Food Chemistry 128 (2011) 543–548

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Geographical origin differentiation of saffron spice (*Crocus sativus* L. stigmas) – Preliminary investigation using chemical and multi-element (H, C, N) stable isotope analysis

Luana Maggi [a], Manuel Carmona [a], Simon D. Kelly [b], Niusa Marigheto [c], Gonzalo L. Alonso [a,*]

[a] Cátedra de Química Agrícola, E.T.S.I. Agrónomos, Universidad de Castilla-La Mancha, Campus Universitario, 02071 Albacete, Spain
[b] Stable Isotope Mass Spectrometry, Food and Environment Research Agency, Sand Hutton, York YO41 1LZ, UK
[c] Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK

ARTICLE INFO

Article history:
Received 3 March 2010
Received in revised form 9 November 2010
Accepted 11 March 2011
Available online 17 March 2011

Keywords:
Saffron
Geographical origin
Isotopic analysis
Chemical analysis

ABSTRACT

A preliminary study of the bulk hydrogen, carbon and nitrogen stable isotope composition of 28 authentic saffron samples produced from *Crocus sativus* L. cultivated in the typical production areas of Western Macedonia in Greece (8), Khorasan Province in Iran (7), Sardinia in Italy (6) and Castilla-La Mancha in Spain (7) is described. A chemical characterisation of 16 key quality parameters was also completed on the same samples by UV–Vis, HPLC and GC analyses. Multivariate analysis of the data revealed that 60.7% of saffron samples could be correctly assigned to their respective production countries using the chemical parameters. However, the combined bio-element stable isotope data reliably classified 100% of the saffron samples according to their respective geographical origins using posterior cross validation. Further work is required to establish the long-term stability of these models with respect to different years of production and other major producers such as India and Morocco.

1. Introduction

Saffron is one of the oldest and most expensive spices in the world. It is obtained from the red dried stigmas of *Crocus sativus* L. that is cultivated in several countries such as Iran, India, Greece, Morocco, Spain and Italy (Ghorbani, 2006). The price of saffron depends on its quality, which is closely related to the terroir of the production area in an analogous way to wine. Saffron quality is also strictly categorised and controlled according to the ISO 3632 (2003). Its main characteristics are colour (Carmona et al., 2005), taste (Carmona, Sánchez, et al., 2007) and aroma (Carmona, Zalacain, Salinas, & Alonso, 2007). The saffron colour is derived from the presence of various water soluble crocetin esters, commonly known as crocins (Carmona, Zalacain, Sánchez, Novella, & Alonso, 2006), while picrocrocin has always been considered as the main compound responsible for saffron's bitter taste (Corradi & Micheli, 1979) and to a lesser extent kaempferols (Carmona, Sánchez, et al., 2007). Saffron's aroma profile is relatively complex, being derived mainly from safranal (Alonso, Salinas, Estéban-Infantes, & Sánchez-Fernández, 1996).

Previously, Martin, Remaud, and Martin (1995) applied carbon stable isotope analysis to distinguish between safranal extracted from authentic saffron and safranal produced by chemical synthesis. One of the two synthetic safranal samples had a $\delta^{13}C‰$ value close to that of safranal extracted directly from saffron. As these two samples of safranal were indistinguishable, Martin et al. (1995) used this as a justification for the site specific $^{2}H/^{1}H$ ratio analysis of safranal by Nuclear Magnetic Resonance (SNIF-NMR™). Significant differences in the $^{2}H/^{1}H$ ratio were observed at each of the six molecular environments measured by SNIF-NMR. However, only one sample of synthetic safranal and one sample of safranal extracted from authentic saffron were reported and no discussion of the possibility of using isotope analysis to distinguish saffron geographical origin was reported. Semiond et al. (1996) also attempted to distinguish between synthetic safranal and safranal extracted from authentic saffron using carbon stable isotope analysis. They reported the successful discrimination of one synthetic safranal sample and five samples of safranal extracted from saffron using methanol and supercritical fluid extraction. However, the $\delta^{13}C‰$ values of extracted safranal differed significantly from those reported by Martin et al. (1995). Semiond et al. (1996) went onto discuss the use of the measured $\delta^{13}C‰$ values of safranal to distinguish geographical origin of saffron. They concluded that carbon isotope ratio analysis did not provide the means to distinguish the geographical origin of saffron. As *C. sativus* L. uses the Calvin cycle to fix carbon dioxide the major carbon isotope fractionation is that which occurs during photosynthesis irrespective of geographical origin. However, carbon isotope ratios from the same plant species are known to vary globally due to the effects of water stress

* Corresponding author. Tel.: +34 967 599310; fax: +34 967 599238.
E-mail address: Gonzalo.Alonso@uclm.es (G.L. Alonso).

0308-8146/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2011.03.063

on stomatal opening and concomitant effect on carbon dioxide diffusion and fractionation. Colder and more humid climates result in relatively depleted $\delta^{13}C‰$ values compared to $\delta^{13}C‰$ values derived from the same plant species growing in relatively hotter and less humid environments.

