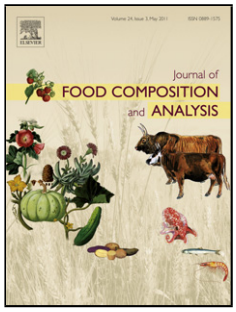
Guijarro-Díez, Miguel et al., 2017. Detection of saffron adulteration with gardenia extracts through the determination of geniposide by liquid chromatography–mass spectrometry. Journal of food composition and analysis, 55, pp.30–37.

Available at https://doi.org/10.1016/j.jfca.2016.11.004



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PII: S0889-1575(16)30194-6
DOI: http://dx.doi.org/doi:10.1016/j.jfca.2016.11.004
Reference: YJFCA 2775

To appear in:

Received date: 27-6-2016
Revised date: 27-10-2016
Accepted date: 9-11-2016

Please cite this article as: Guijarro-Díez, Miguel., Castro-Puyana, María., Crego, Antonio Luis., & Marina, María Luisa., Detection of saffron adulteration with gardenia extracts through the determination of geniposide by liquid chromatography-mass spectrometry.*Journal of Food Composition and Analysis* http://dx.doi.org/10.1016/j.jfca.2016.11.004

Detection of saffron adulteration with gardenia extracts through the determination of geniposide by liquid chromatography-mass spectrometry

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Type of paper: Original Research Article.

Highlights:

- Development of a LCMS methodology for the quality control of saffron.
- Potential of geniposide as adulteration marker of saffron with gardenia extracts.
- LCMS method enabled the detection of up to 0.004 % of adulteration with gardenia.

Abstract

A new and sophisticated saffron adulteration method with gardenia was recently discovered in the European saffron market. In this work, an analytical methodology using liquid chromatography-(quadrupole-time of flight)-mass spectrometry has been developed for the detection of the adulteration of saffron samples with gardenia through the determination of geniposide as adulteration marker. A fused-core C18 column was employed using an isocratic elution with water:acetonitrile (85:15 v/v) containing 0.1 % formic acid. After optimization of the mass spectrometry conditions, the analytical characteristics related to the determination of geniposide in negative electrospray ionization mode were evaluated. Then, it was possible to detect up to 10 ng/mL geniposide after a dilution step of 50-fold of the saffron extract (LOD of 41.7 µg of geniposide per gram of sample analysed (i.e up to 0.004 %)). The developed LC-MS methodology was applied to the analysis of different authentic and suspicious saffron samples.

Keywords: adulteration marker, geniposide, gardenia, liquid chromatography-mass spectrometry, QTOF, quality control, saffron, food analysis, food composition.

1. Introduction

Food safety involves no risk to consumer health. However, this property is impossible to ensure when food adulteration by food producers, manufacturers, processors, distributors, or retailers occurs because in any case it results in a change of the identity and/or purity of the original food using physical or chemical means. One of the risks gaining attention in food safety is the possibility of food poisoning when adulterated with chemical extracts (Moore et al., 2012).

Saffron has been described as one of the most commonly adulterated food ingredients due to its high price and limited quality assurance (Moore et al., 2012; Petrakis et al., 2015). It is produced from the dried stigmas of *Crocus sativus* L. being considered as one of the most expensive spice in the world because of the direct labor required for growing, harvesting and handling as well as its limited production. This spice has been employed for a long time as a flavoring and colorant in food preparation; however, it is also known for a wide range of health benefits, such as offering some protection against heart disease and cancer, and having a high potential as a memory enhancer (Rios et al., 1996; Karimi et al., 2001; Abdullaev, 2002; Hosseinzadeh and Younesi, 2002; Melnyk et al., 2010; Papandreau et al., 2011). In addition to its three main secondary metabolites, crocins (crocin and its derivatives are responsible for coloring strength), picrocrocin (responsible for the saffron taste), and safranal (responsible for the flavor), saffron also contains flavonoids, proteins, sugars, vitamins, amino acids, mineral materials, gums, and other chemical compounds (Winterhalter and Straubinger, 2000; USDA Food composition Database).

