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# Highlights

Food Research International xxx (2015) xxx–xxx

## $^{1}$H NMR-based metabolomics of saffron reveals markers for its quality deterioration

Stella A. Ordoudi [a], Laura R. Cagliani [b], Sofia Lalou [a], Eleni Naziri [a], Maria Z. Tsimidou [a], Roberto Consonni [b,*]

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- NMR based metabolite fingerprinting for saffron quality characterisation
- Identification of storage threshold (4 years) for freshness of saffron
- Identification of specific markers to monitor storage effects



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Q5 Supplementary material.

Food Research International xxx (2015) xxx–xxx

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# [1]H NMR-based metabolomics of saffron reveals markers for its quality deterioration

Stella A. Ordoudi [a], Laura R. Cagliani [b], Sofia Lalou [a], Eleni Naziri [a], Maria Z. Tsimidou [a], Roberto Consonni [b,*]

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ABSTRACT

[1]H NMR-based metabolomics is proposed here for the quality control of saffron upon storage using the ability to detect a wide range of chemical compounds with minimal sample preparation. Saffron quality deterioration is the result of enzymatic, oxidative and hydrolytic reactions that change the content and structure of the glycosides of crocetin and picrocrocin, which are the metabolites responsible for the major sensory properties of this most expensive spice. Several authentic saffron samples (n = 98) of known storage history after processing were examined. The Principal Component Analysis shows a clear-cut separation of samples into two groups based on the storage period regardless of the sample origin. The S-plot derived from the Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) model shows the markers for quality deterioration, i.e., sugars bound to crocetin, glucose in picrocrocin, free sugars and fatty acids. These new markers become critical for the samples that were stored for more than 4 years. The OPLS-DA model was validated with a test set and proved to be properly designed to predict the length of storage after harvest. The answer to the question set in the market of "when" and "why" a saffron sample can no longer be considered fresh is supported by our findings.



## 1. Introduction

Saffron, which is the dried red stigmas of *Crocus sativus* L. flowers, is a highly appreciated spice because it imparts foods with unique colour and flavour traits that are attributed to the high concentrations of certain secondary metabolites (crocetin esters, picrocrocin, safranal, etc.) (Ordoudi & Tsimidou, 2004). In addition, it is also known to exert beneficial health properties and is a valuable candidate in new drug designs (Bathaie & Mousavi, 2010). *C. sativus* L. fresh stigmas are converted to saffron spice by dehydration. Dehydration practices, which are usually traditional, differ among the countries of production (Carmona, Zalacain, & Alonso, 2006) and affect the moisture content of the end product (saffron) and the contents of its secondary metabolites, which determine the initial commercial quality of the product (ISO, 2011). The moisture content and other storage conditions (temperature, exposure to light, oxygen and enzymatic activity) affect the deterioration rate of the saffron quality (Alonso, Varon, Salinas, & Navarro, 1993; Bolandi & Ghoddusi, 2006; Morimoto, Umezaki, Shoyama, Saito, Nishi, & Irino, 1994; Raina, Agarwal, Bhatia, & Gaur, 1996; Tsimidou & Biliaderis, 1997). Kinetic studies at ambient temperature in the dark, which mimicked the best practice for saffron storage, show that its shelf life dramatically depends on the water activity ($a_w$). In particular, at intermediate $a_w$ levels (0.43–0.53), the colouring strength is effectively retained and indicates a possible post-harvest storability of the product of more than one year. Indeed, a half-life period ($t_{1/2}$) of approximately five years was found for ground saffron that was stored in the dark at $a_w$ 0.43 and 25 °C (Tsimidou & Biliaderis, 1997). In this long period of storage, the bitterness is also retained as suggested by the slow kinetics of picrocrocin degradation (Alonso et al., 1993). Under good processing and storage conditions (dark, intermediate $a_w$ and ambient temperature), the content of major metabolites remains intact within one year after production (Raina et al., 1996); thus, the spice can be considered "fresh" during this period. Deterioration of the spice in time as a result of enzymatic, oxidative and hydrolytic reactions leads to severe loss in secondary metabolites, development of off-flavours and tissue changes (Bonazzi & Dumoulin, 2011; Maggi et al., 2010; Morimoto et al., 1994). In all of the above studies, the data for different classes of important metabolites were produced using methods that lack the potential of analytical techniques such as infrared spectroscopy and nuclear magnetic resonance to identify the useful markers in the quality control of this most expensive spice in the world. Indeed, recently, using Fourier-Transform Mid-Infrared (FT-MIR) spectroscopy coupled with chemometrics, it was found that the bands related to free glucose and breakage of glycosidic bonds were useful for the diagnostic monitoring of storage effects (Ordoudi, de los Mozos Pascual, & Tsimidou, 2014). Limitations of the FT-IR technique regarding the structure elucidation of the responsible compounds are expected to be overcome using analytical techniques that are specifically dedicated to structural-elucidation studies.

