beneficial health and medical attributes (Razak, Anwar Hamzah, Yee, Kadir, & Nayan, 2017); it has antidepressant and aphrodisiac properties and shows therapeutic efficacy on many organs, including the endocrine, gastrointestinal and cardiovascular systems (Bukhari, Manzoor & Dhar, 2018). In several studies, scientists have found that saffron has the unique ability to both slow and reverse cancer growth (Finley & Gao, 2017; Razak et al., 2017).

This spice is characterized by a large number of volatile and aroma-yielding compounds recently identified and counted in ca. 150. The main qualitative properties depend also on the presence of many non-volatile components such as polysaccharides, sugars and lipids as well as biologically active components including carotenoids, as crocins, zeaxanthin, lycopene, α-carotenes (Lech, Witowska-Jarosz & Jarosz, 2009).

The saffron plant is cultivated in light soil fields, since flowers might lose their properties when exposed to poor weather conditions for a long period of time (Rubert, Lacina, Zachariasova & Hajslova, 2016) and typical countries where it is mainly produced are Iran, Spain, India and Greece. All post-harvest processes (separation of stigmas from flowers, drying or toasting, and packaging) have a significant effect on the quality and, consequently, the corresponding commercial value of the spice.

The drying process represents a critical step: the applied conditions may widely vary resulting from the producer experience in each production place through trial-and-error approaches, traditions, along with the available resources and drying tools (Carmona et al., 2005). This, however, significantly affects the chemical composition and, as a consequence, the overall quality including the sensory properties such colouring strength, aroma and bitterness. Different drying process have been reported (Acar, Sadikoglu & Okzaymak, 2011; Perez, 1995; Tong et al., 2015).

While high temperatures, during drying process, can facilitate the release of a large amount of secondary metabolites located in chromoplasts (Carmona et al., 2005), with positive effects on the final quality, thermal degradation of pigments can also occur resulting in poor quality products (Raina, Agarwal, Bhatia & Gaur, 1996).

In general, saffron is recognized a high value spice and so frauds and adulterations are increasing by the inclusion of colouring and flavouring substances able to keep similar colour properties than the

* Corresponding authors.
E-mail addresses: msergi@unite.it (M. Sergi), ppittia@unite.it (P. Pittia).
[1] Equally senior authors.

https://doi.org/10.1016/j.foodchem.2018.04.111
Received 15 January 2018; Received in revised form 28 March 2018; Accepted 24 April 2018
Available online 26 April 2018

original. This, however, arises important issues on traceability and authenticity, and reliable quality indices must be defined.

Saffron quality, based on crocins, picrocrocin and safranal, is commonly evaluated by spectrophotometric analysis based on the ISO 3632 standard that allows spice classification in three categories (ISO 3632-1:2011), even if this approach has some limitations when applied for traceability and authenticity. Different analytical techniques were developed and applied to this aim, such as high performance liquid chromatography (HPLC) (D'Archivio, Giannitto, Maggi & Ruggieri, 2016; Masi et al., 2016; Nescatelli et al., 2017), gas chromatography (GC) (Anastasaki et al., 2009), near-infrared spectroscopy (NIRS) (Nescatelli et al., 2017; Rubert et al., 2016), inductively coupled plasma mass spectrometry (ICP-MS) (D'Archivio, Giannitto, Incani & Nisi, 2014) and stable isotope analysis (Maggi, Carmona, Kelly, Marigheto & Alonso, 2011). For authentication purposes, some authors proposed different and alternative analytical methods like the Sequence-Characterized Amplified Regions (SCAR) markers (Marieschi, Torelli & Bruni, 2012; Torelli, Marieschi & Bruni, 2014), Near-Infrared Spectroscopy (Zalacain et al., 2005), Nuclear Magnetic Resonance (NMR) spectroscopy (Schumacher, Mayer, Sproll, Lachenmeier & Kuballa, 2016; Yilmaz, Nyberg, Mølgaard, Asili & Jaroszewski, 2010) and Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) techniques (Masi et al., 2016; Nenadis, Heenan, Tsimidou & Van Ruth, 2016).

Crocins are a group of water soluble-carotenoids, made of various molecules originated from zeaxanthin by enzymatic cleavage, present in saffron in the 6–16% range (on dry matter) depending on variety, growing conditions and processing methods (Gregory, Menary & Davies, 2005; Raina et al., 1996). They are glycosyl esters of crocetin with different glycosyl moieties, such as glucose, gentiobiose, or triglucose and can exist in cis- and trans- isomeric forms. Crocin-4, di(β-D-gentiobiosyl) crocetin ester, is esterified in both terminal carboxylic groups with gentiobiose. Crocin-3, β-D-gentiobiosyl- β-D-glucosyl crocetin ester, exhibits mixed glucosyl–gentiobiosyl structure. Crocin-2 and crocin-2II have identical molecular mass, but different structure. Crocin-2 is the gentiobiosyl derivative of crocetin, and crocin-2II contains two glucosyl moieties, while crocin-1 is esterified with only one glucose molecule. When saffron is exposed to white fluorescent light, trans- isomers change into cis- isomers (Sarfarazi, Jafari & Rajabzadeh, 2015; Strain, 1963).

In literature, some papers deal with the identification and quantification of crocins by HPLC-MS that offers good specificity and sensitivity (Carmona, Zalacain, Sanchez & Novella, 2006; Cossignani et al., 2014; Lech et al., 2009; Tarantilis & Tsoupras, 1995).

Overall these methods allowed to determine the various different crocins isomers, but any of them focused the study on the effect of the processing conditions, in particular drying, on all cis-trans crocins pattern and its evolution. For quality purposes, this needs a deeper understanding in view of a possible use as index to characterise the origin, production methodologies and age of saffron.

The aim of this study was to develop a reliable UHPLC-MS/MS method for the determination of crocins isomers and isoforms in saffron to be used as marker of quality, process and traceability. Forty-two samples of saffron were analyzed for crocins content and overall pattern by using optimized extraction and analytical conditions.

2. Materials and methods

2.1. Saffron samples and chemicals

Forty-two saffron samples of different origin, process and age were collected (Table 1). Samples from Italian regions (n = 31 from Abruzzo, Basilicata, Campania, Lazio, Lombardy and Sardinia) were kindly provided by local farmers and producers. Detailed information on the commercial samples was not available, as blended at production or packaging level, and their origin was then attributed based on either country of purchase or production/packaging locations reported on the corresponding packaging label.

Table 1
List of the saffron samples, origin with different origin, drying process and year of production.

Country	Region	Year	Thermic treatment	Label
Italy	Abruzzo	2012	Hard	1H
Italy	Lombardy	2012	Hard	2H
Italy	Abruzzo	2012	Microwave	A
Italy	Abruzzo	2013	Hard	3H
Italy	Abruzzo	2013	Toasted	B
Italy	Lombardy	2013	Hard	4H
Italy	Lazio	2014	Soft	5M
Italy	Abruzzo	2014	Hard	6H
Italy	Lombardy	2014	Hard	7H
Italy	Sardinia	2014	Hard	8H
Italy	Abruzzo	2015	Soft	9M
Italy	Lombardy	2015	Soft	10M
Italy	Abruzzo	2015	Hard	11H
Italy	Lombardy	2015	Hard	12H
Italy	Sardinia	2015	Hard	13H
Italy	Lazio	2015	Soft	C
Italy	Abruzzo	2015	Embers	D
Italy	Abruzzo	2016	Soft	14M
Italy	Abruzzo	2016	Soft	15M
Italy	Abruzzo	2016	Soft	16M
Italy	Abruzzo	2016	Soft	E
Italy	Abruzzo	2016	Microwave	F
Italy	Campania	2016	Soft	17M
Italy	Abruzzo	2016	Hard	18H
Italy	Lombardy	2016	Hard	19H
Italy	Sardinia	2016	Hard	20H
Italy	Lazio	2016	Soft	G
Italy	Basilicata	2016	Soft	I
Argentina	Unknown	2015	Unknown	ARG
Austria	Unknown	2015	Unknown	AU
Greece	Unknown	2015	Unknown	GR
India	Unknown	2015	Unknown	IND
Iran	Unknown	2015	Unknown	IR
Lebanon	Unknown	2015	Unknown	LEB
Turkey	Unknown	2015	Unknown	TUR
Hungary	Unknown	2015	Unknown	HUN
Italy	Unknown	2016	Unknown	Comm_IT_1
Italy	Unknown	2016	Unknown	Comm_IT_2
Italy	Unknown	2016	Unknown	Comm_IT_3
Spain	Unknown	2016	Unknown	Comm_SP_4
Iran	Unknown	2016	Unknown	Comm_IR_5
Greece	Unknown	2016	Unknown	Comm_GR_6

Eleven samples were collected as powders, while the remaining were as stigmas and upon arrival underwent to manual grinding in a dry box up to a fine and homogeneous powder. After the ISO quality analysis (according to ISO 3632-1:2011, see Section 2.2) all samples were freeze-dried to remove residual moisture and then packed in high barrier metalized plastic bags hermetically closed and stored at −18 °C until analysis.