Multi-element isotopic analysis has been successfully applied to a range of foodstuffs to develop methods that will permit their geographical origins to be determined (Kelly, Heaton, & Hoogewerff, 2005). Carbon isotopes in foodstuffs do exhibit some geographical dependence linked to water stress and humidity during cultivation but the differences are very small in comparison to oxygen and hydrogen isotopes. The measurement of hydrogen and oxygen stable isotopes are applicable to the characterisation of geographical origin because they depend strongly on the latitude, distance from the sea and altitude, due to fractionation in the global hydrological cycle (Yuntseover & Gat, 1981). It has previously been demonstrated that the 'geographical signal' from water is transferred into plant and animal products (Hobson, 1999). Furthermore, nitrogen isotope compositions provide information about marine and terrestrial plants and also regional agricultural practices, especially the use of fertilisers used in organic and conventional agriculture (Bateman & Kelly, 2007).

Other analytical techniques and parameters have been studied to verify saffron origin such as, in terms of aroma by gas chromatography (Kanakis, Daferera, Tarantilis, & Polissiou, 2004), infrared spectroscopy (Anastasaki et al., 2010; Zalacain et al., 2005) or electronic nose (Carmona, Martínez, et al., 2006), free amino acids (Del Campo et al., 2009) and flavonoids content (Carmona, Sánchez, et al., 2007).

The aim of this research was to apply for first time multi-element stable isotope analysis to distinguish the geographical origin of saffron spice by measuring its hydrogen, carbon and nitrogen stable isotope composition. The isotopic composition was assessed along with chemical composition characteristics such as colour, taste and aroma parameters to establish the extent to which the geographical origin of saffron could be reliably determined using multivariate statistical analysis.

2. Materials and methods

2.1. Samples and reagents

This study involved 28 well-defined samples of saffron from leading producers (8 from Greece, 7 from Iran, 6 from Italy and 7 from Spain) from the same harvesting year (2006). Samples were obtained directly from the producers with the guarantee of their origin and lack of adulteration. Saffron is produced in a relatively restricted number of locations in these countries and so the samples used in this study were typically representative of those that would find their way into retail markets and were derived from *C. sativus* L. cultivated in the typical production zones of Western Macedonia (Greece), Khorasan Province (Iran), Sardinia (Italy) and Castilla-La Mancha (Spain). The samples were kept at 4 °C in absence of light until their analysis.

Cyclohexane, *n*-hexane and formic acid were purchased from Panreac (Barcelona, Spain), while acetonitrile from Scharlau (Barcelona, Spain). Safranal with purity of 98% was supplied by Sigma–Aldrich (Madrid, Spain) while water was purified through a Milli-Q System (Millipore, Bedford, MA, USA).

2.2. Chemical characterisation

2.2.1. Saffron working solution

Five hundred milligrams of ground saffron, previously passed through a sieve of 5 mm pore diameter, was placed in a volumetric flask (1 L) and 900 mL of distilled water were added. The solution was stirred by magnetic bar (1000 rpm) for an hour by keeping it away from light exposure. Subsequently, the flask was then filled to 1 L mark and the solution was homogenised through agitation.

2.2.2. Moisture and volatile matter content

Determination of moisture and volatile matter content of saffron was carried out according to the ISO 3632 (2003).

2.2.3. UV–Vis determinations

The analytical procedure employed to prepare the saffron solution and for recording the absorbance variation followed the specifications established at ISO 3632 (2003), were colouring strength was determined as $E^{1\%}_{1\ cm}$ 440 nm, picrocrocin as $E^{1\%}_{1\ cm}$ 257 nm, and safranal as $E^{1\%}_{1\ cm}$ 330 nm.

2.2.4. Identification and quantification of crocetin esters and picrocrocin by HPLC

Identification of crocetin esters has been carried out according to Carmona, Zalacain, et al. (2006). The nomenclature for the crocetin ester identified was: T-4GG (*trans*-crocetin di-(β-D-gentiobiosyl) ester), T-3Gg (*trans*-crocetin (β-D-glycosyl)-(β-D-gentibiosyl) ester), T-2G (trans-crocetin (β-D-gentiobiosyl) ester), C-4GG (*cis*-crocetin di-(β-D-gentiobiosyl) ester) and C-3Gg (*cis*-crocetin (β-D-glycosyl)-(β-D-gentiobioyl) ester).

