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Abstract: High-resolution NMR spectroscopy was employed to analyze Italian Protected Designation of Origin (PDO) saffron from L'Aquila, S. Gimignano and Sardinia and commercial saffron samples available on the Italian market. An extensive resonance assignment of DMSO saffron extract was reported, including glucose and gentiobiose in bound and unbound form for the first time. A multivariate statistical analysis of NMR data led to a clustering of samples by performing unsupervised PCA. OPLS-DA model was successively performed to highlight the markers responsible for this discrimination. An analysis of the corresponding S-plot indicated that picrocrocin and crocins were the most relevant compounds for characterizing Italian PDO saffron, thus confirming the higher quality of these products. By contrast, commercial saffron barely contained these characteristic compounds, and they were primarily enriched in fatty acids.

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Dear Editor

I am very pleased to submit the following paper to your attention in order to be considered for publication in Food Control. The paper title is: “NMR investigations for a quality assessment of Italian PDO saffron (Crocus sativus L.)”. The manuscript deals with the application of NMR metabolite determination combined with multivariate statistical methods for the quality evaluation of Italian PDO and commercial saffron and for comparison among them. PDO products are very expensive in Italy while commercial products are more affordable. This NMR study, highlighted for the first time the compositional differences between the two categories of saffron, allowing a correct quality valorisation. In particular this study revealed the content of crocins and picrocrocin as the dominant compounds in PDO saffron, while commercial products were characterized by larger content of fatty acids, notwithstanding different harvest and date of purchase of samples.

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NMR investigations for a quality assessment of Italian PDO saffron (*Crocus sativus* L.)

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Abstract

High-resolution NMR spectroscopy was employed to analyze Italian Protected Designation of Origin (PDO) saffron from L'Aquila, S. Gimignano and Sardinia and commercial saffron samples available on the Italian market. An extensive resonance assignment of DMSO saffron extract was reported, including glucose and gentiobiose in bound and unbound form for the first time. A multivariate statistical analysis of NMR data led to a clustering of samples by performing unsupervised PCA. OPLS-DA model was successively performed to highlight the markers responsible for this discrimination. An analysis of the corresponding S-plot indicated that picrocrocin and crocins were the most relevant compounds for characterizing Italian PDO saffron, thus confirming the higher quality of these products. By contrast, commercial saffron barely contained these characteristic compounds, and they were primarily enriched in fatty acids.

KEYWORDS Saffron, Italian PDO, Quality, NMR, OPLS-DA

1. Introduction

Saffron, which is obtained from the dried red stigmas of *Crocus sativus* L., is the most expensive spice in the world. It has broad use in the food industry as an additive for coloring and flavoring foods. It is also employed as a drug in traditional medicine and exhibits anti-tumor activity (Hosseinzadeh & Nassiri-Asl, 2013; Magesh, Singh, Selvendiran, Ekambaram, & Sakthisekaran, 2006; Ríos, Recio, Giner, & Máñez, 1996; Sun et al., 2013). The typical color, taste, aroma and flavor of saffron are caused by the following three metabolites: crocins (glycosylated apocarotenoids, glucose, gentiobiose, neapolitanose or triglucoses are the reported saccharidic moieties) are responsible for the strong coloring capacity, picrocrocin (the glucosylated monoterpene precursor of safranal) confers the bitter taste and safranal (a monoterpene aldehyde derived from the chemical or enzymatic dehydration of picrocrocin during saffron handling, drying and storage) gives rise to its characteristic odor and aroma (Kanakis, Daferera, Tarantilis, & Polissiou, 2004; Tarantilis, Polissiou, & Manfait, 1994).

The quality of saffron and its commercial value are determined by specifications described within the ISO/TS-3632 standard (ISO 3632-1, 2011; ISO 3632-2, 2010) that established the spectrophotometric quantification of crocins in aqueous saffron extracts for picrocrocin and safranal by taking absorbance measurements at 440, 257 and 330 nm. Unfortunately, this method presents some disadvantages because safranal is just barely water soluble and also exhibits adsorption in the 320-340 nm range for *cis*-crocin isomers (Kanakis, Daferera, Tarantilis, & Polissiou, 2004; Tarantilis, Tsoupras, & Polissiou, 1995; Zougagh, Ríos, & Valcárcel, 2006). Saffron quality parameters can also be influenced by different harvesting conditions, dehydration procedures (under direct sunlight or at room temperature in ventilated conditions, as in India, Iran and Morocco, at a mild temperature as preferred in Italy and Greece or at high temperatures as adopted in Spain) (Del Campo et al., 2010a), storage conditions and blends with other non-colored parts of the plant, generally stalks.

Additional modifications to the saffron quality could be caused by different geographical origins, thus affecting the aroma and test as well. Saffron is cultivated worldwide, particularly in Morocco, Algeria,

Egypt, China, India, Iran and Turkey, but also within Europe, where Greece, Spain and Italy are the primary producers. In particular, Italian saffron from L'Aquila, S. Gimignano and Sardinia have received the PDO trademark (Reg. CE n° 205 04/02/05 and GUCE L 33 05/03/05 for saffron from L' Aquila and S. Gimignano and Reg. CE n°98 02/02/09 and GUCE L 33 03/02/09 for Sardinia saffron), which indicates the quality, characteristics and properties that are significantly or exclusively determined by the geographical environment, including natural and human factors. In fact, the PDO product must be produced, processed and prepared in the designated region, area or country by using traditional production procedures in accordance to set rules indicated in the PDO disciplinary. Several works concerning different qualitative aspects of saffron are present in the literature; different analytical techniques have been applied, primarily UV-Vis spectrophotometry and NIRS, to determine the characteristic chemical compounds and also to distinguish between the natural or artificial colorants that are added to saffron (Sánchez, 2008; Zalacain et al., 2005a; Zalacain et al., 2005b). Mass spectrometry combined with GC (Alonso, Salinas, & Garijo, 1998; D'Auria, Mauriello, & Rana, 2004; Kanakis, Daferera, Tarantilis, & Polissiou, 2004; Tarantilis & Polissiou, 1997), HPLC (Alonso, Salinas, Garijo, & Sánchez-Fernández, 2001; Lage, & Cantrell, 2009; Li, Lin, Kwan, & Min, 1999) and LC (Verma & Middha, 2010) analysis have focused on the identification of volatile molecules, coloring pigments or taste compounds. Extensive and time-consuming purification procedures were required for the spectroscopic characterization of crocetin derivatives (Van Calsteren et al., 1997) and glycosidic aroma precursors (Straubinger, Bau, Eckstein, Fink, & Winterhalter, 1998). The picrocrocin content has been analyzed by HPLC and FT-NIR (Del Campo et al., 2010b) and by SPE coupled with UV-Vis (Sánchez, Carmona, Del Campo, & Alonso, 2009). A few papers have reported studies of Italian saffron (Anastasaki et al., 2010b ; Maggi et al., 2011; Procida, Pagliuca, & Cichelli, 2009; Sánchez, Carmona, Del Campo, & Alonso, 2009) that were performed with different analytical techniques with the aim of geographical or quality characterization. Only two studies reported the investigation of Sardinian saffron as performed by MIR (Anastasaki et al., 2010a) and by multi-element stable isotope analysis (Maggi, Carmona, Kelly, Marigheto, & Alonso, 2011). D'Auria et al. (D'Auria, Mauriello, Racioppi, &

Rana, 2006; D'Auria, Mauriello, & Rana, 2004) investigated the volatile organic components of saffron from S. Gavino and L'Aquila, evaluating the modifications of aromatic constituents during storage, and other authors analyzed the effects of mild temperatures on the dehydration of PDO Sardinian saffron from S. Gavino on the basis of quality parameters (Del Campo, 2010a). Very recently, the influence of drying conditions on crocins, picrocrocin and safranal contents in saffron from Cascia (central Italy) have been evaluated by UV-Vis, HR-GC (for safranal) and HPLC-DAD-MS (for crocins and picrocrocin) (Cossignani, Urbani, Simonetti, Maurizi, Chiesi, & Blasi, 2014). The need for certified high-quality food products, such as the designation conferred by the PDO, is an increasingly important requirement for both consumers and producers; the reasons can be traced not only to patriotism but also primarily to health benefits or specific organoleptic and culinary qualities associated with regional products, media attention, decreasing confidence in the quality and safety products coming from outside their local region, country or the EU, and concerns about animal welfare and environmentally friendly production methods. In recent years, several authors have focused their efforts on tracing the origin of food products (Luykx & Van Ruth, 2008). There is therefore also a significant interest in developing accurate analytical methods for saffron quality characterization that could be applied to prevent adulteration or false labeling with regards to product origins.