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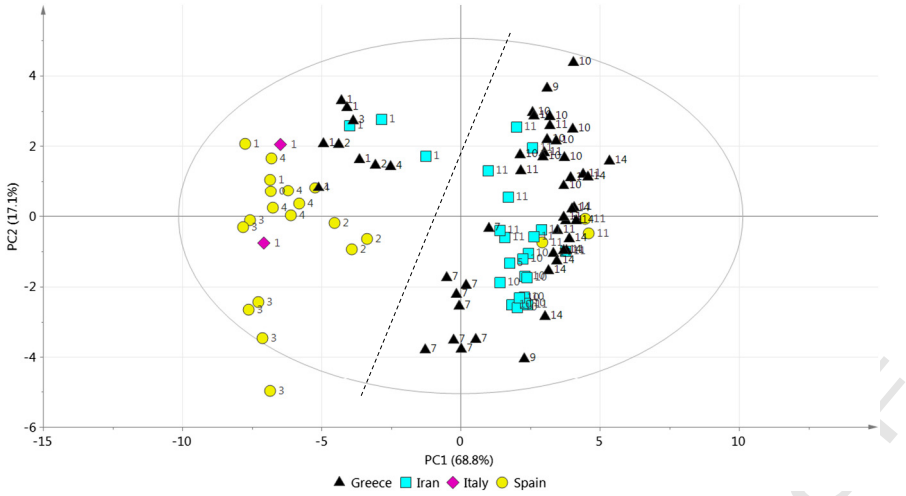


**Fig. 1.** PCA score plot performed considering all (n = 98) saffron samples. PC1 = 68.8%; PC2 = 17.1%. $R^2X = 92.8\%$; $Q^2 = 90.4\%$. The samples are coloured according to the origin: black triangles, light blue boxes, purple diamonds and yellow circles represent Greek, Iranian, Italian and Spanish samples, respectively; the storage period for each sample is reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NMR-based metabolomics, which enables the identification and quantification of all molecules in a biological system, shows a great potential in food science as a tool to monitor the product quality and authenticity among other purposes (Cevallos-Cevallos & Reyes-De-Corcuera, 2012; Mannina, Sobolev, & Capitani, 2012; Wishart, 2008). The large sets of metabolomic data can be handled by