The crocetin esters quantification was also estimated using the method based on the extinction coefficient and the related area calculated. Hence, the crocetin ester concentrations were calculated using the following expression: concentration(mg/100 mg) = $(A\times\ 100/A_t) \times (mw/\varepsilon) \times E^{1\%}_{1\ cm}440\ nm/10$, where the extinction coefficient (ε) was 89,000 $M^{-1}\ cm^{-1}$ and 63,350 $M^{-1}\ cm^{-1}$ for *trans*- and *cis*-crocetin esters, respectively (Speranza, Dadà, Manitto, Monti, & Gramatica, 1984). *A* was the area of the crocetin ester peak in the chromatogram and finally A_t was the total area of the crocetin esters. Finally $E^{1\%}_{1\ cm}440$ nm was the colouring strength of the samples, and mw was the molecular weight of the crocetin ester identified and quantified: T-4GG 977 g mol^{-1}, T-3Gg 815 g mol^{-1}, T-2G 653 g mol^{-1}, C-4GG 977 g mol^{-1}, C-3Gg 815 g mol^{-1}.

The identification of picrocrocin in the samples was carried out by the standard isolated according to Sánchez, Carmona, del Campo, and Alonso (2009). Picrocrocin calibration curve was: picrocrocin amount (mg picrocrocin/100 mg saffron) = (0.0708 ± 0.0004) × 50 × Area/m, R^2 = 0.9998, where Area was the area of the peak of picrocrocin in the chromatogram at 250 nm, *m* was the mass of the saffron and 0.0708 was the slope and 50 was the volume of the sample multiplied by 100 to the units correction.

2.2.5. Identification and quantification of volatile compounds by TD–GC–MS

The determination of volatile compounds was carried out according to Maggi et al. (2009). Identification was carried out by the selection of their respective *m*/*z*, using the NIST library. Not all volatile compounds present in saffron are commercially available as standards, so safranal has been used as external standard. The quantification of volatile compounds was performed with the equation (mg safranal kg^{-1} saffron = 10.88 + 36.76 × $Area_{safranal}$ where $Area_{safranal}$ = safranal peak area/10^6 in the GC chromatogram; R^2 = 0.998).For each sample, every chemical determination was carried out by triplicate.

2.3. Isotopic characterisation

2.3.1. Extraction procedure for isotopic analysis

Two hundred and fifty milligrams of ground saffron were weighed and introduced into a centrifuge tube and 10 mL of

n-hexane added. The tube was placed into the ultrasonic water bath and sonicated for 15 min. The temperature of the sonicated water bath did not exceed 25 °C. The tube was centrifuged for 15 min at 4000 rpm. The organic extract was removed to another tube and concentrated using a nitrogen flow of 1 mL/min. The residue was air-dried and sealed in an amber vial.

2.3.2. Simultaneous carbon and nitrogen stable isotope analysis

Samples of 1 mg of saffron defatted dry mass were weighed into tin capsules (6 mm × 4 mm, Elemental Microanalysis, Okehampton, UK). The tin capsule was sealed and placed inside a 'zero-blank' autosampler attached to a Eurovectar ECS4010 elemental analyser (Milan, Italy). A typical EA combustion/reduction configuration was used to produce carbon dioxide and nitrogen gas for $\delta^{13}C‰$ and $\delta^{15}N‰$ measurement, respectively (Vallet, Arendt, Mabon, Naulet, & Martin, 1991).

Samples were analysed in triplicate and values accepted when precision ($\sigma n-1$, $n = 3$) was <0.3‰ for $\delta^{15}N‰$ analysis and <0.2‰ for $\delta^{13}C‰$ analysis. Nitrogen and carbon isotope data are reported in conventional δ-notation in units of per mil (‰) with respect to atmospheric nitrogen (air) and Pee Dee Belemnite (PDB) respectively and according to Eq. (1),

$$\delta_{ref} = \left(\frac{R_{samp} - R_{ref}}{R_{ref}}\right) \times 1000 \quad (1)$$

where δ_{ref} is the isotope ratio of the sample expressed in delta units (‰, per mil) relative to the reference material. R_{samp} and R_{ref} are the absolute isotope ratios of the sample and reference material, respectively.

2.3.3. Hydrogen stable isotope analysis

Samples of 1 mg of saffron defatted dry mass were weighed into tin capsules (6 mm × 4 mm, Elemental Microanalysis, Okehampton, UK). As labile hydrogen in the saffron protein, which constitutes approximately 15% w/w of the defatted dry mass, is influenced by exchange with water vapour in the laboratory a comparative equilibration was conducted prior to analysis (Wassenaar & Hobson, 2003). This was achieved using an Inter-laboratory Comparison Material (ICM) – casein, which has similar thermodynamic characteristics to the measured protein. After equilibration the tin capsule was sealed and placed inside a 'zero-blank' autosampler attached to a Vecstar silicon carbide furnace maintained at 1300 °C. Thermo-chemical conversion of the sample to gaseous products was performed in an alumina tube over glassy carbon grit (Sigradur G, HTW, Thierhauptan, Germany). The pyrolysis gasses then passed through a GC column packed with molecular sieve (4 mm i.d., length 2 m) heated to 80 °C, which separated H_2, N_2 and CO.

The GC effluent then flowed into the stable isotope ratio mass spectrometer via a 'Conflo III' interface. The hydrogen gas generated from the saffron samples were compared against a hydrogen reference gas of known $^2H/^1H$ ratio previously calibrated against accepted International Atomic Energy Agency reference materials.

