$\delta^{13}C$ data of the total water-soluble fraction and triacylglycerols as related indexes for differentiating the geographical origin of saffron (*Crocus sativus* L.)

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ABSTRACT

Using isotopic ratio mass spectrometry (IRMS) measurements, this study analyzed samples of saffron originating from two distinct geographical regions. We then used the results to distinguish saffron of the two considered origins.

$\delta^{13}C$ data related to the crocin fractions in 48 saffron samples from Western Macedonia (Greece) and 48 samples from Khorasan Province (Iran) were correlated to an index derived from triacylglycerols. Isotopic data could clearly differentiate between samples from the two areas. The isotopic measurements were -28.3 to -26.9 for Greek samples, and -26.1 to -24.5 for Iranian samples. Another index, derived from a gas-chromatographic analysis of the triacylglycerols, successfully determined that the range of isotopic values that characterized Greek samples was 52% larger than the range that characterized Iranian samples. The application of statistical evaluations permitted us to identify the two groups of saffron with confidence and to accurately identify the site of origin of a saffron sample.

1. Introduction

In recent years, various approaches have been adopted to assess saffron authenticity and to characterize saffrons from different geographic origins (Anastasaki et al., 2009; Bosmali, Ordoudi, Tsimidou, & Madesis, 2017; Cagliani, Culeddu, Chessa, & Consonni, 2015, Consonni, Ordoudi, Cagliani, Tsiangali, & Tsimidou, 2016; D'Archivio, Giannitto, Incani, & Nisi, 2014; D'Archivio & Maggi, 2017; Guijarro-Diez, Nozal, Marina, & Crego, 2015; Karabagias, Koutsoumpou, Liakou, Kontakos, & Kontominas, 2015; Nescatelli et al., 2017; Parizad et al., 2019; Petrakis & Polissiou, 2017; Rubert, Lacina, Zachariasova, & Hajslova, 2016; Siracusa et al., 2013; Sobolev et al., 2014, Tahri et al., 2015). The many papers published, including those cited above, demonstrate the interest in analytical methods that provide data to confirm the site of origin of a saffron sample. Controlled supply chain declarations, especially with respect to high market value products, are relevant for commercial and corporate image purposes. There is also growing interest in isotopic data resulting from scientific investigations, given that such data are often influenced by factors linked to the territory and the production environment. The ISO 3632-2 (ISO, 2010) method provides calculations for the various indices for safranal, picrocrocin, and total crocins, based on non-specific spectrophotometric measures. Alternatively, gas chromatography (GC) is commonly used to study the specific chemical compounds present in the saffron matrix with gas-chromatography (GC) (Bononi, Milella, & Tateo, 2015; Condurso, Cincotta, Tripodi, & Verzera, 2017), as are GC in tandem with mass spectrometry (GC/MS) (Kanakis, Daferera, Tarantilis, & Polissiou, 2004; Tarantilis & Polissiou, 1997), isotopic analysis (Semiond, Dautraix, Desage, Majdalan, Casabianca, & Brazier, 1996), near infrared spectroscopy (NIR) (Zalacain et al., 2005), high performance liquid chromatography coupled with mass spectrometry (LC/MS) (Carmona, Sánchez,

Ferreres, Zalacain, Tomas-Barberan, & Alonso, 2007; D'Archivio, Giannitto, Maggi, & Ruggieri, 2017; Verma & Middha, 2010), and GC-olfactometry (Amanpour, Sonmezdag, Kelebek, & Selli, 2015; Culleré, San-Juan, & Cacho, 2011). Other studies have been conducted to measure the total phenolic content, radical scavenging activity, reducing power, and specific compounds in saffron extracts (Assimopoulou, Sinakos, & Papageorgiou, 2005; Kyriakoudi, Chrysanthou, Mantzouridou, & Tsimidou, 2012; Karabagias et al., 2017).

Isotopic analyses with chemometrics are often considered useful for tracing spices (Frank, Dietrich, Kremer, & Mosandl, 1995, Jelínek, Dolečková, Karabín, Hudcová, Kotlíková, & Dostálek, 2012; Ramakrishna & Ravishankar, 2011). Among spices, saffron is one of the most interesting. It is appreciated in cuisine for its flavor, but it has also drawn pharmaceutical interest, because the crocetin sugar esters, or crocins, are thought to exert biological activities. Currently, evaluating the molecular identity of saffron is the preferred strategy for conducting authentication studies. Various techniques have been used, including metabolic fingerprinting (Rubert et al., 2016), species-specific molecular markers (Bosmali et al., 2017), metabolomics with Fourier-transform infrared spectroscopy and nuclear magnetic resonance spectroscopy (Consonni et al., 2016), metabolomic fingerprinting with liquid chromatography/mass spectrometry (LC/MS) (Guijarro-Diez et al., 2015), microwave-assisted extraction – high-performance liquid chromatography (MAE)-HPLC, and near infrared spectroscopy (NIR) methodologies (Nescatelli et al., 2017).