Among all the analytical methods used in food characterization, NMR has garnered general acceptance as a powerful method over the last few years (Consonni & Cagliani, 2008; Monakhova, Kuballa, & Lachenmeier, 2013) as a quality assessment for a wide range of foods. NMR spectra can be considered as a type of a fingerprint for a product that carries qualitative and quantitative information on the composition. Concerning saffron, NMR investigations have been applied only for structural characterizations of crocetin or crocetin derivatives (Assimiadis, Tarantilis, & Polissiou, 1998; Pfister, Meyer, Steck, & Pfander, 1996; Straubinger, Bau, Eckstein, Fink, & Winterhalter, 1998; Straubinger, Jezussek, Waibel, & Winterhalter, 1997a; Van Calsteren et al., 1997). Only recently has NMR been combined with Principal Component Analysis to discriminate between Iranian saffron and commercial samples by analyzing methanol extracts (Yilmaz, Nyberg, Mølgaard, Asili, & Jaroszewski, 2010).

120 The aim of the present study is to evaluate the ability to discriminate among PDO products from
121 commercial samples that are available on the Italian market by NMR and chemometric analysis.

122 **2. Material and methods**

123 *2.1. Saffron Samples*

124 Twenty-two saffron samples were investigated by ^{1}H NMR in two biological replicates, in
125 particular, for 9 Italian PDO and 13 commercial saffron samples. Four representative PDO samples
126 came from L'Aquila (the Abruzzo region, both powder and stigmas), 1 from S. Gimignano (the Tuscany
127 region, stigmas) and 4 from the Sardinia region (S. Gavino and Turri, stigmas). The commercial
128 samples (all powder) were directly purchased on the Italian market at different periods; their origin and
129 harvest years were not reported on the label. All samples were stored in the dark at room temperature
130 before data acquisition. All details about samples are reported in Table 1.

131 *2.2. Sample preparation*

132 4 mg of saffron was extracted with deuterated dimethylsulfoxide (DMSO-d_6, 600 µL), stirred (vortex)
133 for 3 minutes at room temperature, and after 10 minutes, the mixture was centrifuged at 12100 rcf for 10
134 minutes. Five hundred microliters of the supernatant was used directly for NMR analysis. Glucose and
135 gentiobiose standards were purchase from Sigma-Aldrich and dissolved in DMSO-d_6 solvent.

136 *2.3. NMR spectral analysis*

137 All ^{1}H-NMR spectra have been recorded on a Bruker DMX 500 spectrometer (Bruker Biospin
138 GmbH Rheinstetten, Karlsruhe, Germany) operating at 11,7 T and equipped with a 5-mm reverse probe
139 with z-gradient. Monodimensional spectra were recorded at 300 K, with a spectral width of 7500 Hz and
140 32K data points. Residual water suppression was achieved by applying a presaturation scheme with low
141 power radiofrequency irradiation for 1.2 s.

142 All spectra were processed with TOPSPIN software (Bruker BioSpin GmbH, version 1.3, Rheinstetten,
143 Karlsruhe, Germany); an exponential function with a line broadening of 0.3 Hz was applied before
144 Fourier transformation and phase and baseline were manually corrected. Spectra were referenced to

DMSO resonance at 2.50 and 39.5 ppm for ^{1}H and ^{13}C respectively and 1D ^{1}H NMR spectra were reduced to integrated regions (buckets) of equal width of 0.01 ppm each from 0.4 to 10.4 ppm. Buckets were normalized to the total integrals after exclusion of residual solvent and water signals, with ACD/Spec Manager (ACD Labs, version 11, Toronto, Canada). Resonance assignment of saffron was achieved by two dimensional homo and heteronuclear correlation NMR spectra (TOCSY, HSQC, HSQC-TOCSY and HMBC). Bidimensional spectra were typically acquired with 15 and 200 ppm over 2048 and 256 data points in proton and carbon dimensions respectively. TOCSY spin lock was set to 80 ms and the direct heteronuclear coupling constant at 145 Hz. Diffusion Ordered Spectroscopy (DOSY) spectrum was acquired with bipolar pulse longitudinal eddy current delay set to 5 ms. The duration of the magnetic field pulse gradient (δ) was optimized for each diffusion time (Δ) and finally set to 2.2 and 100 ms respectively in order to obtain a residual signal of 1% with the maximum field strength. The pulse gradient was incremented from 5 up to 95% of the maximum gradient strength in a linear ramp; spectrum processing was performed with TOPSPIN software.

2.4. Statistical methods

NMR data were imported into SIMCA-P+ 13 (Umetrics, Umea, Sweden) for Principal Component Analysis (PCA) and Orthogonal Projection to Latent Structure-Discriminant Analysis (OPLS-DA) by using "mean centering" as a data pretreatment. The OPLS technique, which is an extension of the PLS regression method, produces a clearer model interpretation by decomposing the systematic variation in the X block into two parts, that is, the predictive or parallel part, modeling the joint X-Y correlated variation and the non-predictive or orthogonal part, which is not related to Y and is usually defined as "structured noise" (Trygg & Wold, 2002). OPLS can be applied for discrimination purposes by introducing dummy variables. When the dimension of the joint correlated space is one, useful visualization tools, such as the S-plot (Wiklund et al., 2008) and the *line plot* (Cloarec et al., 2005), can be used to highlight the role played by the variables in the model. In this last plot, the variables (buckets) are colored according to the correlation coefficient p(corr) between the corresponding bucket and the Y variable (class, in our case the Italian PDO or commercial saffron). Thus, the higher the

p(corr) value, the more reliable the variables are for discriminating among the samples. T2 and distance to the model (DModX) tests were applied to verify the presence of outliers. In addition, a permutation test on the Y block was performed on the corresponding PLS-DA model to overcome randomness safely or over-fitting in the model. In fact, the permutation plot displays the correlation coefficient between the original Y variable and the permuted Y variable on the X axis versus the cumulative R^2 and Q^2 on the Y axis. The regression line is then used to evaluate the intercept as a measure of the over-fit.

3. Results and discussion

3.1. NMR Spectral Analysis

The advantage of NMR spectroscopy is that it provides global information about all the soluble constituents of a complex matrix with a single experiment without any separation procedure, thus maintaining the original ratio of the components. The typical ^{1}H NMR spectrum of saffron in DMSO solution (Figure 1A) resulted dominated at low field by the singlet of the aldehydic proton at 10.05 ppm of 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde moiety, as typically observed for picrocrocin. The group of broad signals between 6.50 and 7.40 ppm are relative to the conjugated double bonds of crocins. In particular, the comparison among our data and previously reported data (Assimiadis, Tarantilis, & Polissiou, 1998; Speranza & Dadà, 1984; Van Calsteren et al., 1997) allowed us to confirm the presence of the dominant content for *trans* crocins, which was also explained by the lack of methyl resonance for *cis* crocins that typically occurs at 2.00 ppm. Molecular reference structures are depicted in Scheme 1.