Measurements were made on three replicate weighings of the same sample of saffron defatted dry mass. Values were accepted when precision ($\sigma n-1$, $n = 3$) was ⩽3.0‰ for $\delta^2H‰$ analysis. Hydrogen isotope data were reported in conventional δ - notation in units of per mil (‰) with respect to Vienna – Standard Mean Ocean Water and according to Eq. (1) above and corrected for the formation of H_3^+ in the ion source.

2.4. Multivariate analysis of HCN stable isotope and chemical data for authentic saffron

Statistical analysis was performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL). Discriminant analysis was applied with the aim of finding a rule that allocated saffron samples of unknown origin to the correct group.

To verify the power and the stability of the model, a 'leave-one-out' cross validation discriminant analysis was performed. In this test known samples were used as 'unknowns' to validate the model built on the basis of a reduced set of cases. Effectively, one sample is removed from the data set and then classified on the basis of model constructed from the remainder. This process is repeated for each sample in turn and the success of the classification re-calculated by comparison with the known origin.

3. Results and discussion

3.1. Chemical characterisation

The chemical characterisation of 28 saffron samples was reported in the Tables 1 and 2. In all samples, moisture and volatile matter content was lower than 12%, maximum limit established by ISO 3632 (2003) in order for *C. sativus* L. to be considered as saffron spice. The average content ranged between 6.90% for Spain and 8.69% for Italy. Colouring strength is one of the main parameter employed by saffron trade companies in order to determine price and the 28 samples belonged to the category I, since they showed values higher than 190 ucs. With respect to the total crocetin esters content, it varied from 21.93% for Iranian samples to 27.35% for Italian ones, considering the five main crocetin esters. The picrocrocin content ranged between 9.36% and 15.50%, respectively for Iran and Italy, whereas Greece and Spain obtained intermediate values. The trend of crocetin esters and picrocrocin is in agreement with the bibliography (Sánchez et al., 2009). As it can be observed in Table 2, the first four volatile compounds responsible for saffron aroma were reported. Safranal represented the main constituent of saffron volatile composition and its content was 65%, 75%, 81% and 83% of volatile fraction, respectively for Iran, Italy, Greece and Spain with levels similar to those reported by others Kanakis et al. (2004). Therefore, the values for isophorone, 4-ketoisophorone and 2,2,6-trimethyl-1,4-cyclohexanedione were according to the scientific bibliography (Maggi et al., 2009).

Table 1
Quality characteristics, total and individual crocetin esters composition and content of picrocrocin.

Country (#)	Moisture and volatile matter content (%)[a]	Colouring strength (E440 nm)[a]	Crocetin esters (mg/100 mg)						Picrocrocin (mg/100 mg)
			Trans-4GG	*Trans*-3Gg	*Trans*-2G	*Cis*-4GG	*Cis*-3Gg	Total	
Greece (8)	8.30 ± 0.36	245.18 ± 16.33	11.24 ± 1.48	6.66 ± 0.69	1.22 ± 0.61	2.62 ± 0.51	1.40 ± 0.32	23.14 ± 2.13	11.51 ± 2.12
Iran (7)	8.55 ± 1.09	230.22 ± 37.08	11.77 ± 2.95	6.49 ± 1.04	1.18 ± 0.37	1.68 ± 0.58	0.82 ± 0.17	21.93 ± 4.17	9.36 ± 2.13
Italy (6)	8.69 ± 0.60	274.60 ± 17.41	16.52 ± 1.01	7.48 ± 0.56	0.97 ± 0.38	1.64 ± 0.31	0.74 ± 0.15	27.35 ± 1.48	15.50 ± 1.52
Spain (7)	6.90 ± 0.97	236.92 ± 42.32	12.34 ± 2.59	7.14 ± 1.58	0.62 ± 0.24	2.16 ± 1.15	0.94 ± 0.50	23.20 ± 4.49	13.68 ± 2.64

[#]Number of samples analysed for each country.
[a] SD lower than 2%.

Table 2
Individual and total content of main volatile chemical characterisation by GC/MS/MS.

Country (#)	Compound (mg/kg)				
	Safranal	Isophorone	4-Ketoisophorone	2,2,6-Trimethyl-1,4-cyclohexanedione	Total volatile compounds
Greece (8)	1361.49 ± 510.75	239.75 ± 31.69	31.05 ± 9.34	59.00 ± 5.98	1691.28 ± 510.26
Iran (7)	440.87 ± 216.36	150.46 ± 57.01	28.96 ± 7.90	58.49 ± 15.74	678.78 ± 289.64
Italy (6)	1084.79 ± 229.26	233.51 ± 62.90	53.95 ± 15.84	75.77 ± 20.08	1448.02 ± 320.71
Spain (7)	977.67 ± 312.88	114.06 ± 56.31	25.43 ± 9.89	55.76 ± 18.35	1172.92 ± 329.21

[#]Number of samples analysed for each country.