Previous studies characterized saffrons from different countries by measuring specific chemical compounds in the matrix (Carmona et al., 2007; Culleré et al., 2011; D'Archivio et al., 2017; Kanakis et al., 2004; Karabagias et al., 2017; Tarantilis & Polissou, 1997). In particular, the total phenolic content of saffron reflecting the geographical origin and the correlation between specific volatile compounds and antioxidant activity parameters have been studied (Karabagias et al., 2017). Isotopic ratio mass spectrometry (IRMS) was previously

77 adopted to investigate the volatile compound safranal extracted from saffron by two different
78 methods (Semiond et. al, 1996).
79 Our goal was to develop a method that might be useful for routine analyses and to produce a
80 rapid measure with elemental analyzer/isotope ratio mass spectrometry (EA-IRMS).
81 Considering that the most abundant compounds in saffron are crocins, we evaluated the
82 feasibility of using an approach to measure IRMS data correlated to this fraction. We
83 considered that the ISO method uses an aqueous saffron extract that is analysed with
84 ultraviolet-visible spectrophotometry (ISO, 2010) to calculate the crocins index. To simplify
85 the extraction process, we directed our attention to the total water-soluble fraction of saffron,
86 based on the high water solubility of crocins.
87 We performed numerous IRMS measures on genuine samples that were derived from two
88 guaranteed origins (Western Macedonia - Greece and Khorasan Province - Iran). These EA-
89 IRMS data will contribute to current knowledge of the isotopic ratio of saffron components.
90 Previously, $\delta^{13}C$ had been rarely used to support statistical applications or to determine
91 correlations with other analytical data (Maggi, Carmona, Kelly, Marigheto, & Alonso, 2011).
92 To determine correlations with other analytical data, we used the ratio index derived from a
93 gas-chromatography–on column injection (GC-OCI) analysis of triacylglycerols (TAGs), a
94 chemico-physical variable that had not been used previously in combination with the $\delta^{13}C$
95 variable in statistical evaluations. Attention was focused on these two chemicals useful to
96 saffron characterization that, up to now, had not been taken into consideration in the literature.
97 The indexes choice is not coincidental: the $\delta\ ^{13}C$ index is intended to represent the result of the
98 Carbon exchange between the plant and the biosphere, and therefore it can have specificities
99 (soil, air, vegetable species). TAG values can likewise be deemed to be specific indices since
100 they are derived from a system of natural multi-enzymatic synthesis, and so affected by both
101 the vegetable species and the growth environment of the plant.

2. Materials and methods

2.1 Chemicals and reagents

Isooctane for GC was purchased from Sigma Aldrich (Milan, Italy). Hydrophilic polypropylene membrane filters (0.45 mm, GH Polypro, Pall) were purchased from VWR International (Milan, Italy).

2.2 Samples

A total of 96 samples of raw saffron (stigmas dried by the supplier at humidity 10-12%) were collected during the quality evaluation of an Italian leader company in 2015-2017. These samples were derived from guaranteed origins (Western Macedonia – Greece, and North, South, and Razavi Khorasan Provinces - Iran).

2.3 Extraction of the water-soluble fraction

Approximately 200 mL of distilled water was added to 500 mg of ground dried stigmas in a 200 mL glass flask. The mixture was vortexed for 5 min and submitted to ultrasound extraction (USE) for 30 min at 25 °C at a fixed frequency of 35 kHz. The extract was filtered through a polypropylene membrane filter (0.45-μm pores, 25-mm diameter). We stored 100 mL of the filtered solution (stock solution) at −80 °C overnight (12 h), and then lyophilized the frozen solution in a SCANVAC-CoolSafe 100-4 (Colaver, Italy). The lyophilized samples were stored in the dark until needed for the EA-IRMS analyses.