Kaempferol signals were recognized by resonances at 8.05, 6.91, 6.43 and 6.19 ppm, even though they were present at very low amounts (Figure 1B). In particular, the double signal for the aromatic protons of kaempferol occurring at 8.05 and at 8.09 ppm suggested the presence of the two most abundant glycosylated isoforms, which was fully consistent with previously reported data (Carmona et al., 2007; Straubinger, Jezussek, Waibel, & Winterhalter, 1997b). At high field the ^{1}H NMR spectrum is dominated by very intense singlets from the methyls of picrocrocin at 1.16, 1.18 and 2.10 ppm and of crocins at 1.97 and 2.00 ppm. Anomeric protons of saccharides bound to crocetin, primarily gentiobiose

and glucose (Sánchez, 2008), were present in β isomeric form (Sánchez, 2008, Tarantilis, Polissiou, & Manfait, 1994) and both occurred at 5.42 (the glucose and ring A of gentiobiose) and 4.17 ppm (ring B of gentiobiose). This finding was confirmed by HMBC correlation (S1 in Supporting Information) and was consistent with previously reported data (Van Calsteren et al., 1997). Conversely, β glucose bound to 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde moiety (Sánchez, 2008; Tarantilis, Polissiou, & Manfait, 1994) occurred at 4.29 ppm, as supported by the HMBC correlation (S2 in Supporting Information). The use of standard compounds allowed us to identify signals for free saccharides, in particular the α anomeric proton of gentiobiose ring A and glucose overlapped at 4.89 ppm, the β anomeric signals of gentiobiose ring A and the glucose overlapped at 4.25 ppm and the β anomeric signal of gentiobiose ring B overlapped with the same anomeric proton of gentiobiose when in a bound state at 4.17 ppm (Figure 1C). In the anomeric region, other two isolated spin systems at 5.18 ppm (91.4 ppm), 3.54 ppm (72.6 ppm) and 3.18 ppm (73.2 ppm), namely U1 and 4.60 (73.88 ppm), 4.38 ppm (73.88 ppm), 2.88 ppm (73.1 ppm) and 2.50 ppm (74.8 ppm) and U2 were not yet determined. Concerning the less intense resonances, unsaturated fatty acids were easily recognized by signals occurring at 5.33 ppm because of their olefinic protons at 2.74 ppm because of their bis-allylic protons and at 2.01 ppm because of their allylic protons; additional typical signals for the methyl protons of linoleic and linolenic acids were detected at 0.86 and 0.93 ppm, respectively. We could not exclude the presence of oleic acid because of the complete overlapping of its spin system with both linolenic and linoleic acids. Other significant signals for both unsaturated and saturated fatty acids were observed at approximately 2.26 and 1.50 ppm (α and β methylene protons with respect to carboxyl groups, respectively) and centered at 1.23 ppm (methylene protons).

The complete assignment of the previously described compounds was consistent with previously reported data and was confirmed by the use of TOCSY, HSQC, HMBC and HSQC-TOCSY experiments. An additional potential assignment aid is the possibility of identifying different compounds by their diffusion coefficient, which is strictly related to molecular size. In Figure 2, the

DOSY spectrum of a typical PDO saffron sample from L'Aquila reporting the assignment of the primary components is depicted, showing the diffusion-weighted separation that is effective for the primary compounds. Because of differences in the MW, smaller molecules were characterized by higher D values. Crocins and picrocrocin were clearly found to be conjugated to the corresponding saccharidic moieties, thus confirming the previous assignments.

3.2. Multivariate statistical analysis

It should be emphasized that the spectral dataset consisted of samples from different harvest years and different geographical origins. The PCA model was initially explored by considering all analyzed saffron samples. This model resulted in 5 PCs explaining 98% of the total variance (R^2X) within the overall cross-validation coefficient of Q^2=93.7% (PC1=70.3%; PC2=14.9%). In the corresponding score plot (data not shown), a clear differentiation between commercial and PDO samples was achieved. To highlight the variables responsible for sample separation, a classification approach such as OPLS-DA was performed by considering two classes. This model resulted in one predictive (t1=39.5%) and six orthogonal (to 1=31.7%) components; the corresponding scatter plot reported in Figure 3 confirmed the commercial samples as being completely different products from all the Italian PDOs, notwithstanding the consideration of different periods of storage, harvest years (PDO) or dates of purchase (commercial). All the Italian PDO saffron was clustered on the positive values of t1, and the commercial ones grouped to the opposite side of the score plot because affected by negative t1 values. The corresponding *line plot* (Figure 4) can be used to visualize both the covariance p and correlation p(corr) between the metabolites and the modeled class designation. The *line plot* allows for the identification of possible markers for sample differentiation in terms of metabolite contents, on the basis of both contribution and reliability. The positive and negative signals correspond to the metabolites that characterized Italian PDO and commercial samples, respectively. Interestingly, the *line plot* showed very few variables (metabolites) with a low |p(corr)| (<0.3), indicating that almost all the total information derived from the NMR data presented good reliability for discriminating among samples. A generally higher metabolite content in Italian PDO saffron with respect to commercial samples was observed. In particular, PDO products

were characterized by higher levels of picrocrocin and crocins. These results were consistent with previously reported data (Yilmaz, Nyberg, Mølgaard, Asili, & Jaroszewski, 2010), indicating that the picrocrocin and glycosyl esters of crocetin (crocins) are the primary characteristic compounds that are useful for distinguishing authentic Iranian saffron from commercial saffron purchased in different countries. This finding could suggest that authentic saffron, independent of its geographical origin, presents a higher amount of picrocrocin and crocins, two of the primary compounds used to establish the saffron quality, and they are responsible for the bitter taste and coloring capacity. The commercial saffron generally presented a lower content of typical saffron compounds, which were primarily characterized by fatty acids. For a deeper evaluation of the role played by the compounds, a stricter threshold value was chosen for the correlation coefficient ($|p(corr)| \geq 0.8$). In this view, only picrocrocin (buckets from 10.04 up to 10.06 ppm, from 3.92 up to 3.96 ppm, from 2.63 up to 2.66 ppm, from 2.59 up to 2.62 ppm, at 2.23 ppm, from 2.09 up to 2.11 ppm, at 1.76 ppm, from 1.73 up to 1.75 ppm, at 1.43 ppm, at 1.41 ppm, from 1.17 to 1.19 ppm, from 1.15 up to 1.16 ppm and buckets from 4.28 up to 4.31 ppm, at 3.13 ppm, and 2.93 ppm and 2.91 ppm in reference to the corresponding glucosidic moiety) and fatty acids (buckets from 5.24 to 5.27 ppm, at 2.78 ppm, 2.75 ppm, 2.70 ppm, 2.02 ppm, and 2.04 ppm and from 0.92 to 0.95 ppm, all of which refer to unsaturated fatty acids) were the most reliable compounds for discriminating Italian PDO saffron from the commercial saffron.

The OPLS-DA model was validated by performing a permutation test on the corresponding PLS-DA model. The decreased values of both the Q^2 and R^2 parameters (the vertical axis intersection points of the Q^2 and R^2 regression lines were negative and 0.4, respectively) confirmed the statistical validity of the model.

4. Conclusions

The results of this study highlighted that NMR data can provide a large amount of information concerning the metabolite content of saffron, and its combination with chemometrics led to very promising work towards saffron quality characterization. Even if the results are considered preliminary

because of the limited number of analyzed samples, our results demonstrated that despite the consideration of different harvest and date of purchase and different storage periods, a very good discrimination between PDO Italian saffron samples and commercially available saffron from the Italian market was possible. Italian PDO samples were characterized above all by higher amounts of picrocrocin and crocins, two of the primary saffron quality components, and the commercial ones were characterized by fatty acids. Additionally, the solvent employed for this study, namely DMSO, was helpful for monitoring the lipophilic and hydrophilic metabolites simultaneously, without any derivation as required by other analytical methods, thus allowing for the detection of fatty acids and soluble metabolites. The combined use of NMR and chemometrics could also improve the analytical determination of quality control procedures, representing an important answer to emerging requests for quality determinations with rapid analysis and the possibility of monitoring different classes of metabolites simultaneously. More samples will be considered in the future to enforce the obtained results and to analyze the influence of the storage period and harvest year on the metabolic contents of saffron.