3.2. Statistical evaluation of the combined chemical parameters

A stepwise Canonical Discriminant Analysis (CDA), with posterior cross-validation of the model, was completed on the 16 chemical parameters measured in 28 authentic saffron derived from *C. sativus* L. cultivated in Greece, Italy, Iran and Spain. The first two discriminant functions describe 97.6% of the variation in the data and 60.7% of cross-validated grouped cases of saffron were correctly classified according to their geographical origin. About 100% of Italian saffron samples ($n = 6$) were correctly classified. Five of the seven Spanish samples (71.4%) were classified correctly according to their origin, with one sample misclassified as Greek and another misclassified as Iranian. Five of the eight Greek saffron samples (62.5%) were correctly classified according to their geographical origin with the remaining three misclassified as Iranian. Only one out of seven Iranian saffron samples (14.3%) was correctly classified with three classified as Greek, two as Spanish and one as Italian.

Function 1 of the CDA described 62.4% of the variance in the data, was highly correlated with the concentration of 4-ketoisophorone, and separated the Italian saffron samples from the remaining Greek, Iranian and Spanish saffron samples. Function 2 described 35.1% of the variation in the data, was highly correlated with % moisture, picrocrocin and isophorone concentration, and provided some separation between Greek and Spanish saffron but little or no separation of Iranian with respect to Italian saffron.

3.3. Hydrogen, carbon and nitrogen stable isotope characterisation of saffron

3.3.1. Hydrogen isotope data ($\delta^2H‰$)

Hydrogen isotopes in plant tissues reflect the hydrogen isotope ratio of the groundwater available to the plant during its growth, which is in turn, is related to the precipitation water for a given region. However, it is critical to acknowledge any biological isotope fractionation that occurs during the assimilation of the water into the plant tissues during biosynthesis. The size of the fractionation for different plant-derived food components such as intra-cellular water, carbohydrates, proteins and lipids is different. As the substance under investigation in our study is the defatted dry matter (DDM) from saffron, it mainly consists of carbohydrate (ca. 85% w/w) and protein (ca. 15% w/w). Consequently, the bulk hydrogen isotope ratio will be dependent on the relative proportions of these latter two nutrients to some extent.

The DDM mean $\delta^2H‰$ results, along with the samples standard deviation, maximum and minimum for authentic saffron cultivated in Greece, Iran, Italy and Spain are shown in Table 3. The highest deuterium contents observed were for saffron produced in the most Southerly location, Iran, with a mean $\delta^2H‰$ value of −67.8‰ and ranging from −59.9‰ to −70.8‰. These results are consistent with the meteorological conditions of the Iranian saffron production area in the southern and central regions of Khorasan Province. Of the four saffron growing locations studied, this is the most Southerly and experiences the hottest and least humid conditions with approximately 120–210 mm of rainfall per annum, humidity of 40–50%, maximum average temperature of 34 °C and is approximately 500 km from the Indian Ocean. The continental effect of depleted input water is counteracted by the significantly higher rates of evapo-transpiration leading to relatively deuterium-enriched plant components.

The Italian saffron was produced in Sardinia and exhibited the next highest deuterium content with a mean $\delta^2H‰$ value of −72.4‰ and ranging from −70.4‰ to −74.0‰. The growing location near Villanovafranca is the closest to the sea and located more or less at sea level (51 m). Consequently, a significant deuterium depletion associated with continental and altitude effects is not expected. However, local climatic conditions for the growing location in Sardinia exhibit relatively high annual rainfall of approximately 560 mm, humidity of 60–75% and a maximum average temperature of 27 °C and therefore deuterium enrichment due to evapo-transpiration is expected to be relatively low compared to a location such as Khorasan.

DDM derived from saffron cultivated in Castilla-La Mancha, Spain has the next lowest mean $\delta^2H‰$ value of −75.1‰ with a range from −70.9‰ to −80.5‰. This growing location between 500 and 700 m above sea-level, 170 km from the Mediterranean Sea and so the continental effect on groundwater values will be significant. The maximum average temperature is 36 °C, humidity 60–85% and average rainfall around 400 mm per annum.

The Greek saffron possesses the lowest mean $\delta^2H‰$ value of −84.7‰ with a range from −80.1‰ to −87.5‰. This saffron is cultivated in the most Northerly location, in Western Macedonia at an altitude of around 700 m above sea-level and approximately 70 km from the Aegean Sea. It experiences the highest rainfall of the four growing locations studied at approximately 700 mm per annum, relatively high humidity of 60–70% and an equal highest maximum average temperature of 36 °C. The Northerly location and relatively high humidity will contribute to relatively deuterium-depleted

Table 3
Mean value of $\delta^2H‰$, $\delta^{13}C‰$ and $\delta^{15}N‰$ along with the standard deviation (sd), the values maximum (max) and minimum (min) per country.