2.4 Extraction of the triacylglycerol fraction

126 Approximately 100 mg of ground dried stigmas were added to 1 mL of isooctane. The mixture
127 was vortexed for 1 min and submitted to USE for 30 min at 25 °C at a fixed frequency of 35
128 kHz. The extract was filtered through a polypropylene membrane filter (0.45-µm pores, 25-mm
129 diameter). A sample (2 µL) of the isooctane phase was analyzed with GC-on column injection
130 (GC-OCI) to determine triacylglycerol (TAG) content.
131
132 *2.5 EA−IRMS analysis*
133 $\delta^{13}C$ values of saffron extracts were measured according to conditions described previously
134 (Bononi, Quaglia, & Tateo, 2015) with a Flash 2000 EA, coupled to a Delta V Advantage
135 (Thermo Fisher, Bremen, Germany) via a ConFlo IV interface. Briefly, the EA was operated
136 with a 100 mL min^{-1} helium flux and temperatures of 950 °C, in the oxidation tube, and 850
137 °C, in the reduction tube. The outlet was equipped with a column that physically retained CO_2
138 at 70 °C; CO_2 was released by increasing the temperature to 210 °C. The overall experiment
139 duration was 600 s. The $\delta^{13}C$ values (‰) were calibrated to Vienna Pee Dee Belemnite with
140 three pulses of CO_2 reference gas, and then calibrated against the international standard.
141 Calibrations were performed at the beginning of the elution run. Samples were weighed in tin
142 capsules (5 × 9 mm). Samples were analyzed in triplicate, and values were accepted when the
143 precision was <0.3‰ for the $\delta^{13}C$‰ (σ n-1, n=3). Data are expressed in conventional δ
144 notation, in units per mL (‰) (Coplen, 2011).
145
146 *2.6 TAG analysis*
147 The isooctane extract was analyzed according to the conditions described previously (Bononi,
148 Tateo, & Tateo, 2017). Briefly, the extract was introduced manually at 40 °C to the OCI of a
149 HRGC 5160Mega Series (Carlo Erba Instruments), equipped with a bonded phase poly
150 (dimethyl siloxane) Petrocol EX 2887 capillary column (Supelco; 5 m × 0.53 mm i.d. and 0.1

μm film thickness). The oven temperature program was set to 150 °C, then increased to 200 °C at a rate of 10 °C min^{-1}, then increased to 340 °C at a rate of 5 °C min^{-1} (maintained for 30 min). The flame ionization detector temperature was set to 350 °C and the carrier gas was H_2 at 20 kPa pressure. An anhydrous butter fat standard certified by the Community Bureau of Reference (CRM 519) was used for peak identification. Samples were analyzed in triplicate, and the resulting standard deviations were between 0.2 and 0.4. Data are expressed as the ratio between (C52 + C54) and (C36 + C38) area counts (R-TAGs), and each single TAG was identified according to the total carbon number.

2.7 Statistical analysis

We performed a Principle Component Analysis (PCA) of $\delta^{13}C$ and R-TAG data (Tables 1 and 2) with SPSS statistical software (SPSS, Chicago, IL). Before PCA, experimental data were standardized through the application of the auto scaling procedure (mean centering and variance scaling transformation) (Einax, Zwanzinger, & Geib, 1997). PCA method was thus used to develop a classification index:

$$I=\sum_{PC=i}^{n} PCwi \sum_{1}^{2} LVS$$

where PC is the i^{th} principal component, n is the PC number used in the index definition, PCw_i is the equivalent fraction of the total variance (100% is equal to 1), explained by the PC divided by the total variance, and S connotes the value of each indicator.

3. Results and discussion

175 We performed EA-IRMS to characterize 48 saffron samples from Western Macedonia (Greece)
176 and 48 saffron samples from Khorasan Province (Iran) collected during the quality evaluation
177 of an Italian leader company in 2015-2017 (Table 1). The $\delta^{13}C$ ranges were between -28.3 and
178 -26.9 in the Greek samples and between -26.1 and -24.5 in the Iranian samples. Examples of
179 EA-IRMS traces are shown in Fig. 1. The two data series for each year considered provided
180 the first consistent evidence that $\delta^{13}C$ values could differentiate between Greek and Iranian
181 samples.
182 We evaluated R-TAG data for the same 96 saffron samples (Table 2). Examples of TAG traces
183 are shown in Fig. 2. The ratios that characterized the Greek samples ranged between 0.6 and
184 2.2, while for Iranian samples ranged between 1.0 to 3.1. These two fluctuating intervals are
185 only partially overlapped. So, we can consider the R-TAG a useful factor to contribute to
186 differentiation between the samples from the two countries.
187 Saffron harvests coming from Greece and Iran were taken into account because this study must
188 be based on saffron of known origin, and our current availability of these products definitely
189 originated from the aforementioned countries.
190 In this initial phase, this investigation was focused on the potential analytic efficiency of the
191 two statistically joined indices. It was beyond the scope of this study to resolve issues related
192 to the identification of the origin of saffron samples from every country where it is produced.
193 A first attempt to compare the saffron samples considering both indicators was made by using
194 the Principal Component Analysis. PCA is a chemometric method to study the relationships
195 among quantitative variables. When a large dataset is under consideration, PCA allows one to
196 reduce the number of variables, thus simplifying data interpretations, and also to identify the
197 most important parameters affecting difference/similarity among samples.
198 By taking variables X_1, X_2,…, Xi variables that describe n samples, the PCA algorithm
199 calculated X_1,i combinations to produce new latent components, the uncorrelated $PC_{1\text{-}i}$. The