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References

Alonso, G. L., Salinas, M. R., & Garijo, J. (1998). Method to determine the authenticity of aroma of saffron (*Crocus sativus* L.). *Journal of Food Protection, 61*, 1525-1528.

Alonso, G. L., Salinas, M. R., Garijo, J., & Sánchez-Fernández, M. A. (2001). Composition of crocins and picrocrocin from Spanish saffron (*Crocus sativus* L). *Journal of Food Quality*, *24*, 219-233.

Anastasaki, E., Kanakis, C., Pappas, C., Maggi, L., Del Campo, C. P., Carmona, M., Alonso, G. L., & Polissiou, M. G. (2010a). Differentiation of saffron from four countries by mid-infrared spectroscopy and multivariate analysis. *European Food Research Technology, 230,* 571-577.

Anastasaki, E. G., Kanakis, C. D., Pappas, C., Maggi, L., Zalacain, A., Carmona, M., Alonso, G. L., & Polissiou, M. G. (2010b). Quantification of crocetin esters in saffron (*Crocus sativus* L.) using Raman spectroscopy and chemometrics. *Journal of Agricultural and Food Chemistry, 58,* 6011-6017.

Assimiadis, M. K., Tarantilis, P. A., & Polissiou, M. G. (1998). UV-Vis, FT-Raman, and ^{1}H-NMR spectroscopies of cis-trans carotenoids from saffron (*Crocus sativus* L.). *Applied Spectroscopy, 52*, 519-522.

Carmona, M., Sanchez A. M., Ferreres, F., Zalacain, A., Tomás-Barberan, F., & Luis Alonso, G. (2007). Identification of the flavonoid fraction in saffron spice by LC/DAD/MS/MS: comparative study of samples from different geographical origins. *Food Chemistry, 100*, 445-450.

Cloarec, O., Dumas, M. E., Craig, A., Barton, R. H., Trygg, J., Hudson, J., Blancher, C., Gauguier, D., Lindon, J. C., Holmes, E., & Nicholson, J. (2005). Statistical Total Correlation Spectroscopy: an exploratory approach for latent biomarker identification from metabolic ^{1}H NMR data sets. *Analytical Chemistry, 77,* 1282-1289.

Consonni, R., & Cagliani, L. R. (2010). Nuclear Magnetic Resonance and chemometrics to assess geographical origin and quality of traditional food products. *Advances in Food and Nutrition Research, 59,* 87-165.

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'Auria, M., Mauriello, G., Racioppi, R., & Rana, G. L. (2006). Use of SPME-GC-MS in the study of time evolution of the constituents of saffron aroma: modifications of the composition during storage. *Journal of Chromatographic Science*, *44*, 18-21.

D'Auria, M., Mauriello, G., & Rana, G. L. (2004). Volatile organic compounds from saffron. *Flavour Fragrance Journal*, *19*, 17-23.

Del Campo, C. P., Carmona, M., Maggi, L., Kanakis, C. D., Anastasaki, E. G., Tarantilis, P. A., Polissiou, M. G., & Alonso, G. L. (2010a). Effects of mild temperature conditions during dehydration procedures on saffron quality parameters. *Journal of Agricultural and Food Chemistry*, *90*, 719-725.

Del Campo, C. P., Carmona, M., Maggi, L., Kanakis, C. D., Anastasaki, E. G., Tarantilis, P. A., Polissiou, M. G., & Alonso, G. L. (2010b). Picrocrocin content and quality categories in different (345) worldwide samples of saffron (*Crocus sativus* L.). *Journal of Agricultural and Food Chemistry*, *58*, 1305-1312.

Hosseinzadeh, H., & Nassiri-Asl, M. (2013). Avicenna's (Ibn Sina) the Canon of Medicine and Saffron (*Crocus sativus*): A Review. *Phytotheraphy Research*, *27*, 475-483.

ISO 3632-1. *Saffron (Crocus Sativus L.): Specifications* (2003 revised 2011) *and* ISO 3632-2 *Saffron (Crocus Sativus L.): Test Methods* (2003 revised 2010), Geneva, International Standards Organization, Switzerland.

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*, 4515-4521.

Lage, M., & Cantrell, C. L. (2009). Quantification of saffron (*Crocus sativus* L.) metabolites crocins, picrocrocin and safranal for quality determination of the spice grown under different environmental Moroccan conditions. *Scientia Horticulturae – Amsterdam, 121*, 366-373.

Li, N., Lin, G., Kwan, Y. W., & Min, Z. D. (1999). Simultaneous quantification of five major biologically active ingredients of saffron by high-performance liquid chromatography. *Journal of Chromatography A, 849*, 349-355.

Luykx, D. M. A. M., & Van Ruth, S. M. (2008). An overview of analytical methods for determining the geographical origin of food products. *Food Chemistry, 107*, 897-911.

Magesh, V., Singh, J. P. V., Selvendiran, K., Ekambaram, G., & Sakthisekaran, D. (2006). Antitumour activity of crocetin in accordance to tumor incidence, antioxidant status, drug metabolizing enzymes and histopathological studies. *Molecular and Cellular Biochemistry, 287,* 127-135.

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*, 543-548.

Maggi, L., Sánchez, A. M., Carmona, M., Kanakis, C. D., Anastasaki, E., Tarantilis, P. A., Pilissiou, M. G., & Alonso, G. L. (2011). Rapid determination of safranal in the quality control of saffron spice (*Crocus sativus* L.). *Food Chemistry, 127,* 396-373.

Monakhova, Y. B., Kuballa, T., & Lachenmeier, D. W. (2013). Chemometric Methods in NMR spectroscopic analysis of food products. *Journal of Analytical Chemistry, 68,* 755-766.

Pfister, S., Meyer, P., Steck, A., & Pfander, H. (1996). Isolation and structure elucidation of carotenoid-glycosyl esters in gardenia fruits (*Gardenia jasminoides* Ellis) and saffron (*Crocus sativus* Linne). *Journal of Agricultural and Food Chem*istry, *44*, 2612-2615.

Procida, G., Pagliuca G., & Cichelli, A. (2009). Geographical differentiation between Italian and Spanish saffron based on volatile fraction composition. A preliminary study. *Agro Food Industry Hi-Tech, 20,* 48-53.

Ríos, J. L., Recio, M. C., Giner R. M., & Máñez, S. (1996). An update review of saffron and its active constituents. *Phytotheraphy Research, 10,* 189-193.

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.

Sánchez, A. M., Carmona, M., Zalacain, A., Carot, J. M., Jabaloyes, J. M., & Alonso, G. L. (2008). Rapid determination of crocetin esters and picrocrocin from saffron spice (*Crocus sativus* L.) using UV-Visible spectrophotometry for quality control. *Journal of Agricultural and Food Chemistry, 56,* 3167-3175.

Speranza, G., & Dadà, G. (1984). 13 *cis*-Crocin: a new crocinoid of saffron. *Gazzetta Chimica Italiana, 114,*189-192.

Straubinger, M., Bau, B., Eckstein, S., Fink, M., & Winterhalter, P. (1998). Identification of novel glycosidic aroma precursors in saffron (*Crocus sativus* L.). *Journal of Agricultural and Food Chemistry, 46,* 3238-3243.

Straubinger, M., Jezussek, M., Waibel, R., & Winterhalter, P. (1997a). Novel glycosidic constituents from saffron. *Journal of Agricultural and Food Chemistry, 45,* 1678-1681.

Straubinger, M., Jezussek, M., Waibel, R., & Winterhalter, P. (1997b). Two kaempferol sophorosidews from *Crocus Sativus*. *Natural Product Letters, 10,* 213-216.