All saffron samples were preliminarily classified by age and drying process intensity. 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 was kindly provided by producers and/or based on conventional drying conditions in the country of origin and age estimated based on the "best consumption date", taking as reference a 5 years-commercial shelf-life.

Drying is generally carried out in electric oven, but other technologies were also used (sun-drying, microwave). Temperature process conditions allow to classify samples in two main categories: samples undergone to hard/intense treatments ("H" samples) in electric ovens at temperatures > 45° up to 120 °C, and samples undergone to mild treatments ("M") carried out at a temperature ≤45 °C.

Some saffron samples were collected in the same region, same year of production and dried with the same drying treatment, but from different local farmers. The commercial samples were purchased in

different countries; the Italian ones with different brands. The crocetin esters identified in this work have been named to abbreviate the names of crocetin esters with the word of the corresponding isomer (cis- and trans-) followed by the total number of glucose moieties esterified at both extremes of crocetin. The glucidic components are represented by triglucose, gentiobioside and glucoside.

Analyses were carried out in 2016 and saffron produced in 2015–2016 were considered as "fresh" age: 1 year maximum); the remaining samples (age > 1 year) were classified overall as "aged". The complete sample list and corresponding acronym is given in Table 1.

Methanol (MeOH), Acetonitrile (ACN), formic acid (HCOOH) and water, all UHPLC-MS grade, were purchased from Fluka Bio Chemika (Milano, Italia), while crocin standard mix (CAS No. 42553-65-1) from Sigma Aldrich (St. Louis, MO, USA).

2.2. Quality evaluation by ISO 3632-1:2011

Samples were analyzed according to ISO 3632-1 procedure with some modifications; an aliquot of saffron (10 mg) was dispersed in 2 mL distilled water and subjected to Ultrasound Assisted Extraction (USAE) for 15 min at T = 25 °C (Armellini, Compagnone, Scamicchio & Pittia, 2017). The extracts were centrifuged and analysed by a spectrophotometer (Perkin-Elmer Lambda Bio 20 UV–Visible, US). The absorbance at 257, 330 and 440 nm of the saffron extract, diluted at 1% w/w in water, was evaluated in a 1 cm pathway quartz cell; distilled water was used as reference.

Then, an aliquot of 15 mg of each sample was exactly weighted and put in an oven at 103 °C for 16 h. Moisture and volatile content is expressed as a percentage of the initial sample computed by the following Eq. (1):

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

ISO quality indexes, were then computed according to the following Eq. (2):

$$A^{1}_{1\,cm}(\lambda_{max}) = \frac{D - 10000}{m * (100 - w_{MV})} \quad (2)$$

where D is the specific absorbance at 257, 330 and 440 nm; m is the mass of the saffron sample (in g); W_{MV} is the moisture and volatile content of the sample, expressed as a mass fraction.

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

2.3. Extraction and analysis of crocins by UHPLC-MS/MS

Two-steps (screening and optimization) experimental design was applied to optimize extraction of crocins isomers from saffron samples by means of Ultrasound Assisted Extraction (USAE). Initially, three independents variables of were investigated: extraction solvent; sonication time and sonication temperature, as described in par. 3.2.

Optimized extraction was carried out on 5 mg of saffron powder dispersed in 1.25 mL of MeOH/H20 (50:50, v/v) in a 1.5 mL Epperdorf vial and subjected to USAE. Thereafter, the extract was centrifuged and filtered through a 0.22 μm polytetrafluoroethylene (PTFE) syringe filter with a diameter of 4 mm and put in an autosampler vial for UHPLC-MS/MS analysis. An UHPLC equipment composed by a Nexera LC20AD XR apparatus, with autosampler, vacuum degasser and column oven (Shimadzu, Tokyo, Japan) coupled with a 4500 Qtrap mass spectrometer (Sciex, Toronto, Canada) equipped with a Turbo V ESI source, was used for UHPLC-MS/MS analysis.

The target analytes were separated using a reverse-phase Kinetex C_{18} column from Phenomenex (Torrance, CA, USA) packed with Core Shell particles (1.7 μm, 2.1 × 100 mm). The mobile phases were 5 mM formic acid in water (phase A) and 5 mM formic acid in acetonitrile (phase B); flow rate was 0.3 mL min^{-1} entirely transferred into the ion source. The analyte separation was performed using a gradient elution: for 0.1 min, phase B is maintained at 5%, then, increased to 99% in 13 min and kept at 99% for 4 min; the system is brought back to the initial conditions in 3.30 min. The analyte separation occurs in 10 min. All the substances were detected in negative ionization with a capillary voltage of −4500 V, nebulizer gas (air) at 50 psi, turbo gas (nitrogen) at 40 psi, and 450 °C.

Table 2
Instrumental parameters of all crocins with each MRM transition (Q1: precursor ion mass; DP: declustering potential; EP: entrance potential; Q3: product ion mass; CE: collision energy; CXP: cell exit potential; amu: atomic mass unit).

Analyte	Rt (min)	Q1 (amu)	DP (V)	EP (V)	Q3 (amu)	CE (V)	CXP (V)
cis1	8.69	489.1	−50	−11	282.9	−30	−16
		489.1	−50	−11	489.1	−10	−6
cis2	7.89	651.1	−50	−5	327.1	−30	−16
		651.1	−50	−9	651.1	−8	−8
cis2II	8	651.1	−50	−5	327.1	−30	−16
		651.1	−50	−9	651.1	−8	−8
cis3	7.45	813.1	−50	−6	762.8	−34	−30
		813.1	−50	−5	813.1	−6	−10
cis4	6.67	975.3	−50	−6	651.0	−32	−8
		975.3	−50	−6	975.3	−11	−12
trans1	7.56	489.1	−50	−11	282.9	−30	−16
		489.1	−50	−11	489.1	−10	−6
trans2	7.56	651.1	−50	−5	327.1	−30	−16
		651.1	−50	−9	651.1	−8	−8
trans2II	7.41	651.1	−50	−5	327.1	−30	−16
		651.1	−50	−9	651.1	−8	−8
trans3	5.79	813.1	−50	−6	762.8	−34	−30
		813.1	−50	−5	813.1	−6	−10
trans4	5.42	975.3	−50	−6	651.0	−32	−8
		975.3	−50	−6	975.3	−11	−12

Source and instrumental parameters for the analytes of interest were optimized by using crocin standard (Sigma Aldrich, St. Louis, USA) dissolved in methanol/water (50:50, v/v) at a concentration of 10 ng mL^{-1} at a flow of 10 μL min^{-1} by a syringe pump. Peak areas for selected ions were determined using Multiquant Software 3.0 (Sciex, Foster City, CA). The selected MRM transitions and UHPLC–MS/MS parameters are reported in Table 2 and extracted ion currents for all analytes are reported in Supplementary Material (Fig. S2).