Common fraudulent practices aimed to saffron adulteration include the addition of different plant materials with similar color and morphology in order to increase its weight and/or to improve its colour properties (or enhance its colour when foreign matter has been added) using natural or synthetic components (Melnyk et al., 2010).

To certificate saffron quality in the international trade market, it is classified by its aroma, flavor, and color strength using the ISO 3632-1: 2011 method, which combines spectrophotometric measurements of picrocrocin and safranal, and chromatographic profiles of pigments (crocins) and apolar dyes that can be toxic (as Sudan dyes) (ISO 3632-1; ISO 3632-2). Nevertheless, this legislation is being revised due to the fact that it is not able to detect saffron adulterations by plant foreign matter with similar color and morphology. In fact, it has recently been demonstrated that saffron adulterants (safflower, marigold or turmeric) up to 20% (w/w) were not detected by the ISO normative (Sabatino et al., 2011).

Several analytical methodologies have been developed to detect plants adulterants in saffron samples. Chromatographic (Sampathu et al., 1984; Alonso et al., 1998; Lozano et al., 1999; Haghighi et al., 2007; Sabatino et al., 2011) and molecular techniques (Ma et al., 2001; Javanmardi et al., 2011; Marieschi et al., 2012; Babaei et al., 2014; Torelli et al., 2014) have been employed with this purpose and have originated encouraging results. For instance, the use of DNA markers enabled the detection of low amounts (up to 1%) of various materials including safflower and turmeric (Javanmardi et al., 2011; Marieschi et al., 2012). Some non-targeted metabolomic studies have also been carried out to discover new authenticity saffron markers but the proposed markers do not allow the identification of the type of the plant used for saffron adulteration (Yilmaz et al., 2010; Cagliani et al., 2015; Guijarro-Díez et al., 2015).

None of the above-mentioned methods enabled to detect saffron adulterations with chemical extracts of *Gardenia jasminoides Ellis* L. (gardenia), a new and more sophisticated type of adulteration than those previously used and difficult to detect because this plant shares with saffron a large number of crocins and flavonoids (responsible for the yellow color) (Pfister et al., 1996; Van Calsteren et al., 1997; Carmona et al., 2006;). In fact, a large number of saffron

adulterations using gardenia extracts has been discovered in the European market. Due to the morphological differences of gardenia and saffron stigmas, adulteration mainly occurs when saffron is in powder form since gardenia extract can be more easily hidden (Guijarro-Díez et al., 2015). Recently, a metabolite fingerprinting strategy based on the use of NMR (using chemometric strategies for classification of samples) has shown to be able to differentiate authentic saffron samples from saffron samples adulterated with 20% of gardenia, turmeric, safflower, and saffron stamens (Petrakis et al., 2015). However, there is an ongoing demand for the development of rapid, simple and sensitive analytical methodologies enabling the detection of saffron adulteration with low amounts of plant adulterants.

Several analytical methods were proposed to find out the fingerprint of Gardenia fruit including HPLC and GC (Yan et al., 2006; Zhou et al., 2010; Ding et al., 2010; Yang et al., 2011; Li et al., 2015; Han et al., 2015). The principal active constituents of gardenia are the iridoid glycosides: geniposide, gardenoside, genipin-1-β-gentiobioside, geniposidic acid, acetilgeniposide, and gardoside (Wang et al., 2004). Among them, geniposide has been recognized as the major iridoid component. Carmona *et al*., described the presence of geniposide in gardenia and its lack in saffron when they studied differences in the chromatographic profile of both samples (Carmona et al., 2006). This fact is of high relevance since it points out the possibility of using this compound as a marker of adulteration of saffron with cheaper gardenia extracts. Even though different HPLC and CE methodologies have been developed to determine geniposide in the Gardenia fruit (Tsai et al., 2002; He et al., 2006; Hou et al., 2007; Bergonzi et al., 2012; Gao et al., 2013; Coran et al., 2014; Lee et al., 2014; Wang et al., 2015), no studies have been reported until now on the determination of geniposide in saffron samples which could enable to propose geniposide as a novel adulteration marker of saffron with gardenia extracts.