Country ()[a]	$\delta^2H‰$	Max	Min	$\delta^{13}C‰_{PDB}$	Max	Min	$\delta^{15}N‰_{AIR}$	Max	Min
Greece (8)	−84.7 ± 2.6	−80.1	−87.5	−26.6 ± 0.2	−26.3	−26.8	1.2 ± 0.6	2.0	0.6
Iran (7)	−67.8 ± 3.9	−59.9	−70.8	−24.5 ± 0.2	−24.2	−24.8	2.2 ± 1.0	3.4	0.7
Italy (6)	−72.4 ± 1.5	−70.4	−74.0	−26.6 ± 0.1	−26.5	−26.8	5.3 ± 0.7	6.2	4.3
Spain (7)	−75.1 ± 3.2	−70.9	−80.5	−24.5 ± 0.4	−23.9	−25.1	4.2 ± 0.6	5.0	3.4

[a] Number of samples analysed for each country.

input water into biosynthesis and relatively deuterium-depleted plant tissues.

3.3.2. Carbon isotope data ($\delta^{13}C‰$)

Carbon isotope fractionation in plants is dominated by the photosynthetic pathway utilised by the plant to fix atmospheric carbon dioxide during photosynthesis (discrimination between C3 and C4 plants) and on the plant age and level of maturation (Farquhar, Ehleringer, & Hubick, 1989; Smith & Epstein, 1971) and can be influenced to a much lesser extent by several environmental factors, such as relative humidity, temperature, amount of precipitation and water stress (O'Leary, 1995). *C. sativus* L., like the majority of terrestrial plants, is discriminated against $^{13}CO_2$ and leads to characteristic $\delta^{13}C‰$ values. The saffron DDM measured here reflects that pathway with values ranging from −23.9‰ in Spanish saffron to −26.8‰ observed in Italian and Greek saffron (see Table 3). Despite the relatively narrow range of $\delta^{13}C‰$ values measured in the saffron samples the usefulness of this parameter, in combination with $\delta^{2}H‰$, to resolve the origin of the saffron can be seen in Fig. 1. The Italian and Greek saffron samples are clearly separated from the Iranian and Spanish samples on the basis of their respective $\delta^{13}C‰$ (x-axis).

3.3.3. Nitrogen isotope data ($\delta^{15}N‰$)

The $\delta^{15}N‰$ values of saffron will depend to a large extent on the agricultural practices used during cultivation such as application of fertilisers and precedent land use (Bateman & Kelly, 2007; Bateman, Kelly, & Jickells, 2005). For example, Spanish *C. sativus* L. is cultivated in a 4 years cycle and is only manured once before planting at a rate of 15–20,000 kg/ha in the months of March and April. This is usually the only application of nutrients in the 4 years cultivation cycle (Perez-Bueno, 1995).

The relatively low mean $\delta^{15}N‰$ values in Greek and Iranian saffron DDM samples (1.2‰ and 2.2‰, respectively) suggests that the crocuses may have been fertilised with synthetic nitrogen. This is because the nitrogen present in synthetic fertiliser originates from air, via the Haber process, and this produces ammonium and nitrate with $\delta^{15}N‰$ values between +2‰ and −2‰ which is translocated from the soil to the plant material (Bateman & Kelly, 2007). However, it is possible that these values could be obtained after a crop rotation, or application of green-manure, involving nitrogen fixing plants such as clover. Conversely the Italian and Spanish saffron DDM samples possess mean $\delta^{15}N‰$ of 5.3‰ and 4.2‰ respectively, which are typical of materials derived from plants that have been fertilised with animal manures. This parameter permits the Italian and Greek saffron samples to be clearly distinguished in Fig. 1. The Iranian and Spanish samples are the most closely related in terms of their isotopic values but they are just resolved by their respective $\delta^{15}N‰$. However, it is acknowledged that examination of a larger set of samples may result in a higher degree of overlap in $\delta^{15}N‰$ values.

3.4. Statistical evaluation of the combined HCN stable isotope data

A three-dimensional scatter-plot of the combined $\delta^{2}H‰$, $\delta^{15}N‰$ and $\delta^{13}C‰$ data for the authentic saffron DDM obtained from *C. sativus* L., cultivated in Greece, Iran, Italy and Spain shows that the combined HCN stable isotope data has the capability to resolve the four countries of origin. However, to quantitatively assess this observation a Canonical Discriminant Analysis (CDA) was performed with posterior cross-validation of the model constructed from the combined HCN stable isotope data. One hundred percent of the cross-validated grouped cases were correctly classified on the basis of geographical origin. Function 1 described 79.2% of the variance in the data and separated Italy and Greece from Spain and Iran. Function 1 was correlated with the isotopic parameters in the order carbon, hydrogen and nitrogen. Function 2 described 18% of the variance in the data and resolves Italian saffron from Spanish, Greek and Iranian saffron. Function 2 was correlated with the isotopic parameters in the order nitrogen, hydrogen and carbon.