2.4. Thermal ageing of saffron

To understand the results of the process impact on crocins and to evaluate the effect of the drying conditions on the crocins fate, saffron stigmas were subjected to different heat treatments. The "I" saffron sample was chosen based on the initial quality characteristics and crocins content. Before heat treatments, filaments were rehydrated at a_w = 0.50 to increase the moisture content to a value similar to the fresh samples.

Aliquots (50 mg) of saffron stigmas were inserted in aluminum pans and subjected to heat treatments in an electric oven at different temperature (100, 125, 150 and 200 °C) for different times (5 and 10 min). Samples were then immediately put in a desiccator with silica gel to decrease the temperature and then left there to equilibrate for 24 h. Before crocins analysis, samples were ground as already described and extracted with the conditions optimized by experimental design reported in 2.3.

Effect of heat treatment on crocins isomers content and pattern has been computed as variation (%), taking as reference the corresponding value of the initial, no aged dried sample according to the following Eq. (3):

$$\text{Crocin variation}\ (\%) = [(A_i - A_f)/A_i] \times 100 \quad (3)$$

where Af = final area of the sample after heating treatment, Ai = initial area of the no-aged.

Table 3
Quality parameters and category of different saffron samples according to ISO 3632 (2011): picrocrocin λ_{max} = 257 nm; safranal λ_{max} = 330 nm and crocins λ_{max} = 440 nm. The coefficient of variation (CV) is under 0.3% for all samples.

Label	$E^{1\%}_{1cm}$			
	Flavour strength 257 nm	Aroma strength 330 nm	Colouring strength 440 nm	ISO Category
1H	64	31	121	III
2H	67	32	177	II
A	64	31	111	n.c.
3H	76	34	154	III
B	77	34	155	III
4H	119	54	216	I
5M	98	42	206	I
6H	85	23	193	II
7H	96	32	235	I
8H	88	32	204	I
9M	82	21	190	II
10M	85	26	208	I
11H	117	33	293	I
12H	101	21	180	II
13H	118	37	268	I
C	84	33	191	II
D	94	39	241	I
14M	72	24	165	III
15M	88	34	236	I
16M	88	22	222	I
E	92	25	223	I
F	100	28	233	I
17M	100	27	243	I
18H	107	29	277	I
19H	131	27	270	I
20H	122	24	275	I
G	100	39	238	I
I	99	36	211	I
ARG	/	/	/	n.c.
AU	43	19	74	n.c.
GR	55	35	78	n.c.
IND	40	22	80	n.c.
IR	72	37	165	III
TUR	31	26	15	n.c.
LEB	59	31	120	III
HUN	22	19	11	n.c.
Comm_IT_1	58	27	152	III
Comm_IT_2	51	28	97	n.c.
Comm_IT_3	112	55	276	I
Comm_SP_4	72	36	181	II
Comm_IR_5	89	40	197	II
Comm_GR_6	111	38	288	I

2.5. Statistical analysis

The results of the chromatographic analysis were analysed by means of principal component analysis (PCA) and ANOVA using XLSTAT2016. Data have been autoscaled (zero mean and unitary variance). PCA was applied without any preliminary assumptions to have a data overview from a higher to a lower dimensional space.

3. Results and discussion

3.1. Quality evaluation of saffron by conventional indices

Generally, the quality of saffron is related to the content of three main components (picrocrocin, safranal and crocins). Presence and concentration of these compounds varies largely as a function of several factors, including climatic conditions, harvesting, drying process, and storage (Carmona et al., 2005). Saffron has to comply with the requirements of the standard ISO 3632-1:2011 by which its quality, authenticity and purity is commonly evaluated via parameters determined by spectrophotometric analysis.

Table 3 reports the values of the quality parameters for the different samples and the corresponding category of each sample. Overall, a wide range of values was obtained with indices of the colouring strength ($E^{1\%}_{1cm}$ = 440 nm) ranging from 15 to 293; aroma strength ($E^{1\%}_{1cm}$ = 330 nm) from 19 to 54 and flavouring strength ($E^{1\%}_{1cm}$ = 257 nm) from 22 to 131. Thus, twenty samples were classified as first (higher) quality category, seven in the second, seven in the third (lower) ones. Eight samples resulted unclassified due to the very low absorbance values and among them, one (ARG, from Argentina), didn't show any absorption at the selected wavelengths, a possible index of adulteration. Independently on age, Italian samples showed a higher colouring strength ($E^{1\%}_{1cm}$ = > 121), related to the crocins content, than saffron from other countries.

Interesting is also to evidence that the sample dried by microwave (A), despite the same age of 1H and 2H, exhibited very low ISO quality values and, overall, was unclassified. Similar result was found for the saffron from India, a sample obtained by sun-drying and this may have impaired the overall quality attributes (Raina et al., 1996).

3.2. Optimization of analytical procedure

Three independents variables were investigated for the extraction performances in a two-level full factorial screening design: extraction solvent (water/methanol volume ratio: 25% and 75% v/v); sonication time (5 and 20 min) and sonication temperature (25 °C and 65 °C). Then, a three levels full factorial optimization design was performed taking as variables extraction solvent and sonication time. Statistical procedures were carried out using MATLAB R2015a. All experiments were carried out by using one batch of saffron powder (Basilicata, label "I").

The best mathematical relationship between the three independent variables and the instrumental results of the eight crocins were fitted by second order polynomial functions without quadratic terms, to choose the optimal value of each factor allowing the highest simultaneous extraction of all the compounds of interest (Table S1). For all target compounds, sonication temperature was inversely proportional to the crocins extraction yield whilst time was significantly and positively correlated with crocins extraction; considering solvent composition, a higher amount of H_2O affected proportionally the concentration of the extracted trans- isomers with the exception of the trans-4. On the contrary, methanol contributed to extract cis- form.

To provide the best value of compromise in the desirable joint response, Derringer's desirability function was applied (Vera-Candioti, De Zan, Cimara & Goicoechea, 2014). The overall option was given by the geometric mean of the individual response functions combination resulting in optimized solvent extraction of 50% H_2O/Methanol to extract simultaneously cis and trans isomers (Supplementary document, Fig. S3). The experimental responses obtained under those optimized conditions were statistically comparable to those predicted by the model.

The optimized extraction procedure and the UHPLC-MS/MS analysis allowed to determine the crocin isomers pattern of the saffron samples. In a preliminary step, both positive and negative ion mode were tested for the MS detection; the latter allowed the identification of ten crocins. In the mass spectra, crocins were observed as quasi-molecular ions $[M\text{-}H]^-$ and also as fragment ions formed by the loss of glycoside units.

In some works, the determination of crocetin glycosides of saffron is carried out by mass spectrometry with electrospray ionization (ESI–MS); MS crocins spectra in positive ion mode (ESI+) and different MS fragments and adducts (Na^+, K^+ and $NH4^+$) were obtained, whereas when, in ESI- mode, quasi-molecular ions (deprotonated ions) and fragments formed by the loss of glycosides, a variety of adducts with components of chromatographic mobile phases were also registered and identified (Guijarro-Díez, Catro-Puyana, Crego & Marina, 2017; Lech et al., 2009).

In this work *quasi*-molecular ions $[M–H]^-$ of the crocin-4, di(β-D-gentiobiosyl) crocetin ester, in both trans- and cis-configurations, were

Table 4
The % relative area (computed on the total area of the crocins) of the ten isomers of all saffron samples under investigation. The relative standard deviation (RSD) is expressed in brackets.