200 eigenvalue (w_i) data described the contribution of $PC_{1\text{-}I}$, in explaining the system variance
201 (Tabachnick & Fidell, 2001).
202 PC_1 and PC_2 explained the percentages of the system total variability: 67.4% and 32.6%,
203 respectively (Fig. 3). Each PC_i was a linear combination of starting parameters *i*, whose
204 contribution is expressed as the correlation coefficient PC vs. parameters for each PC and
205 named Loading Values (LV).
206 PC_1 depended above all on $\delta^{13}C$ (loading value = 0.984), while a lesser contribution was
207 ascribed to R-TAG (loading value = 0.177); for PC_2, the contribution of the parameters was
208 the opposite with the greatest dependence associated with R-TAGs.
209 Plotting the samples in the PC_1 vs. PC_2 space (Fig. 3), a clear separation between Greek and
210 Iranian saffron occurred on PC_1 that translates into the great importance of $\delta^{13}C$. By
211 considering production years, however, samples were scattered for PC_2 indicating the high
212 variability of the experimental data.
213 PCA gave a qualitative indication of the similarity/dissimilarity of saffron, but did not allow
214 us to quantitatively classify the degree of similarity/dissimilarity. With the aim to valorize both
215 parameters an additive index based on PCA has been developed.
216 This approach has already been applied (Dunjó, Pardini, & Gispert, 2003; Scaglia & Adani,
217 2008): weighting the parameters by using PC importance (*w*) and loading values (LV).
218 The index was calculated as follow:
219
220 $I = 0.674*[(0.984*\delta^{13}C_{\text{autocentered\_value}})+0.177*\text{R-TAGs}_{\text{autocentered\_value}})]+0.326**[(0.984*\delta^{13}C$
221 $_{\text{autocentered\_value}})+0.177*\text{R-TAGs}_{\text{autocentered\_value}})]$
222

From a statistical point of view, the above index I was able to distinguish G and I samples (ANOVA bootstrap, $p<0.01$). The three indexes were successively used to build the box plot in Fig. 4.

Conclusion

In the current study, we showed that the IRMS measurements of crocins, the most abundant component in saffron, represented a useful method to differentiate the two origins of this spice and confirmed the advantage of IRMS data for the isotopic characterization of saffron components. These data extend the limited amount of information available thus far.

Moreover, the TAG composition, and, in particular, a derived index identified as R-TAGs, a chemico-physical variable that has not been previously used for the quality control of this spice, can be adopted as a correlate variable for PCA statistical evaluation useful to highlight two distinct sites of origin of saffron.

The results of this research were sufficiently adequate for the purpose of this preliminary work. We think that more numerous application of the proposed method can confirm these preliminary results.

The extension of the work with the above indices to other saffron samples of different provenance will be carried out at a later stage, when samples of positively identified origin from other geographical sites have been acquired.

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Table 1. EA-IRMS data for the $\delta^{13}C$ (‰) in the water-soluble fractions of 96 genuine saffron samples derived from guaranteed origins: Western Macedonia-Greece (G) and North, South, and Razavi Khorasan Provinces - Iran (I). Samples were collected in 2015-2017. All analyses were performed in triplicate.