Sun, Y., Xu, H. J., Zhao, Y. X., Wang, L. Z., Sun, L. R., Wang, Z., & Sun, X. F. (2013). Crocin exhibits antitumor effects on human leukemia HL-60 cells in vitro and in vivo. *Evidence-Based Complementary and Alternative Medicine,* doi: 10.1155/2013/690164.

Tarantilis, P. A., & Polissiou, M. G. (1997). Isolation and identification of the aroma components from saffron (*Crocus sativus*). *Journal of Agricultural and Food Chemistry, 45,* 459-462.

Tarantilis, P. A., Polissiou, M., & Manfait, M. (1994). Separation of picrocrocin, *cis*-*trans*-crocins and safranal of saffron using high-performance liquid chromatography with photodiode-array detection. *Journal of Chromatography A, 664*, 55-61.

Tarantilis, P. A., Tsoupras, G., & Polissiou, 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*, 107-118.

Trygg, J., & Wold, S. (2002). Orthogonal projections to latent structures (O-PLS). *Journal of Chemometrics, 16*, 119-128.

Van Calsteren, M. R., Bissonnette, M. C., Cormier, F., Dufresne, C., Ichi, T., LeBlanc, J. C. Y., Perreault, D., & Roewer, I. (1997). Spectroscopic characterization of crocetin derivatives from *Crocus sativus* and *Gardenia jasminoides*. *Journal of Agricultural and Food Chemistry, 45*, 1055-1061.

Verma, R. S., & Middha, D. (2010). Analysis of Saffron (*Crocus sativus* L. Stigma) components by LC-MS-MS. *Chromatographia, 71*, 117-123.

Wiklund, S., Johansson, E., Sjöström, L., Mellerowicz, E. J., Edlund, U., Shockcor, J. P., Gottfries, J., Moritz, T., & Trygg, J. (2008). isualization of GC/TOF-MSBased Metabolomics Data for Identification of Biochemically Interesting Compounds Using OPLS Class Models. *Analytical Chemistry, 80*, 115-122.

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

Zalacain, A., Ordoudi, S. A., Blázquez, I., Díaz-Plaza, E. M., Carmona, M., Tsimidou, M. Z., & Alonso, G. L. (2005a). Screening method for the detection of artificial colours in saffron using derivative UV-Vis spectrometry after precipitation of crocetin. *Food Additives & Contaminants A, 22*, 607-615.

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

Zougagh, M., Ríos, A., & Valcárcel, M. (2006). Determination of total safranal by *in situ* acid hydrolysis in supercritical fluid media: application to the quality control of commercial saffron. *Analytica Chimica Acta*, *578*, 117-121.

448 **Figure captions**

449 Figure 1. NMR spectra of DMSO extract of Italian PDO saffron from L'Aquila. (A) One dimensional
450 proton spectrum with primary assignments reported. (B) Expansion of the aromatic region of TOCSY
451 spectrum reporting the resonances assignment of kaempferol. (C) Anomeric region of HSQC spectrum
452 reporting the assignment for bound and unbound saccharides.

453 Figure 2. DOSY spectrum of PDO saffron from L'Aquila. Single compounds are indicated with boxes
454 of the same colour connected together. LogD (m^2/s) are reported in F1 axis.

455

456 Figure 3. OPLS-DA score plot performed by considering all saffron samples analyzed: R^2X=98.4%,
457 R^2Y=98.8% and Q^2=84.6%. Red dots and black diamonds represent Italian PDO and commercial saffron
458 samples respectively.

459

460 Figure 4. *Line plot* of OPLS-DA performed by considering all saffron samples analyzed. Positives and
461 negatives signals correspond to metabolites that characterized Italian PDO and commercial samples
462 respectively. The colour scale indicates the correlation coefficient p(corr). The variables (buckets) with
463 high $|p(corr)|$ (> 0.8) indicate the most reliable variables/metabolites for discriminating the samples.