Label	Crocin isomer (%)									
	cis1	cis2	cis2 II	cis3	cis4	trans1	trans2	trans2 II	trans3	trans4
1H	0.4 (7)	3.9 (9)	3.5 (2)	3.5 (5)	20.2 (8)	0.8 (2)	2.9 (12)	1.0 (8)	6.0 (9)	57.9 (9)
2H	0.2 (6)	0.8 (8)	0.8 (8)	1.5 (6)	45.6 (8)	0.2 (9)	1.7 (7)	0.8 (8)	2.8 (14)	45.6 (7)
A	0.4 (11)	2.4 (2)	1.5 (11)	4.4 (14)	9.5 (2)	0.8 (9)	4.9 (7)	1.0 (10)	8.4 (6)	66.7 (8)
3H	0.4 (4)	2.4 (5)	1.9 (3)	4.2 (2)	14.4 (8)	0.5 (6)	3.0 (5)	0.8 (6)	6.5 (6)	65.9 (10)
B	2.5 (2)	18.7 (3)	16.0 (7)	4.2 (5)	25.0 (7)	1.4 (7)	11.2 (7)	1.0 (13)	2.6 (14)	17.4 (14)
4H	0.3 (7)	1.0 (2)	1.0 (2)	3.1 (1)	13.7 (3)	0.6 (14)	2.9 (8)	1.0 (2)	5.2 (7)	71.2 (12)
5M	0.3 (2)	1.7 (8)	1.7 (7)	3.1 (2)	17.7 (10)	0.5 (4)	2.0 (8)	0.6 (7)	3.7 (2)	68.6 (11)
6H	0.2 (7)	1.6 (4)	1.0 (10)	4.0 (10)	10.3 (11)	0.6 (9)	2.8 (1)	0.6 (7)	7.7 (5)	71.1 (9)
7H	0.2 (12)	0.6 (4)	0.6 (2)	2.7 (13)	13.3 (8)	0.4 (5)	1.9 (14)	0.6 (5)	3.8 (1)	75.7 (7)
8H	0.2 (6)	1.8 (13)	0.9 (10)	3.6 (7)	11.9 (5)	0.4 (8)	2.6 (10)	0.7 (8)	8.8 (6)	69.0 (12)
9M	0.2 (14)	3.0 (3)	2.7 (6)	5.1 (7)	18.0 (3)	0.4 (10)	2.2 (5)	0.7 (10)	3.9 (10)	63.9 (7)
10M	0.1 (9)	2.2 (11)	1.4 (10)	1.7 (7)	14.1 (8)	0.5 (11)	3.6 (6)	1.3 (7)	4.6 (7)	70.5 (4)
11H	0.2 (4)	1.8 (8)	1.2 (14)	2.9 (3)	8.1 (12)	0.5 (14)	3.5 (2)	0.7 (1)	7.0 (5)	74.1 (8)
12H	0.2 (5)	3.2 (7)	2.6 (2)	5.4 (12)	21.4 (5)	0.6 (9)	1.9 (5)	0.8 (3)	3.1 (4)	60.8 (9)
13H	0.6 (6)	4.8 (8)	3.1 (14)	5.6 (12)	21.1 (11)	0.6 (11)	3.6 (3)	0.7 (8)	5.8 (6)	54.1 (9)
C	0.2 (12)	2.7 (13)	2.1 (11)	3.8 (3)	18.2 (14)	0.5 (3)	2.6 (4)	1.0 (9)	3.8 (12)	65.0 (10)
D	0.4 (11)	2.8 (1)	2.7 (10)	3.6 (6)	21.7 (11)	0.5 (4)	2.1 (8)	0.5 (4)	3.6 (4)	62.1 (1)
14M	0.2 (3)	2.7 (4)	1.0 (10)	2.6 (4)	9.6 (3)	0.4 (10)	3.2 (8)	1.3 (14)	7.0 (10)	71.9 (1)
15M	0.2 (13)	2.9 (12)	1.7 (2)	5.7 (11)	13.1 (9)	0.5 (2)	3.5 (4)	1.1 (1)	5.4 (3)	65.7 (6)
16M	0.2 (4)	1.8 (8)	0.8 (3)	2.9 (9)	8.8 (2)	0.5 (7)	3.4 (5)	1.1 (12)	7.3 (12)	73.2 (8)
E	0.1 (7)	2.0 (15)	0.9 (6)	3.0 (8)	9.1 (6)	0.5 (9)	1.9 (7)	0.9 (7)	7.0 (12)	74.6 (9)
F	0.4 (3)	4.5 (4)	3.3 (10)	5.7 (14)	16.8 (3)	0.5 (10)	3.9 (8)	1.2 (14)	5.7 (10)	57.9 (1)
17M	0.2 (8)	3.6 (5)	1.3 (2)	5.8 (9)	13.3 (4)	0.5 (7)	3.3 (4)	1.0 (7)	7.4 (4)	63.7 (1)
18H	0.2 (4)	4.5 (14)	1.6 (2)	3.4 (4)	16.1 (8)	0.4 (4)	4.3 (9)	1.5 (8)	4.8 (13)	63.2 (1)
19H	0.2 (12)	2.5 (6)	0.7 (12)	5.1 (2)	12.0 (9)	0.5 (4)	3.1 (6)	1.2 (2)	6.8 (8)	67.8 (13)
20H	0.2 (1)	3.3 (3)	0.7 (9)	3.9 (2)	9.2 (14)	0.4 (11)	3.7 (3)	1.5 (6)	9.5 (5)	67.5 (9)
G	0.3 (7)	3.5 (4)	1.7 (2)	4.6 (11)	18.1 (10)	0.6 (2)	4.0 (9)	1.1 (7)	4.5 (12)	61.7 (4)
I	0.3 (8)	3.3 (13)	1.9 (5)	7.0 (13)	21.7 (8)	0.6 (5)	3.2 (5)	0.9 (4)	6.2 (14)	54.8 (2)
ARG	/	/	/	12.9 (2)	42.5 (1)	/	/	/	9.7 (4)	35.0 (7)
AU	/	4.8 (6)	3.4 (5)	5.8 85)	30.3 (7)	/	3.1 (6)	0.9 (13)	3.1 (8)	48.6 (7)
GR	/	4.8 (7)	4.3 (8)	3.5 (6)	22.2 (9)	/	2.7 (8)	1.0 (12)	2.5 (6)	59.0 (4)
IND	/	8.9 (4)	6.6 (7)	7.5 (8)	22.4 (9)	/	3.9 (4)	4.4 (15)	5.7 (4)	40.7 (7)
IR	/	3.2 (1)	2.8 (2)	3.5 (8)	22.8 (4)	/	2.6 (2)	0.8 (3)	2.9 (11)	61.3 (2)
LEB	/	4.0 (4)	3.2 (10)	4.1 (11)	19.6 (9)	/	2.7 (8)	0.7 (8)	4.1 (7)	61.7 (8)
TUR	/	6.5 (7)	3.4 (14)	3.9 (3)	29.3 (8)	/	3.4 (12)	3.0 (8)	4.3 (12)	46.2 (13)
HUN	/	6.1 (4)	6.1 (4)	6.9 (14)	26.8 (8)	/	6.1 (4)	6.1 (4)	7.1 (6)	35.0 (8)
Comm_IT_1	0.5 (1)	3.6 (11)	2.4 (6)	5.7 (10)	21.9 (8)	1.0 (12)	4.0 (7)	1.2 (11)	4.6 (13)	55.2 (9)
Comm_IT_2	0.5 (14)	3.6 (3)	2.3 (7)	4.5 (8)	18.8 (9)	1.1 (1)	4.0 (11)	1.0 (4)	5.6 (12)	58.6 (15)
Comm_IT_3	0.5 (4)	4.4 (6)	2.0 (4)	6.7 (3)	18.8 (7)	1.2 (1)	5.1 (5)	1.3 (3)	4.1 (4)	55.8 (10)
Comm_SP_4	0.7 (2)	4.3 (13)	3.0 (10)	6.6 (7)	17.6 (11)	0.9 (11)	5.4 (2)	1.4 (2)	7.1 (10)	53.1 (10)
Comm_IR_5	0.8 (3)	5.7 (5)	3.2 (19	6.6 (5)	24.7 (7)	1.1 (11)	5.1 (1)	1.3 (5)	5.5 (4)	46.0 (11)
Comm_GR_6	0.4 (3)	3.0 (2)	1.3 (6)	5.7 (11)	18.0 (9)	1.1 (4)	3.8 (8)	0.8 (8)	5.4 (7)	60.7 (8)

recorded at m/z 975.3 and the loss of one gentiobiosyl unit was observed as a signal at m/z 651.1 $[M–H–Gnt]^-$. All crocins, except the crocin-1, were detected at the MRM transition 651.1-327.1; this transition represents the common fragmentation of all analytes by the loss of glucoside units. All MRM transitions were confirmed with the respective precursor-precursor transition. Being the crocin standard mix not an analytical standard, which concentration is certified, but a microscopy standard, it was not possible to evaluate the Limits of Detection (LODs) and Quantification (LOQs), neither perform an absolute quantification of the single crocins.