sample	2015		2016		2017	
	G	I	G	I	G	I
1	-27.1	-25.2	-27.4	-25.9	-27.9	-25.2
2	-27.5	-25.5	-27.6	-25.8	-28.2	-25.5
3	-27.6	-24.7	-27.9	-25.8	-27.6	-24.7
4	-27.1	-25.6	-27.0	-25.2	-27.6	-24.5
5	-27.6	-25.0	-28.1	-25.2	-27.3	-25.2
6	-28.2	-25.7	-26.9	-25.4	-27.3	-25.6
7	-27.6	-25.4	-27.1	-25.6	-27.1	-25.0
8	-27.3	-25.0	-27.3	-25.2	-27.2	-25.4
9	-27.6	-24.7	-27.4	-25.6	-27.0	-25.1
10	-28.1	-25.4	-27.4	-25.9	-27.9	-26.1
11	-27.6	-24.8	-27.6	-25.1	-28.0	-25.0
12	-27.1	-26.1	-28.3	-24.8	-27.7	-26.1
13	-27.8	-24.5	-27.4	-25.2	-28.2	-24.5
14	-28.2	-25.8	- 27.0	-26.1	-27.5	-25.6
15	-27.8	-26.0	-27.9	-25.1	-27.2	-24.8
16	-26.9	-24.2	-26.9	-25.0	-27.9	-24.7

Table 2. Ratios of triacylglycerol area counts (C52 + C54)/(C36 + C38) in 96 samples of saffron derived from guaranteed origins: Western Macedonia - Greece (G) and North, South, and Razavi Khorasan Provinces - Iran (I). Samples were collected in 2015-2017. All analyses were performed in triplicate.

sample	2015		2016		2017	
	G	I	G	I	G	I
1	0.7	1.4	1.3	1.5	1.9	3.1
2	0.7	1.3	1.9	1.3	1.6	1.9
3	1.4	1.0	1.5	1.3	1.6	1.8
4	1.9	1.3	0.9	1.7	1.8	2.2
5	2.0	1.0	1.7	1.4	1.6	2.5
6	1.6	1.4	1.3	1.8	1.3	2.6
7	1.0	1.6	1.6	2.3	1.3	2.4
8	1.4	1.8	1.4	1.9	1.2	1.9
9	1.0	1.7	1.7	1.8	1.9	2.1
10	1.2	1.7	1.9	1.4	1.8	2.5
11	1.2	1.3	2.0	1.9	1.4	2.6
12	1.4	1.9	2.2	1.4	2.2	1.8
13	1.9	1.8	1.4	1.3	1.7	2.1
14	1.3	1.5	0.8	1.5	1.8	2.6
15	0.6	1.8	0.9	1.8	2.1	2.6
16	0.7	2.1	1.2	2.1	1.4	2.4

Fig. 1. EA-IRMS traces for two saffron samples collected in 2015 (see Table 1). G: Greece; I: Iran

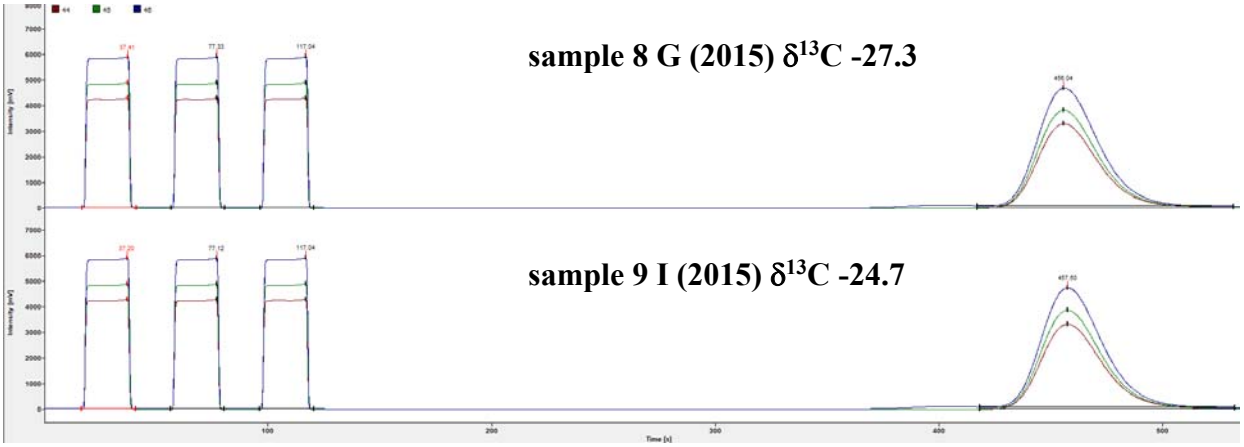

Fig. 2. Examples of TAG profiles obtained with gas-chromatographic analyses of two saffron samples collected in 2015 (see Table 2). G: Greece; I: Iran; R-TAG: the ratio of TAG area counts (C52 + C54)/(C36 + C38)