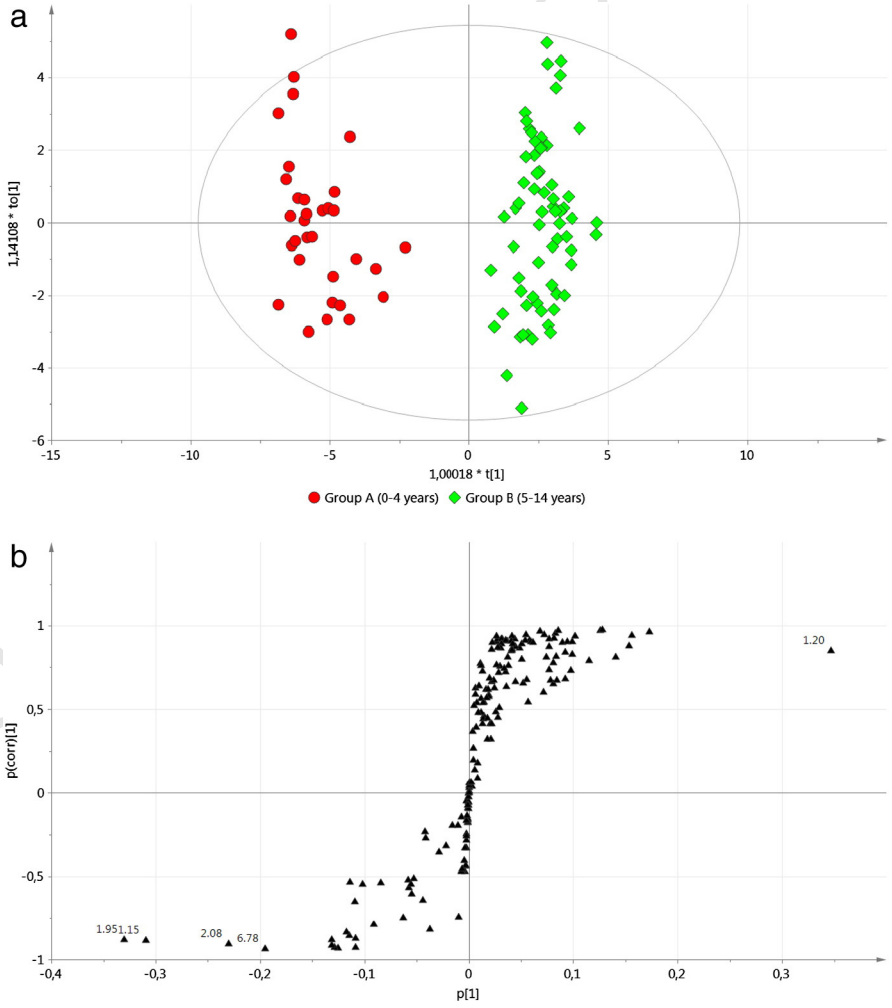


**Fig. 2.** Score plot (a) and S-plot (b) of OPLS-DA, which was performed considering all saffron samples: group A (saffron stored up to 4 years) and group B (saffron stored for more than 4 years). $R^2X = 88.2\%$, $R^2Y = 94.6\%$, and $Q^2 = 93.7\%$. The ppm values were only indicated for the relevant metabolites.

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chemometric tools, which enable the evaluation of differences or similarities in the analysed samples, classification of the samples and development of predictive models (Consonni & Cagliani, 2010).

Several studies in the literature focused on the food quality and authenticity assessment using NMR metabolite fingerprinting with chemometrics (Alonso-Salces, Moreno-Rojas, Holland, Reniero, Guillou, & Heberger, 2010; Cagliani, Pellegrino, Giugno, & Consonni, 2013; Consonni & Cagliani, 2010; Spraul et al., 2009). Concerning saffron, NMR has been applied to isolated constituents or saffron extracts mainly for identification purposes (Assimiadis, Tarantilis, & Polissiou, 1998; Calsteren et al., 1997; Pfister, Meyer, Steck, & Pfander, 1996; Straubinger, Jezussek, Waibel, & Winterhalter, 1997). The metabolite fingerprinting of saffron extracts using $^1$H-NMR spectra and chemometrics has been reported for the authentication of Iranian and Italian saffron (Cagliani, Culeddu, Chessa, & Consonni, 2015; Yilmaz, Nyberg, Molgaard, Asili, & Jaroszewsk, 2010) and adulteration with certain plant adulterants (Petrakis, Cagliani, Polissiou, & Consonni, 2015). The potential of applying the NMR-based metabolomic approach in controlling the saffron quality deterioration has not been examined.

The present study aimed to further elucidate the importance of sugars in free or bound forms to monitor the quality deterioration of saffron upon storage. The importance of other metabolites for the same objective was also investigated. Several authentic saffron samples (n = 98) of known storage history after processing were examined using $^1$H NMR and chemometrics. The long-term benefit of this investigation is to answer the question set in the market of "when" and "why" a saffron sample can no longer be considered "fresh".

## 2. Materials and methods

### 2.1. Saffron samples

In total, 98 saffron samples were investigated with $^1$H NMR. In particular, the saffron of Spanish (n = 21), Greek (n = 51) and Iranian (n = 24) origin belonged to the sample collection of the laboratory of Food Chemistry and Technology (AUTh); the Italian samples (n = 2; PDO products from L'Aquila and from Sardinia) were kindly gifted by the producers. Details of the geographical origin, harvest year, storage conditions and time elapsed until the NMR analysis are provided as Supplementary data (Table S-1). The samples that were analysed within the first year after harvesting are considered to be "fresh".