Based on the optimized method, it was possible, by ESI-MS in negative ion mode, the differentiation and the identification of the ten crocins isomers based on their quasi-molecular weight and fragment formed by loss of glycosides units in ESI-MS; this result is in agreement with Lech et al. (2009).

3.3. Crocin isomer composition of saffron samples

In Table 4, the values of the relative area (computed as % on the total area of all crocins) of the ten isomers of saffron samples characterized by different process, age and origin, are reported. The reproducibility was calculated for each analyte from 6 independent samples submitted to the whole analytical process and reported in Table 4 as relative standard deviation (%).

The total chromatographic area of the ten crocins, from which is possible to highlight the different total content of crocins along with the total relative area of cis- and trans-isomers, is reported as Supplementary Material (Table S2).

In all saffron samples the relatively most abundant crocin is the trans -4, that was found in a rather wide range of concentration (35.0–75.7%) followed by the cis-4, except for the sample B (toasted), that showed a higher concentration of both isomers of crocin-2, particularly the cis isomers. In general, all the fresh saffron (year production: 2016), had a content of crocins higher than the older samples. The prevalent occurrence of trans- isomers of crocins for most of the samples was expected considering the lower energy of trans- isomer than cis- isomers and, thus the higher stability of the former (Zechmeister, 1944).

Interesting, the toasted saffron (B) had the highest content of cis-isomers; this can be attributed to the peculiar drying conditions. In fact, the treatment, carried out at rather high temperature for a short time may have limited the carotenoid isomerization.

A similar result was obtained for the Argentina saffron (ARG). The latter sample did not show any absorption at 440 nm with the ISO procedure; at this wavelength crocins are expected to absorb light, then, a possible adulteration with some other saffron mimicking substances

was suspected. UHPLC-MS/MS data identified cis-4 (42.5% of the total) and cis-3 (12.5%), in a relative higher concentration than the trans-4 crocin (35%). This result, along with the low overall chromatographic area of crocins (Table S2) confirm that some saffron is present, likely mixed with other components; alternatively, the effect of an intense thermal treatment and/or a long storage can be supposed.

A higher content of total crocins (in particular, trans-4 and trans-3) in all Italian saffron samples with respect to other countries has been noticed (Table S2) in agreement with other recent studies (Masi et al., 2016).

In general, the effect of the different drying methodology and intensity seems not to directly affect the overall crocins content (Table S2), while a different distribution of the isomers has been found (Table 4). In particular, the toasted sample (B) showed a total content of crocins similar to other samples, with the total cis- isomers content higher than the trans- content. The Argentina sample (ARG), having a lower total crocins content than Italian samples, had the same behavior with a higher presence of cis- isomers.

These variations may be attributed to different drying processes and storage conditions under which the spice has been processed, kept and packed in each country. All processing steps and storage are factors that affect glycosidic carotenoids, thermally labile and photochemically sensitive components (Tarantilis and Tsoupras, 1995).

Correlation analysis between the total content of crocins (Table S2) computed as total UHPLC-MS/MS area and the colouring strength (ISO 3632-1: 2011) led to a poor correlation ($R^2 = 0.70$). This difference is likely due to the presence in saffron of other interfering substances with absorbance at 440 nm and considering that the UV–Vis is not a specific technique that could mask any differences that exist between samples. No correlation was also found between the ISO colouring strength values and the total cis- and trans- crocins content (Table S2) ($R^2 = 0.42$ and $R^2 = 0.69$, respectively), particularly for the former, because at 440 nm absorb only the trans- isomers.

Some works deal with the comparison of crocetin esters in saffron and gardenia extracts and showed that the latter is characterized by the same crocins pattern in different amounts, except for trans-4, high in both species. The trans2 II is not present in gardenia extract (Carmona et al., 2006; Guijarro-Díez et al., 2017). This allows to characterise the two different materials (gardenia and saffron) for authentication purposes.

Trans2 II was detected in all saffron samples under study, also in the commercial ones, that is a possible evidence of their authenticity.

In literature, there are some studies about the effect of drying method on saffron constituents (Acar et al., 2011; Carmona et al., 2005; Gregory et al., 2005; Raina et al., 1996; Tong et al., 2015) and dehydration treatment is accepted as being responsible for saffron organoleptic characteristic. Moreover, despite these other studies deal with crocins analysis of saffron, none of them deepen the evaluation on the fate of each crocins isomers that can be used for quality characterisation and traceability purposes.

3.4. Multivariate analysis of crocins isomers

All data obtained with UHPLC-MS/MS analysis were processed by Principal Component Analysis (PCA); before applying the PCA algorithm, data were linearly normalized and autoscaled (zero mean and unitary variance) in order to remove differences in signal range. The dataset consisted of a 42×10 matrix, made by forty-two saffron samples having different geographic origin, processing and storage conditions and, as variables, the ten crocins isomers. The data variance of the two principal components was 87.50% (Fig. 1A). The loadings, cis- and trans- crocins isomers, contributed in sample separation on the PC2 accounting for 19.63% of the total variance. The majority of cis-crocins was in the first quadrant of the PC1-PC2 cartesian plane, except the cis-3, and the most trans-crocins appeared closely localized in the second one.

The PCA did not show a clear separation of all samples; only the toasted sample (B) displayed a clear different behavior (high content of cis-crocins with a low molecular weight, up to right side of the PCA) and the Comm_GR_6 (that high content of trans-crocins, down, on the right side). The non-Italian samples, characterized by a lower concentration of crocins than Italian samples, are all located in the left side of the graph. Moreover, the non-Italian commercial samples showed a higher crocins content than the non-Italian samples and have, in general, a crocins concentration similar to the Italian samples with traced origin.

To better characterise the samples based on the criteria under investigation (origin, age and drying intensity), additional statistical analysis was made on selected set of data.

3.4.1. Effect of origin

Saffron corms of different origins, grown in the same experimental field, produce daughter corms with different dimensions and produce stigma samples with different pigment profiles. Thus, daughter corm dimensions and pigment profile may be related to the origin of the sample, and, therefore, pigments can be used as chemotaxonomic markers (Siracusa et al., 2013). The series of samples analyzed in this study were mostly from Italy or other countries and collected from local producers that confirm the specific origin while others were from both commerce (but bought in Italy) and no Italian countries without a clear traceability in terms of origin except for the information data of the packaging label. Principal Component Analysis (PCA) was carried out on samples at similar drying intensity process (mild) and commercial ones were not included for their uncertain geographic origin. A dataset of a 15×10 matrix, made of the selected fifteen saffron samples having different geographic origin, and as variables the ten crocins isomers was analysed.

The data variance of the two principal components was 93.01% (Fig. 1B). The PCA showed a clear separation between Italian saffron and the other samples. The PC1 describing 83.50% of the variability, differentiated the Italian samples from the other countries on the basis of the crocins concentration. Moreover, the cis-trans crocins differences was also important to distinguish saffron from Austria, Greece, Iran and Lebanon (high cis-crocins content) from saffron samples from Argentina, India, Turkey and Hungary (high content of trans-crocins).