The aim of this work was to develop a sensitive LC-MS methodology enabling the determination of geniposide as adulteration marker of saffron with gardenia extracts which could be a powerful tool to be applied in the routine quality control to detect adulterations of saffron with gardenia extracts.

2. Materials and Methods

2.1 Chemicals and samples

Acetonitrile, ethanol, and formic acid of HPLC grade were purchased from Scharlab (Barcelona, Spain), while water was purified through a Milli-Q system (Millipore, Bedford, MA). Geniposide standard (purity ≥98%), sodium tetraborate, ammonium formate, and ammonium acetate were obtained from Sigma (St. Louis, MO, USA).

A total of eight samples (stigmas and powered) of authentic saffron from Iran and Spain were provided by “Carmencita" (Alicante, Spain). All these samples were of Commercial Category I and their quality and authenticity were checked according to ISO 3632. The low number of these samples can be explained by the fact that they were supplied with the guarantee of their origin and authenticity (lack of adulteration). One powdered gardenia extract (with an estimated geniposide content of 37.5 mg/g extract) and ten saffron samples (stigmas and powered) suspected of being adulterated according to the criteria of the market based on their low cost and/or questionable origin were also provided by “Carmencita” company.

2.2 Standard and sample preparation

A stock standard solution of geniposide was prepared by dissolving it in acetonitrile up to a final concentration of 1 mg/mL. This solution was stored at 4 ºC and different aliquots were diluted in Milli-Q water to get solutions with different concentrations of geniposide.

Saffron stigmas were finely ground in a mortar with stainless balls Ultra Turrax (IKA, Staufen, Germany) for 2 min. 0.3 g of ground or powdered saffron samples and gardenia extract were extracted under optimized conditions with 25 mL of ethanol:borate buffer at pH 9.0 (50:50 v/v) by using an ultrasonic-assisted solid-liquid extraction for 15 min at room temperature. After centrifugation (15 min, 4000*g* and 25 ºC) the supernatant fraction was diluted 1/50 with Milli-Q water and 4 mL of this solution were ultra-filtered through a 3 kDa cut-off filter (Amicon Ultra Filters, Merck, Darmstadt, Germany) to remove carbohydrates and proteins. These solutions were stored at 4ºC and warmed at room temperature before use.

2.3 LC-MS analysis

LC analysis were carried out in a 1100 series LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a mass spectrometer via an orthogonal electrospray ionization source (ESI) with Jet Stream thermal focusing technology (6530 series, Agilent Technologies, Palo Alto, CA, USA). MS detection was performed in a quadrupole time offlight (QTOF) series 6530 (Agilent Technologies, Palo Alto, CA, USA). MS control, data acquisition, and data analysis were performed by using the Agilent Mass Hunter software (B.040.00).

Two different columns supplied by Sigma (Sigma, St. Louis, MO, USA), namely an Ascentis Express Fused-core C18 column and an Ascentis Express Fused-core Cyano column, both 100 mm×2.1 mm, fused-core® particles with 0.5 µm thick porous shell and 2.7 µm particle

size, were tested. Both separation columns were protected using C18 and cyano pre-columns, respectively (Ascentis Express guard column (5 x 2.1 mm) from Sigma).