### 2.2. NMR analysis

#### 2.2.1. Sample preparation

The NMR saffron sample was obtained by stirring (vortex) the deuterated dimethylsulfoxide (DMSO-$d_6$, 600 μL) extract of approximately 4 mg of ground stigmas for 3 min at room temperature. After 10 min, centrifugation at 12,100 *g* for 10 min was performed, and 500 μL of the supernatant was used for the NMR analysis.

#### 2.2.2. Spectrum acquisition and processing

All $^1$H-NMR spectra were obtained using the 11.7 T NMR spectrometer (Bruker DMX 500, Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany), which was equipped with a 5-mm reverse probe with z-gradient. The data were obtained with the following acquisition parameters: sample temperature 300 K, spectral width 8012 Hz and 32 K data points. The residual water suppression was achieved by applying a presaturation scheme with low-power radiofrequency irradiation for 1.2 s. A resolution enhancement function with an exponential multiplication of 0.5 Hz for the line broadening was applied; all $^1$H NMR spectra were carefully phased and baseline-adjusted with the TOPSPIN3.0® software (Bruker BioSpin GmbH, version 1.3, Rheinstetten, Karlsruhe, Germany). The spectra were referenced to the solvent signal at 2.50 ppm and subjected to manual bucketing in the range of 0.4–10.4 ppm according to the resonance assignment. The bucket normalisation was performed with respect to the total integral value using the ACD/Spec Manager (ACD Labs, version 11, Toronto, Ontario,

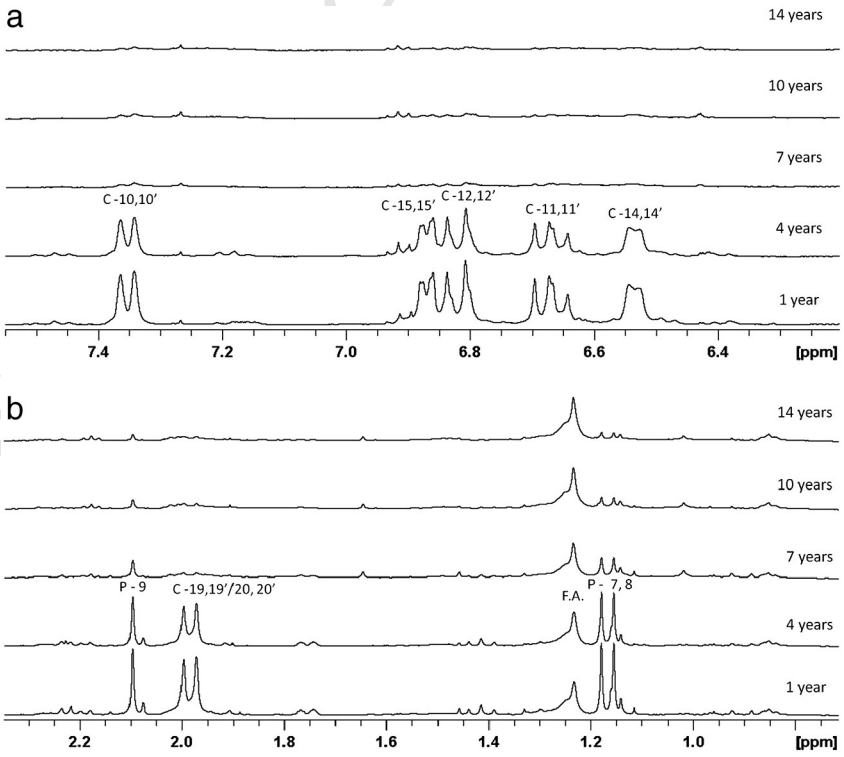


**Fig. 3.** From bottom to top: selected regions of DMSO-$d_6$ extracts of the $^1$H-NMR spectra of an Italian saffron that was stored for 1 year, a Spanish saffron stored for 4 years, a Greek saffron stored for 7 years, an Iranian saffron stored for 10 years and a Greek saffron stored for 14 years. (a) Aromatic region with the signals of crocetin esters and (b) aliphatic region with methyl signals of picrocrocin at 1.16, 1.18 and 2.10 ppm, methyl signals of crocetin esters at 1.97 and 2.00 ppm, and methylene protons of fatty acids at 1.23 ppm. The signals are numbered according to the chemical structures provided in the supplementary data (Figure S-1): P, C and F.A. stand for picrocrocin, crocetin esters and fatty acids, respectively.