3.4.2. Effect of drying method and age

An additional PCA was performed to highlight differences among Italian saffron samples with a known drying process conditions (temperature and time) based on the crocins content and pattern. In this case, analysis was performed on a dataset of 20 saffron samples with different thermal treatment and year of production. PCA results are reported in Fig. 1C: data total variance of the two principal components was 85.12%; PC1 described 63.84% of the variability, differentiating samples on the basis of crocins content. The thermal treatment influenced the samples variability particularly for the more recent samples with clear separation between the saffron subjected to mild and hard drying conditions. This behavior was found also in the aged samples (samples of years 2012–2014). All aged samples from years 2012 to 2014 were located in the left part of the biplot chart followed by the samples of year 2015 and in the right part by the high treated samples of year 2016. The fresh saffron, produced in 2016, showed a higher content of crocins than other samples.

The fresh (2016), dried samples by the more intense drying (electric oven at $T > 45^{\circ}$ up to 120 °C), showed in general a high content of all crocin isomers.

Some authors highlighted that thermal treatments carried out at high temperature for short time minimizes losses of crocins as inactivation of enzymes related to the crocins degradation may occur (Gregory et al., 2005). This was confirmed in our study by the high content of the sum of crocins isomers subjected to hard drying conditions (Table S2) and by the lower concentration of crocins of the mild

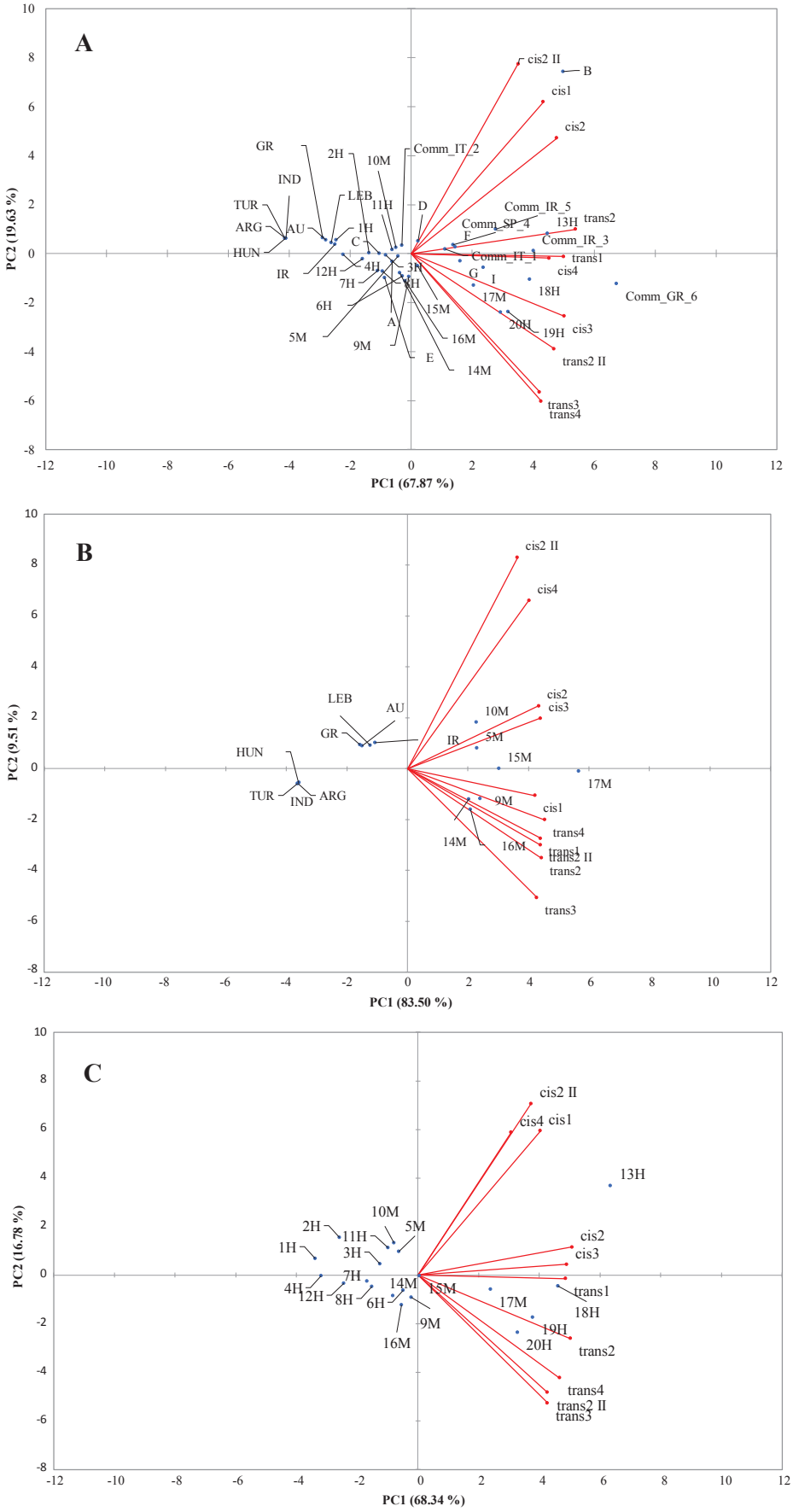

Fig. 1. A) PCA on ten crocins results obtained from 42 saffron samples samples (from Italy and other countries, labels in Table 1). Biplot of the first two components (explained variance = 87.50%). In both PCA the data were autoscaled (zero mean and unitary variance). B) PCA on ten crocins results obtained from 15 saffron samples (from Italy and other countries, labels in Table 1). Biplot of the first two components (explained variance = 93.01%). In both PCA the data were autoscaled (zero mean and unitary variance). C) PCA of ten crocins results obtained from 20 saffron samples (Italian origin, labels in Table 1). Biplot of the first two components (explained variance = 85.12%). A progressive labels numbering was used to highlight the year of production. For the thermal treatment, the following labels were used: H = Hard treatment-; M = Mild treatment.

dried samples (~45 °C) where a higher enzymatic activity may have caused their degradation.

This can be observed also in the aged samples (year production < 2016) where the effect of the drying treatment was also observed along with the effect of the storage conditions that causes an overall reduction of the crocins content.

Tsimidou and Biliaderis (1997) hypothesizes that carotenoid loss may occur at temperatures above the Tg, because of limited molecular diffusion in the matrix at sub-Tg temperatures; both increasing temperature and relative humidity exert a strong influence on the degradation kinetics, accelerating the rate of pigment decomposition (Tsimidou & Biliaderis, 1997).

The crocins isomers (cis- and trans-) contributed in sample separation on the PC2 accounting for 16.78% of the total variance. Cis-crocins were all in the first quadrant of the PC1-PC2 cartesian plane and trans-crocins appeared closely localized in the second one. The cis-trans crocins differences were correlated to the thermal treatment. The majority of the "mild" samples had higher trans-crocins isomers, on the contrary a mix of cis and trans configuration was found in the samples with "H" saffron. It was not possible to distinguish samples from different Italian regions.

Results of the crocins isomers in our samples highlighted an important role of process and age/year of production. It is recognized that post-harvest and drying procedures, in particular temperature and time, significantly affect the amount of key quality constituents and lead to different metabolite patterns.

There is few information about the impact of the temperature on the fate of the crocins in general as well as the specific isomers and their kinetics. Data on the loss of carotenoids during food processing and storage are somewhat conflicting, but carotenoid degradation is known to increase with the destruction of the food cellular structure, increase of surface area or porosity, length and severity of the processing conditions, storage time and temperature, transmission of light and permeability to O_2 of the packaging (Rodriguez-Amaya, 1999).

To deepen the understanding of our analytical results, an accelerated thermal ageing under specific temperature and time combinations were investigated. Basilicata saffron, a fresh, first class ISO quality sample dried under mild thermal conditions (45 °C, 15 min) was selected for these tests, as characterized by the presence at a relative high concentration of all crocins isomers.

Heat treatments were carried out on saffron stigmas rehydrated at $a_W = 0.50$ to increase the moisture content and increase system mobility (Tsimidou and Biliaderis, 1997). In dried/low moisture content matrices, diffusion limited reactions are hindered by the glassy, immobile state where only rotational and vibrational movements are allowed (Fan & Roos, 2017). Moreover, different thermal ageing conditions were selected to reproduce a wide range of drying conditions that potentially could be used, from the milder ones, up to those applied to toasting (~200 °C) in Spain.