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Figures

Figure 1A

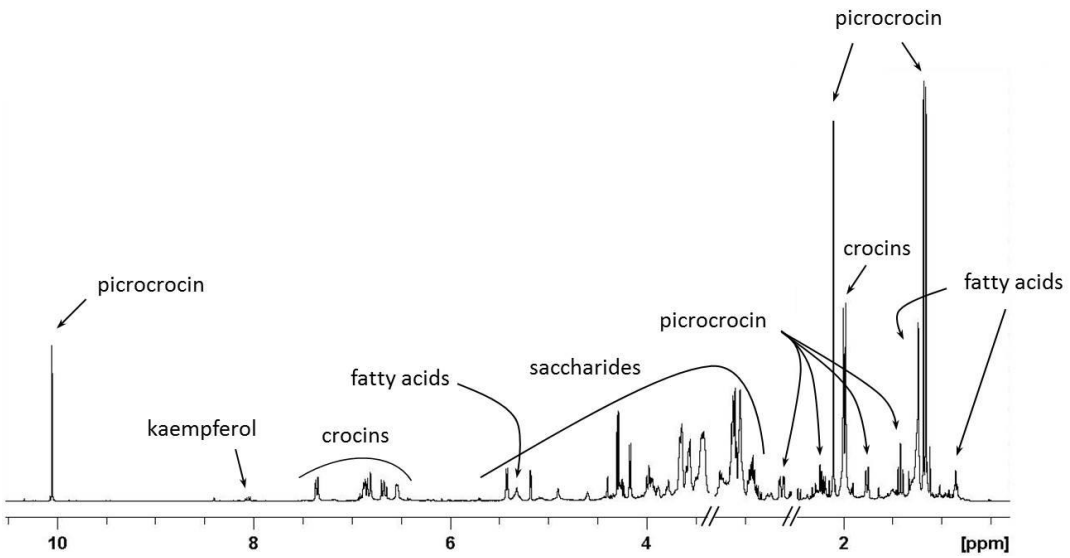


Figure 1B

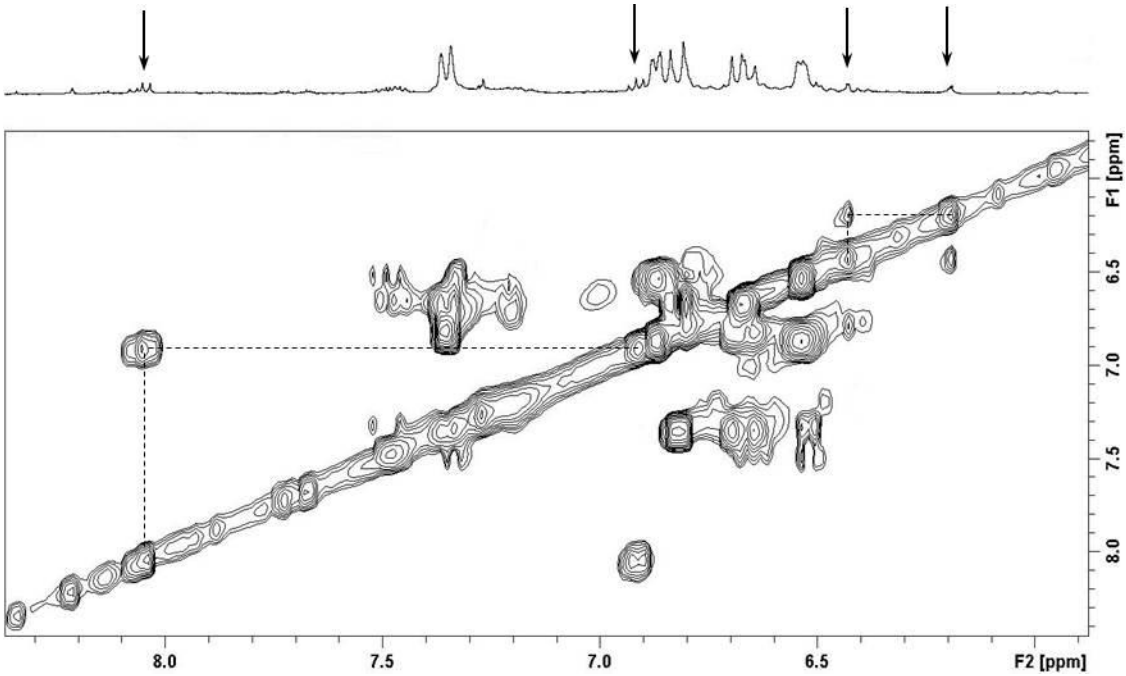


Figure 1C

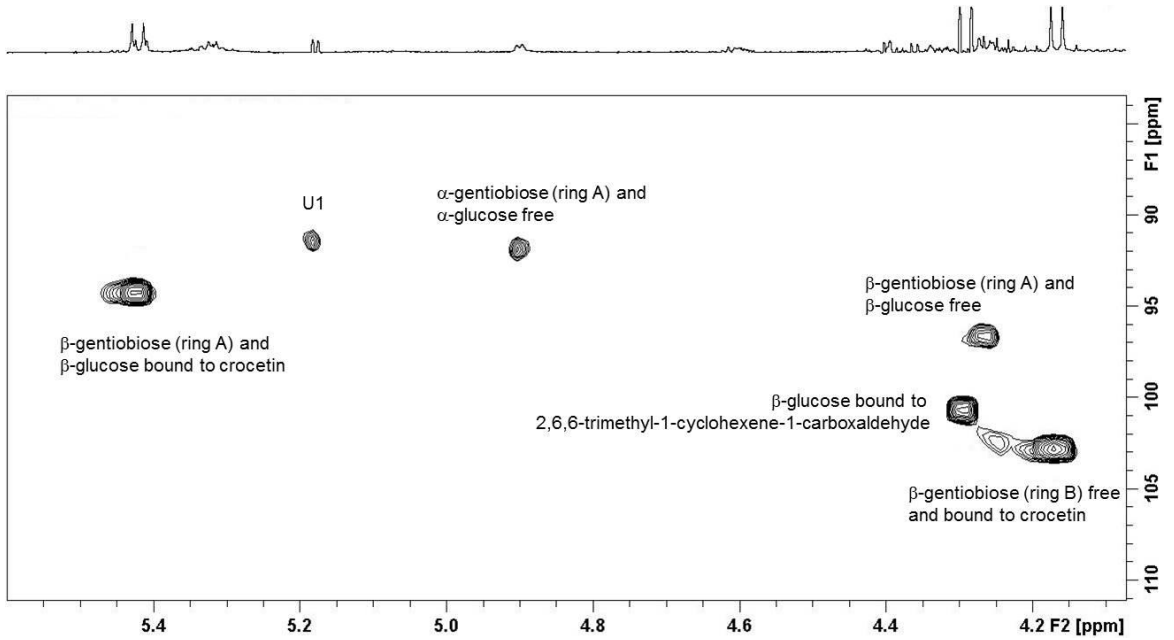


Figure 2

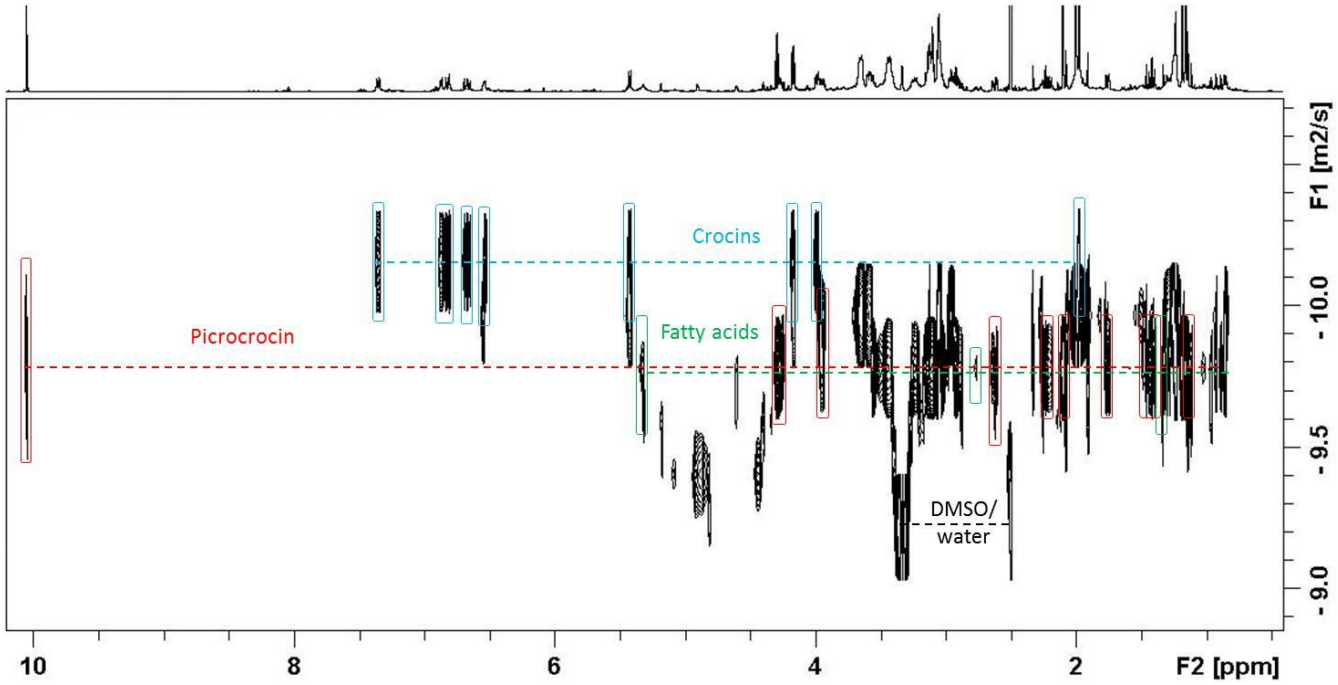


Figure 3

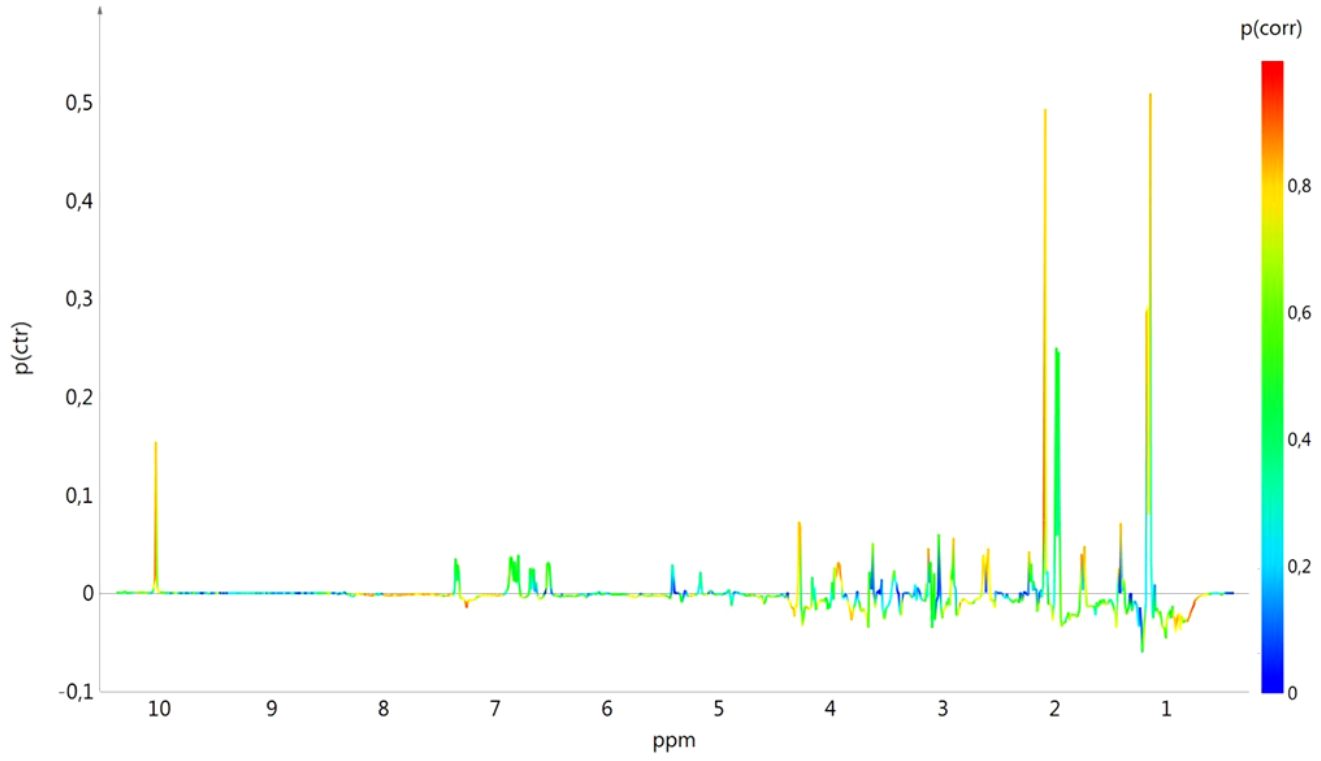
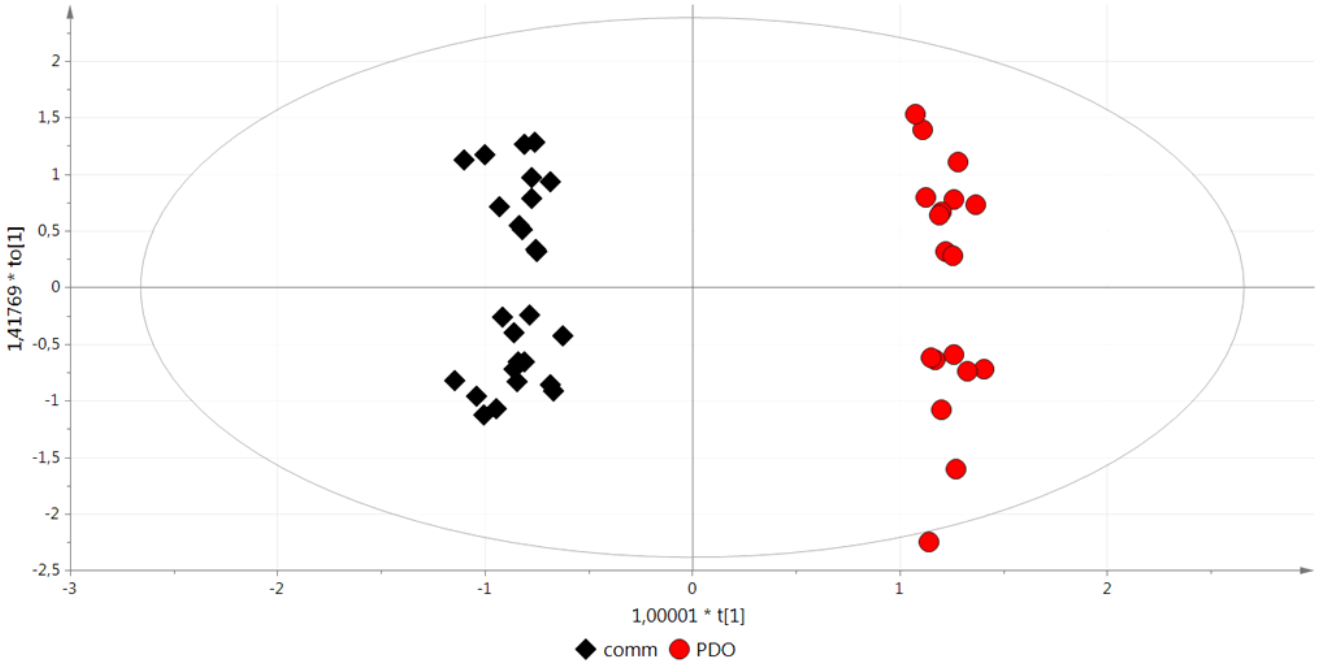
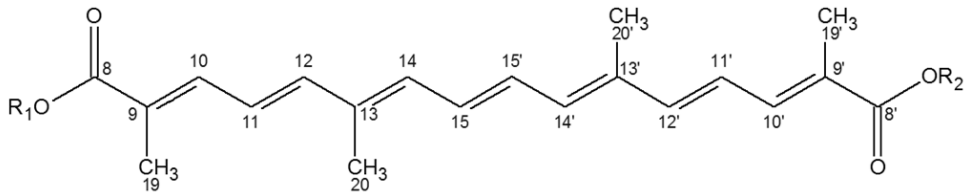


Figure 4



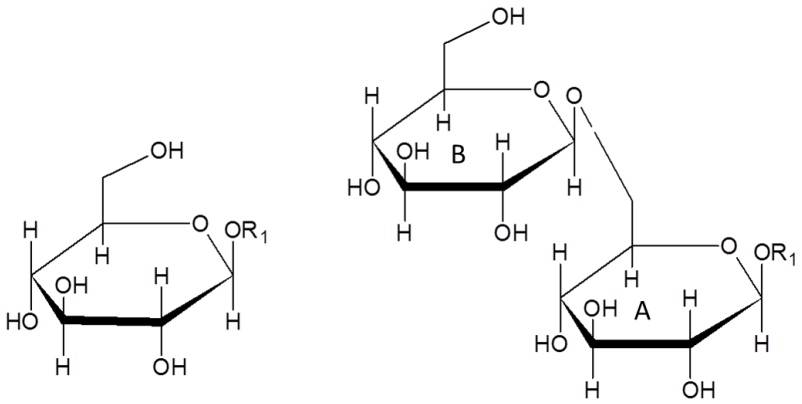
Scheme 1: Structures of crocins, saccharides moieties and picrocrocin under discussion



Crocins

R_1=R_2= β-D-gentiobiose

R_1=β-D-glucose, R_2=β-D-gentiobiose



a b

Saccharidic moieties: (a) β-D-glucosyl; (b) β-D-gentiobiosyl.

Picrocrocin

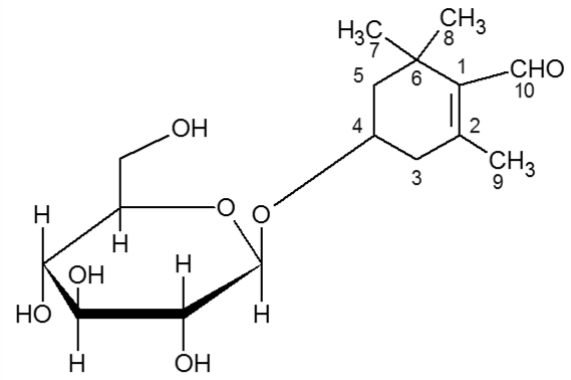


Table 1

List of Italian PDO saffron and commercial saffron bought in Italian market. Harvest (for PDO) or date of purchase (for commercial) of samples are reported together with the period of storage at the time of analysis for each sample.

n° sample	PDO/Comm	Harvest/Purchase	Storage (years)