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Canada), where the exclusion of solvent signal was 2.48–2.53 ppm, and the residual water signal was 3.30–3.37 ppm.

*2.2.3. Statistical methods*

SIMCA-P + 13 (Umetrics, Umea, Sweden) was used for the Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) of the NMR data. Data pretreatment was performed with "Pareto" scaling. The OPLS is well suited to decompose the systematic variation in the X block into two parts: parallel and orthogonal parts. The former is also known as the predictive part, which models the joint X–Y correlated variation, whereas the latter is not related to Y, usually defined as "structured noise" with unpredictive capability and removed (Trygg & Wold, 2002). The OPLS can be used in discriminant analysis by introducing dummy variables. A useful visualisation graph, which is the so-called S-plot (Wiklund et al., 2008), was used to highlight the role of the variables in the model. T2 and the distance to the model (DModX) tests were applied to check for outliers and evaluate whether the test set samples fall within the model applicability domain. The model was validated with the permutation test on the Y block to safely overcome randomness or overfitting to the model.

*2.2.4. Training and test set selection*

To investigate the predictive capability of the statistical model, a balanced training set and a test set were extracted from the candidate set based on the PCA model.

*2.3. GC-MS analysis of fatty acid methyl esters*

Fatty acid methyl esters, which were prepared using a cold alkali transesterification procedure, were analysed on an Agilent 6890A gas chromatograph (Palo Alto, CA, USA), which was equipped with an MSD 5973 mass spectrometer (MS) under the following conditions: HP FFA column (15 m × 200 μm × 0.30 μm); carrier gas, helium (1.1 mL/min); oven temperature 50 °C (3 min), increased at 15 °C/min to 200 °C (10 min) and at 20 °C/min to 220 °C (6 min); injector temperature 230 °C; detector temperature 230 °C. The MS was operated in the electron-impact mode (MS-EI) at 70 eV to scan the range 35–350 m/z at a scan rate of 2 scans/s. The samples were injected with a split ratio of 1:30. The transfer line and ion source temperature were set at 280 °C and 230 °C, respectively. Identification was based on the comparison of retention times and mass spectra with the standard ones. The data were analysed using the MSD ChemStation software.

## 3. Results and discussion

The $^1$H NMR data of all samples were initially explored using the unsupervised PCA; four components (PC1 = 68.8%, PC2 = 17.1%) explain 92.8% of the total variance ($R^2X$), and the prediction goodness parameter is $Q^2$ = 90.4%. The corresponding score plot in Fig. 1 shows a clear clustering of the samples into two groups along the first principal component according to the storage period. The samples that were stored up to four years (hereafter named Group A) are negatively correlated with PC1; conversely, the samples that were stored for 5–14 years (hereafter named Group B) positively correlate with PC1. This inherent grouping appears regardless of the sample origin (Table S-1). Then, a two-class OPLS-DA model was performed to better highlight the responsible variables of this clustering. This model results in one predictive and two orthogonal components (Fig. 2a). The corresponding S-plot (Fig. 2b) illustrates the most relevant variables that affect the sample differentiation between the two classes. The buckets at 1.95 ppm because of the methyl groups, 6.78 ppm because of the conjugated double bonds of the hydrocarbon skeleton of crocetin esters and 1.15 and 2.08 ppm because of the methyl groups of picrocrocin characterise the clustering in Group A (Fig. 3). In addition to the resonances because of the aglycon moieties of crocetin esters and picrocrocin, considering the previous finding (Ordoudi et al., 2014) about the fate of glycosidic bonds, the signals related to β-glucose bound to the 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde moiety of picrocrocin (bucket at 4.28 ppm, |p| = 0.12 and |p(corr)| = 0.83) and β-glucose and β-gentiobiose bound to crocetin (bucket at 5.38 ppm, |p| = 0.11 and |p(corr)| = 0.93) were also identified as the contributors to the clustering of Group A. The anomeric signals of α-glucose and α-gentiobiose in unbound form, which overlap in the bucket at 4.86 ppm (|p| = 0.08 and |p(corr)| = 0.82) (Cagliani, et al., 2015), account for the allocation of the samples to Group B. This particular finding, which is completely consistent with previous ones based on FT-IR measurements (Ordoudi et al., 2014), provides some evidence for the hydrolytic type of reactions that faster occur in the storage period of 0–4 years and significantly decrease the desired colour and taste attributes that are abundant in the "fresh" spice. Thus, much lower amounts of crocetin esters and picrocrocin appear in samples that are stored for more than four years (Group B). In the spectra of Group B, the bucket at 1.20 ppm, which was recently assigned to methylene protons of saturated and unsaturated fatty acids (Cagliani et al., 2015), accounts for the most differentiation of Group B samples. The GC-MS analysis of