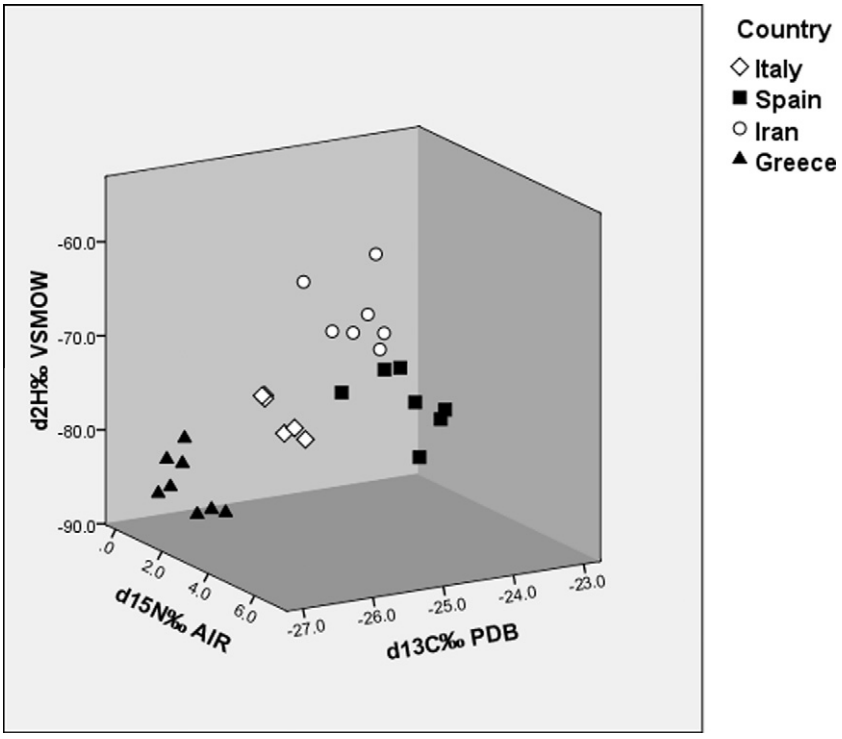

Fig. 1. Three dimensional plot of data obtained from isotopic characterisation of authentic saffron spice, derived from *Crocus sativus* L., cultivated in Spain, Italy, Greece and Iran.

Function 3 described 2.8% of the variance in the data and provided the separation between the Spanish and Iranian saffron samples. Function 3 was correlated with the isotopic parameters in the order hydrogen, carbon and nitrogen.

4. Conclusion

Although the limited number of samples from only 1 year, the chemical analysis of saffron provides important parameters for the classification of its quality according to ISO 3632 and permits quantification of compounds that give rise to its characteristic colour, taste and aroma. These parameters, in some instances, can provide unique chemical markers for saffron from a particular country such as the concentration of 4-ketoisophorone, which is highly characteristic of the Italian saffron samples analysed in this study. However, analysis of the stable isotopes of the bio-elements hydrogen, carbon and nitrogen has proved to be a very reliable means of discriminating the geographical origin of saffron, in this study, derived from *C. sativus* L. cultivated in the typical production zones of Western Macedonia in Greece, Khorasan Province in Iran, Sardinia in Italy and Castilla-La Mancha in Spain. Further work is required to establish the long-term stability of these models with respect to different years of production and other major producers such as India and Morocco. Undoubtedly, the incorporation of sulphur isotope, strontium isotope and trace element data would lead to further refinement of the geographical origin classification model for this important spice.

Acknowledgements

We thank the Consejería de Educación y Ciencia of the JCCM and the European Social Fund for funding this work with the Exp. 09/09-C; the Ministerio de Educación y Ciencia and FEDER (CE) for the AGL2007-64092/ALI project.

References

Alonso, G. L., Salinas, M. R., Estéban-Infantes, F. J., & Sánchez-Fernández, M. A. (1996). Determination of safranal from saffron (*Crocus sativus* L.) by thermal desorption–gas chromatography. *Journal of Agricultural and Food Chemistry, 44*, 185–188.

Anastasaki, E., Kanakis, C., Pappas, C., Maggi, L., del Campo, C. P., Carmona, M., et al. (2010). Differentiation of saffron from four countries by multivariate analysis of mid-infrared spectroscopy. *European Food Research and Technology, 230*, 571–577.

Bateman, A. S., Kelly, S. D., & Jickells, T. D. (2005). Nitrogen isotope relationship between crops and fertilizer: Implications for using nitrogen isotope analysis as an indicator of agricultural regime. *Journal of Agricultural and Food Chemistry, 53*(14), 5760–5765.

Bateman, A. S., & Kelly, S. D. (2007). Fertilizer nitrogen isotope signatures. *Isotopes in Environmental and Health Studies, 43*, 237–247.

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

Carmona, M., Zalacain, A., Sánchez, A. M., Novella, J. L., & Alonso, G. L. (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*, 973–979.