In Table 5, the variation (%) of the crocins isomers concentration, computed on the corresponding initial concentration value, is reported. For the sake of clarity, a positive value corresponds to an increased concentration of the isomer in respect to the initial saffron sample and, on the contrary, a negative value to a decrease of the content due to thermal degradation.

Results of Tukey's HSD post hoc test showed that there is a significant evolution of crocins with the different thermal treatment.

All crocins isomers showed a decrease when subjected to relatively mild heat treatments (100 and 125 °C), but trans-2II, whose presence remained unaffected. This result could be due to a higher stability of this isomer characterized by an esterification with two glucose moieties at opposite side, different in respect to the trans-2 where a gentiobiose unit (two glucoses moieties) in one extreme.

By increasing the temperature of the heat treatment (above the 125 °C/10 min), crocins tend to lose glucose and this seems to result to an increase both cis- and trans- isomers with a low number of glucose moieties (crocin-2 and crocin-1); the cis-1 is, acutely, the isomer that increases more due to the heat treatment; however, also cis-4 increased during the treatment.

Crocin-3 (both cis- and trans-isomers) and trans-4 isomers decreased during the dehydration process; On the other side the intense heat treatment at 200 °C caused the degradation of all crocins.

In the majority of foods, the all-trans configuration of most carotenoids predominates, although cis-isomers have been documented to be biochemically synthesized and found in unprocessed fruits and vegetables (Kopec, Cooperstone, Cichon & Scwartz, 2012). In agreement with this also in our case, trans isomers were more favorite than cis for all crocins except for crocin-1.

These data evidence that a mild heat treatment results in a higher content of trans-4 and trans-3, i.e. the crocins with higher number of glucose moieties. Increasing the temperature (hard treatment), samples showed a higher content of crocins with a lower number of glucose moieties especially in cis configuration that is particularly evident by the toasted sample (B) that was dried by a very intense heat treatment (Table 4).

4. Conclusion

A simple and fast procedure for the simultaneous determination of crocins has been developed by UHPLC-MS/MS, allowing the detection of all crocins isomers and isoforms in a single run. The extraction method was developed and optimized using experimental design in order to find the best conditions for all crocetin esters: MeOH/H_2O (50:50, v/v) solution provided a better extraction of all crocins, including the less polar ones; on the contrary, only water (according to ISO 3632-1 procedure) was not able to extract these molecules, especially crocin-1, which has only one glucose moiety.

The experimental data confirmed that geographical traceability can be obtained by crocins concentration and isomers pattern: i.e. Italian samples had a higher content of crocins than other countries. Moreover, important differences were also noticed among saffron samples from Austria, Greece, Iran and Lebanon that showed a high cis-crocins

Table 5
Variation (%, in respect to the initial) of crocins isomers content of Basilicata saffron subjected to different heat treatment conditions; results followed by the same case-letter, where not significantly different according to Turkey HSD post hoc test ($p > 0.05$).

Temperature (°C)	Crocin	cis1	cis2	cis2II	cis3	cis4	trans1	trans2	trans2II	trans3	trans4
	Time (min)										
100	5	-41.3^{cd}	-81.3^{cd}	-80.5^{cd}	-93.2^{c}	-70.4^{c}	-68.1^{c}	-78.3^{c}	112.0^{bc}	-80.6^{ab}	-63.3^{a}
100	10	-39.1^{cd}	-83.2^{cd}	-86.3^{cd}	-93.2^{c}	-74.2^{c}	-75.9^{c}	-75.3^{c}	73.5^{c}	-85.1^{a}	-63.8^{a}
125	5	-19.8^{cd}	-79.2^{cd}	-79.0^{cd}	-94.2^{c}	-65.6^{c}	-74.1^{c}	80.7^{c}	129.9^{bc}	-87.7^{ab}	-79.2^{b}
125	10	78.2^{c}	-59.2^{c}	-47.9^{c}	-92.3^{c}	-40.1^{b}	-58.0^{c}	-69.5^{c}	206.7^{ab}	-87.0^{ab}	-77.5^{b}
150	5	503.4^{b}	52.7^{b}	67.6^{b}	-82.9^{b}	-7.8^{a}	27.1^{b}	52.2^{b}	358.0^{a}	-78.4^{ab}	-74.3^{b}
150	10	867.2^{a}	113.7^{a}	191.5^{a}	-65.5^{a}	-26.0^{b}	97.6^{a}	186.3^{a}	264.5^{a}	-82.8^{ab}	-83.4^{b}
200	5	-47.9^{cd}	-96.8^{d}	-93.5^{d}	-96.7^{c}	-99.3^{d}	-91.8^{c}	-93.0^{c}	-79.3^{d}	-99.5^{b}	-99.6^{c}
200	10	-83.4^{d}	-99.2^{d}	-98.3^{d}	-99.4^{c}	-99.6^{d}	-90.0^{c}	-98.7^{c}	-93.5^{d}	-99.1^{ab}	-99.7^{c}

content and samples from Argentina, India, Turkey and Hungary that had high content of trans-crocins.

Multivariate analysis could be also used to distinguish saffron samples with different storage: fresh sample (< 1 year) have a high content of crocins with a higher number of glucose moieties than the other aged samples. The thermal treatment can influence the quality of saffron and results indicates that, increasing the temperature of the treatment, a degradation of saffron pigments with high number of glucose moieties occurs.

All crocins isomers showed a decrease when subjected to relatively mild heat, except for the trans-2II. By increasing the temperature of the heat treatment, crocins tend to lose glucose moieties increasing both cis- and trans- isomers with a low number of glucose units.

Results of the present study confirmed the feasibility of crocins isomers and isoforms in saffron to be used as marker of quality, process and traceability.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2018.04.111.

References

Acar, B., Sadikoglu, H., & Ozkaymak, M. (2011). Freeze drying of saffron (*Crocus sativus* L.). *Drying Technology, 29*(14), 1622–1627.

Anastasaki, E., Kanakis, C., Pappas, C., Maggi, L., del Campo, C. P., Carmona, M., & Polissiou, M. G. (2009). Geographical differentiation of saffron by GC-MS/FID and chemometrics. *European Food Research and Technology, 229*(6), 899–905.

Armellini, R., Compagnone, D., Scampicchio, M., & Pittia, P. (2017). Hydrogen and atom transfer activity of saffron extracts by square wave voltammetry. *Electroanalysis, 29*(2), 521–528.

Bukhari, S. I., Manzoor, M., & Dhar, M. K. (2018). A comprehensive review of the pharmacological potential of *Crocus sativus* and its bioactive apocarotenoids. *Biomedicine and Pharmacotherapy, 98*, 733–745.

Carmona, M., Zalacain, A., Pardo, J. E., López, E., Alvarruiz, A., & Alonso, G. L. (2005). Influence of different drying and aging conditions on saffron constituents. *Journal of Agricultural and Food Chemistry, 53*(10), 3974–3979.

Carmona, M., Zalacain, A. M., Sanchez, J. L., & Novella, G. L. A. (2006). Crocetin esters, picrocrocin and its related compounds present in *Crocus sativus* stigmas and gardenia jasminoides fruits. Tentative identification of seven new compounds by LC-ESI-MS. *Journal of Agricultural and Food Chemistry, 54*(3), 973–979.

Cossignani, L., Urbani, E., Simonetti, M. S., Maurizi, A., Chiesi, C., & Blasi, F. (2014). Characterisation of secondary metabolites in saffron from central Italy (Cascia, Umbria). *Food Chemistry, 143*, 446–451.

D'Archivio, A. A., Giannitto, A., Incani, A., & Nisi, S. (2014). Analysis of the mineral composition of Italian saffron by ICP-MS and classification of geographical origin. *Food Chemistry, 157*, 485–489.