**Table 1**
Prediction of group of storage for the test set samples (n = 46) considering the training set (n = 52), which had samples of different geographical origins in Group A (0–4 years of storage) and Group B (5–14 years of storage). The classification threshold was 0.6.

| Origin | Group | Y predicted for Group A | Y predicted for Group B |
|---|---|---|---|
| Greece | A | **0.9** | 0.1 |
| Greece | A | **1.0** | 0.0 |
| Greece | A | **1.3** | −0.3 |
| Greece | A | **0.6** | 0.4 |
| Greece | B | 0.1 | **0.9** |
| Greece | B | −0.1 | **1.1** |
| Greece | B | 0.1 | **0.9** |
| Greece | B | 0.1 | **0.9** |
| Greece | B | 0.1 | **0.9** |
| Greece | B | −0.1 | **1.1** |
| Greece | B | 0.1 | **0.9** |
| Greece | B | 0.0 | **1.0** |
| Greece | B | 0.0 | **1.0** |
| Greece | B | −0.1 | **1.1** |
| Greece | B | 0.0 | **1.0** |
| Greece | B | 0.0 | **1.0** |
| Greece | B | 0.0 | **1.0** |
| Greece | B | −0.1 | **1.1** |
| Greece | B | 0.0 | **1.0** |
| Greece | B | 0.0 | **1.0** |
| Greece | B | −0.1 | **1.1** |
| Greece | B | −0.1 | **1.1** |
| Greece | B | 0.0 | **1.0** |
| Greece | B | −0.2 | **1.2** |
| Iran | A | **0.9** | 0.1 |
| Iran | B | 0.1 | **0.9** |
| Iran | B | 0.1 | **0.9** |
| Iran | B | 0.0 | **1.0** |
| Iran | B | 0.2 | **0.8** |
| Iran | B | 0.1 | **0.9** |
| Iran | B | 0.0 | **1.0** |
| Iran | B | 0.0 | **1.0** |
| Iran | B | 0.0 | **1.0** |
| Iran | B | 0.1 | **0.9** |
| Iran | B | 0.1 | **0.9** |
| Spain | A | **1.1** | −0.1 |
| Spain | A | **0.9** | 0.1 |
| Spain | A | **0.9** | 0.1 |
| Spain | A | **0.9** | 0.1 |
| Spain | A | **1.0** | 0.0 |
| Spain | A | **1.0** | 0.0 |
| Spain | A | **1.0** | 0.0 |
| Spain | A | **1.0** | 0.0 |
| Spain | B | −0.2 | **1.2** |
| Italy | A | **0.7** | 0.3 |
| Italy | A | **1.1** | −0.1 |