1	PDO L'Aquila	2005	5
2	PDO L'Aquila	2005	5
3	PDO L'Aquila	2005	5
4	PDO L'Aquila	2012	1
5	PDO S. Gimignano	2006	4
6	PDO Sardinia	2010	1
7	PDO Sardinia	2010	1
8	PDO Sardinia	2012	1
9	PDO Sardinia	2012	1
10	Comm	2006	4
11	Comm	2006	4
12	Comm	2006	4
13	Comm	2006	4
14	Comm	2006	4
15	Comm	2010	1
16	Comm	2010	1
17	Comm	2011	1
18	Comm	2011	1
19	Comm	2011	1
20	Comm	2012	1
21	Comm	2012	1
22	Comm	2012	1

HIGHLIGHTS

- Quality assessment of Italian PDO saffron samples by NMR metabolite analysis
- Discrimination between Italian PDO and commercial saffron bought in Italian market
- Identification of unbound glucose and gentiobiose anomeric signals

Supplementary data

Figure S1

Overlay of HSQC (red) and HMBC (blue) expansions of the anomeric and aromatic regions, showing the long range heteronuclear correlation between anomeric proton of β-gentiobiose and β-glucose at 5.42 ppm and carbonyl C8 of crocins at 168.3 ppm. This carbonyl shows a long range correlation with proton H10 of crocins at 7.35 ppm.