D'Archivio, A. A., Giannitto, A., Maggi, M. A., & Ruggieri, F. (2016). Geographical classification of Italian saffron (*Crocus sativus* L.) based on chemical constituents determined by high-performance liquid-chromatography and by using linear discriminant analysis. *Food Chemistry, 212*, 110–116.

Fan, F., & Roos, Y. H. (2017). Glass transition-associated structural relaxations and applications of relaxation times in amorphous food solids: A review. *Food Engineering Reviews, 9*(4), 257–270.

Finley, J. W., & Gao, S. (2017). A perspective on *Crocus sativus* L. (saffron) constituent crocin: A potent water-soluble antioxidant and potential therapy for Alzheimer's disease. *Journal of Agricultural and Food Chemistry, 65*(5), 1005–1020.

Gregory, M. J., Menary, R. C., & Davies, N. W. (2005). Effect of drying temperature and air flow on the production and retention of secondary metabolites in saffron. *Journal of Agricultural and Food Chemistry, 53*(15), 5969–5975.

Guijarro-Díez, M., Castro-Puyana, M., Crego, A. L., & Marina, M. L. (2017). Detection of saffron adulteration with gardenia extracts through the determination of geniposide by liquid chromatography–mass spectrometry. *Journal of Food Composition and Analysis, 55*, 30–37.

ISO 3632-1:2011. Spices -Saffron (Crocus sativus L.) – Part 1: Specification.

Kopec, R. E., Cooperstone, J. L., Cichon, M. J., & Schwartz, S. J. (2012). Analysis methods of carotenoids. *Analysis of Antioxidant-Rich Phytochemicals, 4*, 105–148.

Lech, K., Witowska-Jarosz, J., & Jarosz, M. (2009). Saffron yellow: Characterization of carotenoids by high performance liquid chromatography with electrospray mass spectrometric detection. *Journal of Mass Spectrometry, 44*(12), 1661–1667.

Maggi, L., Carmona, M., Zalacain, A., Kanakis, C. D., Anastasaki, E., Tarantilis, P. A., & Alonso, G. L. (2010). Changes in saffron volatile profile according to its storage time. *Food Research International, 43*(5), 1329–1334.

Maggi, L., Carmona, M., Kelly, S. D., Marigheto, N., & Alonso, G. L. (2011). Geographical origin differentiation of saffron spice (*Crocus sativus* L. stigmas) – Preliminary investigation using chemical and multi-element (H, C, N) stable isotope analysis. *Food Chemistry, 128*(2), 543–548.

Marieschi, M., Torelli, A., & Bruni, R. (2012). Quality control of saffron (*Crocus sativus* L.): Development of SCAR markers for the detection of plant adulterants used as bulking agents. *Journal of Agricultural and Food Chemistry, 60*(44), 10998–11004.

Masi, E., Taiti, C., Heimler, D., Vignolini, P., Romani, A., & Mancuso, S. (2016). PTR-TOF-MS and HPLC analysis in the characterization of saffron (*Crocus sativus* L.) from Italy and Iran. *Food Chemistry, 192*, 75–81.

Nenadis, N., Heenan, S., Tsimidou, M. Z., & Van Ruth, S. (2016). Applicability of PTR-MS in the quality control of saffron. *Food Chemistry, 196*, 961–967.

Nescatelli, R., Carradori, S., Marini, F., Caponigro, V., Bucci, R., De Monte, C., & Secci, D. (2017). Geographical characterization by MAE-HPLC and NIR methodologies and carbonic anhydrase inhibition of saffron components. *Food Chemistry, 221*, 855–863.

Perez, M. (1995). *El Azafrán. Historia, cultivo, comercio, gastronomía.* Madrid, Spain: Mundi-Prensa.

Raina, B. L., Agarwal, S. G., Bhatia, A. K., & Gaur, G. S. (1996). Changes in pigments and volatiles of saffron (*Crocus sativus* L.) during processing and storage. *Journal of the Science of Food and Agriculture, 71*(1), 27–32.

Razak, S. I. A., Anwar Hamzah, M. S., Yee, F. C., Kadir, M. R. A., & Nayan, N. H. M. (2017). A review on medicinal properties of saffron toward major diseases. *Journal of Herbs, Spices and Medicinal Plants, 23*(2), 98–116.

Rodriguez-Amaya, D. (1999). Changes in carotenoids during processing and storage of foods. *Archivos Latinoamericanos de Nutricio, 49*, 38–47.

Rubert, J., Lacina, O., Zachariasova, M., & Hajslova, J. (2016). Saffron authentication based on liquid chromatography high resolution tandem mass spectrometry and multivariate data analysis. *Food Chemistry, 204*, 201–209.

Sarfarazi, M., Jafari, S. M., & Rajabzadeh, G. (2015). Extraction optimization of saffron nutraceuticals through response surface methodology. *Food Analytical Methods, 8*(9), 2273–2285.

Schumacher, S., Mayer, S., Sproll, C., Lachenmeier, D., & Kuballa, T. (2016). Authentication of saffron (Crocus sativus L.) using 1H nuclear magnetic resonance (NMR) spectroscopy. In Proceedings of the XIII International Conference on the Applications of Magnetic Resonance in Food Science 2016, Karlsruhe, DE.13.

Siracusa, L., Gresta, F., Avola, G., Albertini, E., Raggi, L., Marconi, G., & Ruberto, G. (2013). Agronomic, chemical and genetic variability of saffron (*Crocus sativus* L.) of different origin by LC-UV-vis-DAD and AFLP analyses. *Genetic Resources and Crop Evolution, 60*(2), 711–721.

Strain, H. H. (1963). cis-trans isomeric carotenoids, vitamins a and arylpolyenes. *Journal of the American Chemical Society, 85*(7) 1025–1025.

Tarantilis, P., & Tsoupras, G. P. M. (1995). Determination of saffron (*Crocus sativus* L.) components in crude plant extract using high-performance liquid chromatography-UV-visible photodiode-array detection-mass spectrometry. *Journal of Chromatography A, 699*(1–2), 107–118.

Tong, Y., Zhu, X., Yan, Y., Liu, R., Gong, F., Zhang, L., & Wang, P. (2015). The influence of different drying methods on constituents and antioxidant activity of saffron from China. *International Journal of Analytical Chemistry*. http://dx.doi.org/10.1155/2015/953164 953164.

Torelli, A., Marieschi, M., & Bruni, R. (2014). Authentication of saffron (*Crocus sativus* L.) in different processed, retail products by means of SCAR markers. *Food Control, 36*(1), 126–131.

Tsimidou, M., & Biliaderis, C. G. (1997). Kinetic studies of saffron (*Crocus sativus* L.) quality deterioration. *Journal of Agricultural and Food Chemistry, 45*(8), 2890–2898.

Tsimidou, M., & Tarantilis, A. P. (2017). Special issue "saffron (*Crocus sativus*, L.): Omics and other techniques in authenticity, quality, and bioactivity studies". *Molecules, 22*(10).

Vera-Candioti, L., De Zan, M. M., Cimara, M. S., & Goicoechea, H. C. (2014). Experimental design and multiple response optimization. Using the desirability function in analytical methods development. *Talanta, 124*, 123–138.

Yilmaz, A., Nyberg, N. T., Mølgaard, P., Asili, J., & Jaroszewski, J. W. (2010). 1H NMR metabolic fingerprinting of saffron extracts. *Metabolomics, 6*(4), 511–517.

Zalacain, A., Ordoudi, S. A., Diaz-Plaza, E. M., Carmona, M., Blazquez, I., Tsimidou, M. Z., & Alonso, L. A. (2005). Near-infrared spectroscopy in saffron quality control: Determination of chemical composition and geographical origin. *Journal of Agricultural and Food Chemistry, 53*(24), 9337–9341.

Zechmeister, L. (1944). Cis-trans isomerization and stereochemistry of carotenoids and diphenyl-polyenes. *Chemical Reviews, 34*(2), 267–344.