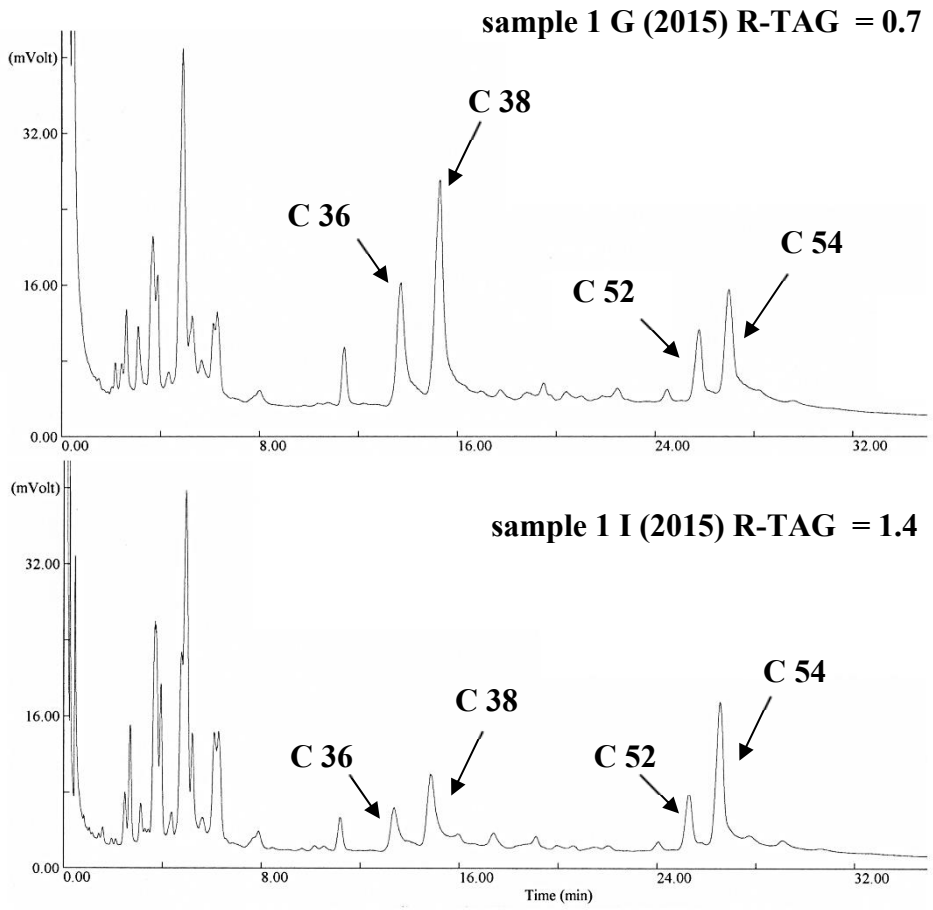

Fig. 3. PCA score loading biplot of 48 saffron samples from Greece (●) and 48 saffron samples from Iran (■), described with two variables: the $\delta^{13}C$ and the R-TAG.

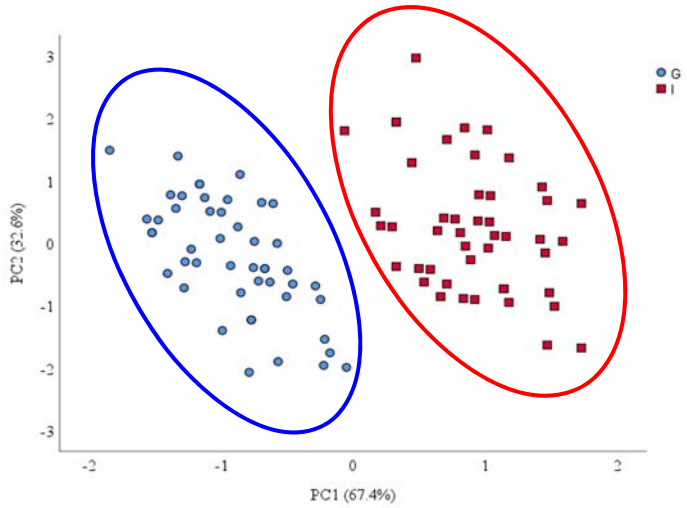

Fig. 4. Box plot of R-TAGs, δ ^{13}C and index I of 48 saffron samples from Greece (G) and 48 saffron samples from Iran (I).