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fatty acid methyl esters of randomly selected fresh and aged saffron shows that the major constituents are palmitic (16:0), oleic and its isomers (18:1, 9c- and 11c-), linoleic (18:2) and linolenic (18:3) acids. The importance of fatty acids as saffron quality deterioration markers has not been evaluated so far, most likely because of the relatively low lipid content of the spice (Carmona et al., 2006). The $^1$H NMR spectral analysis uses their presence as markers to control the saffron quality deterioration when metabolic profiling approaches are employed. Saffron samples that were stored at temperatures below 20 °C under controlled $a_w$ conditions were found to be well preserved up to four years. This result is fully consistent with literature findings, which were discussed in the Introduction (Alonso et al., 1993; Bolandi & Ghoddusi, 2006; Morimoto et al., 1994; Raina et al., 1996; Tsimidou & Biliaderis, 1997). As noted by Tsimidou and Biliaderis (1997), "unlike the typical effect of $a_w$ on lipid oxidation kinetics, where the rate increases below the monolayer $a_w$ value (i.e., in the dry state), as well as above 0.3–0.4 $a_w$, oxidative degradation of saffron pigments increases with increasing $a_w$, within the range of 0.11–0.64 $a_w$. Water appears to have a plasticising effect, lowering the *Tg* (glass transition temperature) of the material and thereby enhancing the mobility of the reactants." Moreover, according to Raina et al. (1996), during extended storage (e.g., 36 months), the processing conditions have weaker effects than those of the natural degradation of metabolites in the plant cells. As a result, it is more difficult to differentiate the origin of old harvest samples than recent ones because of the biochemical changes and physical damage of the tissues (Bonazzi & Dumoulin, 2011). The representative $^1$H NMR spectra of saffron of different origins and storage periods are shown in Fig. 3. A combination of FT-IR and NMR spectral data and multivariate analysis was recently used to assess the metabolic differences in processed plant material such as conventional and genetically modified potatoes (Kim et al., 2009). In that case, primary metabolites such as amino acids and carbohydrates were proposed as the markers of quality deterioration after only one week of storage.

To check the predictive capability of the OPLS-DA model, balanced training and test sets were selected based on the PCA score plot: in particular, 52 samples (17 from Group A and 35 from Group B) and 46 samples (15 from Group A and 31 from Group B) were selected. The new two-class OPLS-DA model, which was obtained with the training set samples, resulted in one predictive and three orthogonal components ($R^2X = 92.2\%$, $R^2Y = 96.0\%$ and $Q^2 = 94.0\%$). The permutation test that was performed on the corresponding PLS-DA validated the model. The classification list in Table 1 shows the predictive performance of the model: all test samples were correctly classified when 0.6 was used as the classification threshold. It was noticed that although there was no Italian sample in the training set, they were properly classified according to their age.