Carmona, M., Martínez, J., Zalacain, A., Rodríguez-Méndez, M. L., de Saja, J. A., & Alonso, G. L. (2006b). Analysis of saffron volatile fraction by TD–GC–MS and e-nose. *European Food Research and Technology, 223*, 96–101.

Carmona, M., Sánchez, A. M., Ferreres, F., Zalacain, A., Tomás-Barberán, F., & Alonso, G. L. (2007). Identification of the flavonoid fraction in saffron spice by LD/DAD/MS/MS: Comparative study of samples from different geographical origins. *Food Chemistry, 100*, 445–450.

Carmona, M., Zalacain, A., Salinas, M. R., & Alonso, G. L. (2007). A new approach to saffron aroma. *Critical Reviews in Food Science and Nutrition, 47*, 145–159.

Corradi, C., & Micheli, G. (1979). Determinazione spettrofotometrica del potere colorante, amaricante ed odoroso dello zafferano. *Bollettino Chimico Farmaceutico, 118*, 553–562.

Del Campo, C. P., Garde-Cerdán, T., Sánchez, A. M., Maggi, L., Carmona, M., & Alonso, G. L. (2009). Determination of free amino acids and ammonium ion in saffron (*Crocus sativus* L.) from different geographical origins. *Food Chemistry, 114*, 1542–1548.

Farquhar, G., Ehleringer, J., & Hubick, K. (1989). Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology, 40*, 503–537.

Ghorbani, M. (2006). The economics of saffron in Iran. II. International symposium on saffron biology and technology. *ISHS Acta Horticulturae, 739*, 321–332.

Hobson, K. A. (1999). Tracing origins and migration of wildlife using stable isotopes: A review. *Oecologia, 120*, 314–326.

ISO 3632-1/2 (2003). Technical specification. Saffron (*Crocus sativus* L.). Part 1 (specification) and part 2 (test methods). Genève, Switzerland: International Organisation for Standardization.

Kanakis, C. D., Daferera, D. J., Tarantilis, P. A., & Polissiou, M. G. (2004). Qualitative determination of volatile compounds and quantitative evaluation of safranal and 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) in Greek saffron. *Journal of Agricultural and Food Chemistry, 52*(14), 4515–4521.

Kelly, S. D., Heaton, K., & Hoogewerff, J. (2005). Tracing the geographical origin of food: The application of multi-element and multi-isotope analysis. *Trends in Food Science and Technology, 16*, 555–567.

Maggi, L., Carmona, M., del Campo, C. P., Kanakis, C. D., Anastasaki, E., Tarantilis, P. A., et al. (2009). Worldwide market screening of saffron volatile composition. *Journal of the Science of Food and Agriculture, 89*, 1950–1954.

Martin, G., Remaud, G., & Martin, G. J. (1995). Food flavours – Generation, analysis, and process influence. In C. Charalambous (Ed.), *Proceedings of the 8th international flavour conference, 6–8 July 1994, Cos, Greece, international flavour conference* (pp. 355–378). Amsterdam, New York: Elsevier.

O'Leary, M. (1995). In E. Wada, T. Yoneyama, M. Mingawa, T. Ando, & B. D. Fry (Eds.), *Stable isotopes in the biosphere* (pp. 78–91). Kyoto: Kyoto University Press.

Perez-Bueno, M. (1995). *El azafrán*. Madrid: Mundi-Prensa.

Sánchez, A. M., Carmona, M., del Campo, C. P., & Alonso, G. L. (2009). Solid-phase extraction for picrocrocin determination in the quality control of saffron spice (*Crocus sativus* L.). *Food Chemistry, 116*, 792–798.

Semiond, D., Dautraix, S., Desage, M., Majdalani, R., Casabianca, H., & Brazier, J. L. (1996). Identification and isotopic analysis of safranal from supercritical fluid extraction and alcoholic extracts of saffron. *Analytical Letters, 29*, 1027–1039.

Smith, B. N., & Epstein, S. (1971). Two categories of $^{13}C/^{12}C$ ratios for higher plants. *Plant Physiology, 47*, 380–384.

Speranza, G., Dadà, G., Manitto, P., Monti, D., & Gramatica, P. (1984). 13-cis-Crocin: A new crocinoid of saffron. *Gazzetta Chimica Italiana, 114*, 189–192.

Vallet, C., Arendt, M., Mabon, F., Naulet, N., & Martin, G. J. (1991). Combination of mass-spectrometry and site-specific NMR isotope analyses in the characterization of amino-acids. *Journal of the Science of Food and Agriculture, 56*(2), 167–185.

Wassenaar, L. I., & Hobson, K. A. (2003). Comparative equilibration an online technique for determination of non-exchangeable hydrogen of keratins for use in animal migration studies. *Isotopes in Environmental and Health Studies, 39*(3), 211–217.

Yuntseover, Y., & Gat, J. R. (1981). Atmospheric waters. *IAEA technical reports series 210-stable isotope hydrology* (pp. 103–142).

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