Figure S2

Overlay of HSQC (red) and HMBC (blue) expansions of the anomeric region, showing the long range heteronuclear correlation between anomeric proton of β-glucose at 4.29 ppm and carbon C4 of the picrocrocin moiety at 68.8 ppm.

Figure S1

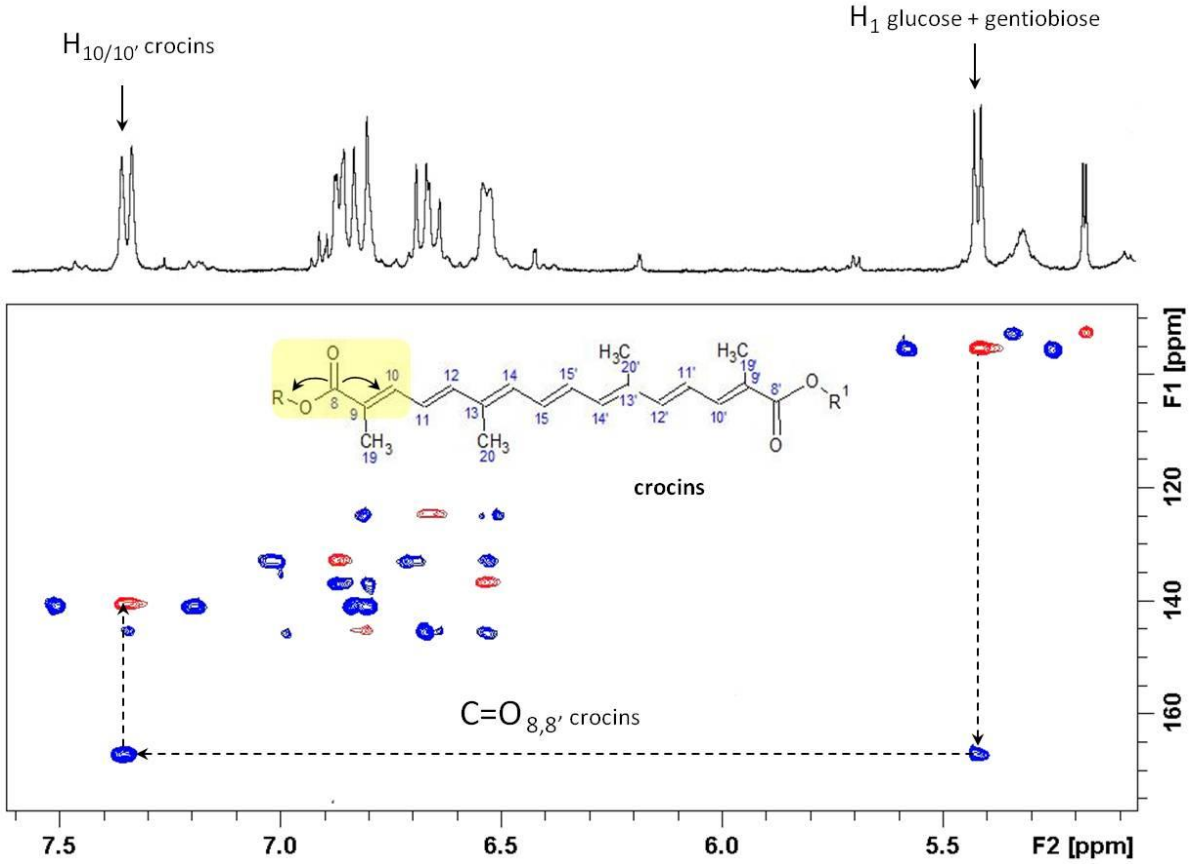
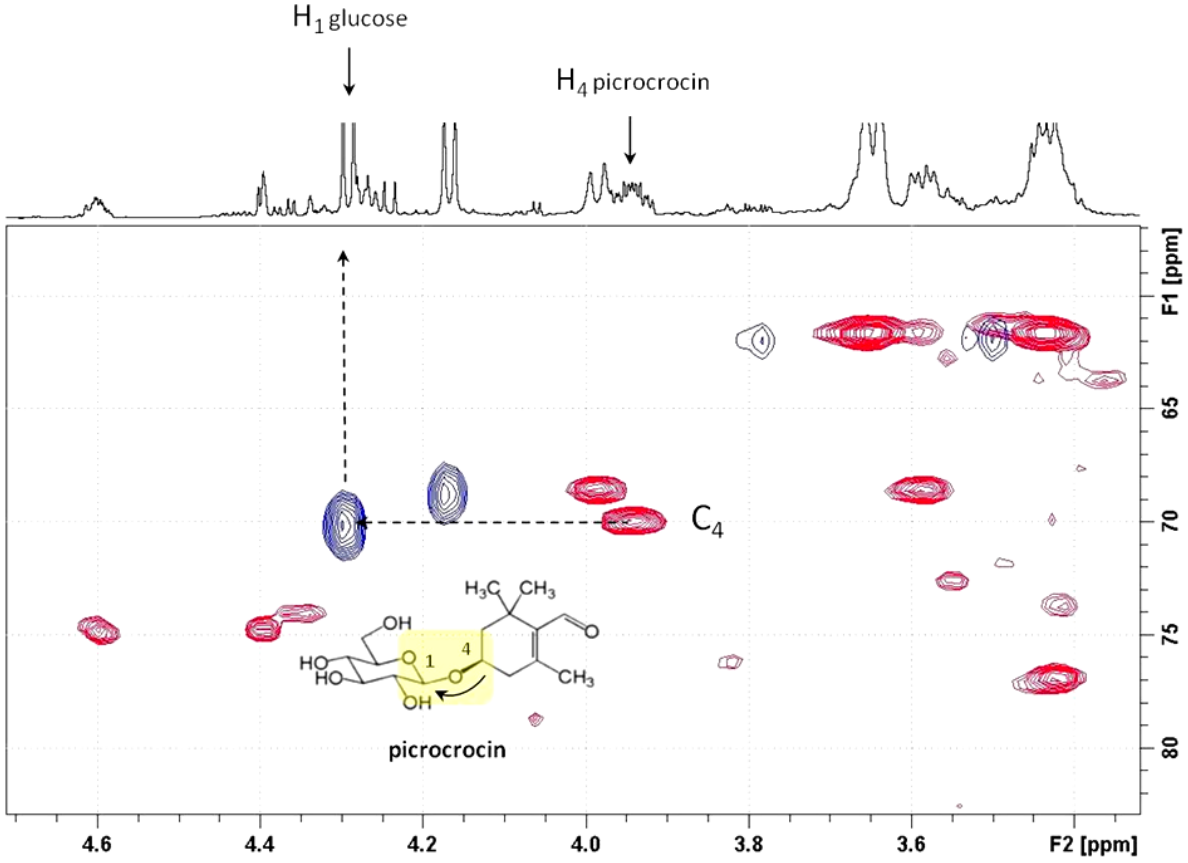

H10/10′ crocins
H1 glucose + gentiobiose
crocins
C=O 8,8′ crocins
F1 [ppm]
120
140
160
7.5
7.0
6.5
6.0
5.5
F2 [ppm]

Figure S2


H1 glucose
H4 picrocrocin
C4
picrocrocin
F1 [ppm]
F2 [ppm]
4.6
4.4
4.2
4.0
3.8
3.6
65
70
75
80