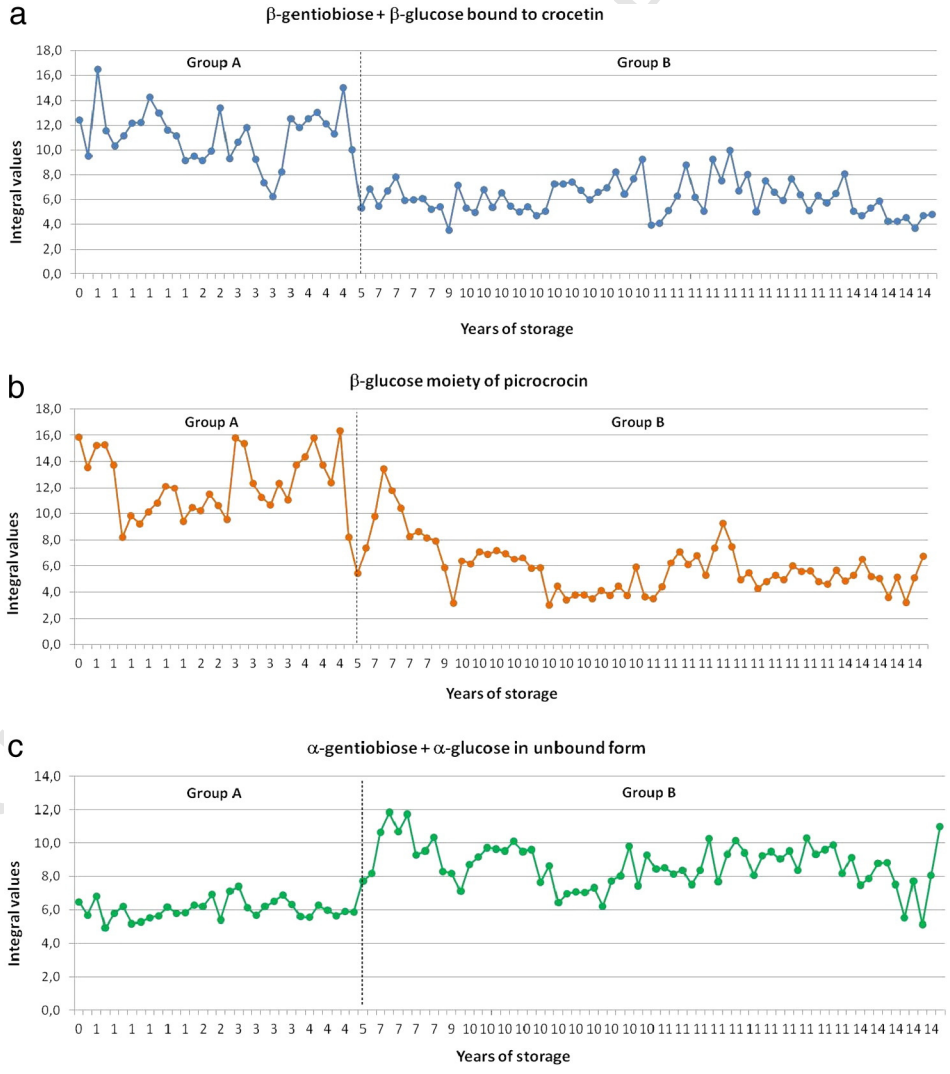


**Fig. 4.** a–c. Evolution of the integral for the bucket at (a) 5.38 ppm, which refers to the anomeric proton of β-glucose and β-gentiobiose (ring A) bound to crocetin, (b) 4.28 ppm, which refers to the anomeric proton of β-glucose moiety of picrocrocin and (c) 4.86 ppm, which refers to the anomeric proton of α-glucose and α-gentiobiose (ring A) in free form. The integrals were scaled to the solvent signal. The samples are distributed according to the sequence reported in the supplementary data (Table S-1).

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Ordoudi et al. (2014) proposed that the IR band at 1028 $cm^{-1}$, which is associated with the presence of glucose moieties and the band in the region of 1175–1157 $cm^{-1}$ that refers to the breakage of glycosidic bonds, are useful tools to monitor the effects of storage on the saffron quality. In the present study, further insight towards the nature and fate of glycosidic bonds was gained. In particular, the potential of $^1H$ NMR to resolve glucose moieties that are bound to crocetin from those in picrocrocin allows one to monitor the changes in content of the two most abundant saccharides (gentiobiose and glucose) in bound and unbound forms for all samples. Fig. 4a shows the evolution of the integral value for the bucket at 5.38 ppm concerning both anomeric proton of β-glucose and β-gentiobiose bound to crocetin. The average value of this integral calculated for the samples of Group B is approximately 45% lower than that of the samples of Group A. The same trend was observed for the β-glucose moiety of picrocrocin, where the average value of the bucket integral at 4.28 ppm is approximately 52% lower than that in Group A samples (Fig. 4b). Conversely, the glucose and gentiobiose contents in the unbound state (bucket at 4.86 ppm referred to the α-isoform for both saccharides), which remain nearly constant for the samples that were stored until four years, clearly increase after this period by approximately 44% (Fig. 4c).

## 4. Conclusions

The results show that $^1H$ NMR is a potent tool to control the saffron quality deterioration and presents specific advantages over FT-IR. Using the NMR-based metabolomic approach, we gained insights to the structural changes in crocetin esters and picrocrocin and differentiated the sugars that were bound to each of them. Moreover, the signals assigned to fatty acids became prominent in the spectra of old saffron. These compounds are not usually examined in the quality control studies of this spice because of their notably low concentration in this matrix. The OPLS-DA of data shows that bound or free forms of glucose and gentiobiose and fatty acids can be used as markers of the quality deterioration of this precious commodity. The answer to the question of "when" and "why" a saffron sample can no longer be considered "fresh" is strongly supported by our findings. The latter is in line with those of model kinetic studies which suggest that the saffron that was produced under optimum processing conditions and appropriately stored may retain its valuable characteristics for more than one year and up to four years.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2015.01.021.

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