LC analyses with gradient elution were carried out by using a mobile phase of water containing 0.1 % formic acid (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B) from 5 to 17.5 % B in 10 min, from 17.5 to 5 % B in 1 min, and 5 % B for 10 min in order to re-equilibrate the column at the initial conditions. Isocratic conditions were based on the use of a mobile phase water:acetonitrile (85:15 v/v) containing 0.1 % formic acid. In both cases, (gradient or isocratic elution), the flow rate was 0.4 mL/min, the injection volume was 5 μL, and the temperature was 40 ºC.

MS analysis was performed in positive and negative ESI modes with the mass range set at *m/z* 100-1700 (extended dynamic range) in full scan resolution mode with an acquisition rate of 2 spectra/s (mass resolution greater than 5000 on the 118 m/z and 10000 on the 1522 m/z according to the instrument specifications). MS conditions employed in a preliminary study to select the ESI polarity were: capillary voltage, 3000 V; nozzle voltage, 0 V; drying gas conditions, 10 L/min and 300ºC; nebulizer pressure, 1.7 bar; sheath gas conditions, 6.5 L/min and 300ºC; fragmentator, 150 V; skimmer, 60 V; octapole voltage, 750 V. Once the ESI polarity was chosen and after optimizing MS parameters, the ionization conditions were: ESI negative mode, capillary voltage, 3500 V with a nozzle voltage, 500 V; drying gas conditions, 10 L/min and 350 ºC; nebulizer pressure, 1.7 bar; sheath gas of jet stream, 7.5 L/min and 350 ºC; fragmentator voltage, 150 V. Skimmer and octapole voltages were automatically tuned by the instrument and their values were 60 V and 750 V, respectively. A commercial mixture from Agilent Technologies with m/z values between 112.985587 and 1633.949786 m/z was used to carry out the external calibration of the TOF-MS. In addition, m/z 121.0508 ($C_5H_4N_4$)) and

922.0097 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for ESI positive, and m/z 112.9856 ($C_2O_2F_3(NH_4)$) and 966.0007 ($C_{18}H_{18}O_6N_3P_3F_{24}$ + formate) for ESI negative, from a reference compound solution from Agilent, were employed as references masses during all analysis to allow constant mass correction to obtain accurate masses.

2.4. Method validation

The developed LC-MS methodology was validated in terms of linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision.

Linearity was evaluated using the external standard calibration method with five calibration levels ranging from 0.8 to 8 μg/mL of geniposide standard, and calculating the correlation coefficient after linear regression analysis.

LOD and LOQ values were determined as the geniposide concentrations corresponding to signal to noise ratios of 3 and 10, respectively.

Accuracy was evaluated as the recovery obtained when geniposide standard (1 μg/mL) was added to three authentic saffron samples (injected in duplicate).

Instrumental repeatability and intermediate precision were chosen to evaluate the precision of the LC-MS method developed. Both were determined using geniposide standard solutions at two concentration levels (0.8 and 8 μg/mL) and an authentic saffron sample adulterated with percentages of 10 and 90 % of gardenia extract.

2.5 Data treatment

Mass Hunter MS software was used to calculate the S/N ratio considering the peak height as the signal and noise as five times the standard deviation of the background. Experimental data

analysis and parameters calculation were achieved using Office Excel 2010 (Microsoft, Redmon, WA, USA) and Statgraphics Plus® version 5.1 (StatPoint, Herndon, Virginia, USA) software.

3. Results and discussion

3.1 Development of a LC-MS methodology for the determination of geniposide in saffron.

To develop a LC-MS methodology for the determination of geniposide in saffron samples, a preliminary study was carried out in order to investigate the effect of ESI polarity (+ or -) on the detection sensitivity of geniposide. To do that, a gardenia extract was analysed by slightly modifying the extraction and LC conditions employed previously by our research group in a metabolomic study of saffron to discover new saffron authenticity markers (Guijarro-Díez, et al., 2015). On the one hand, LC analysis was performed using a C18 column and mobile phases based on water containing 0.1 % formic acid (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B) following an elution gradient from 5 to 17.5 % B in 10 min. On the other hand, the gardenia extract analyzed in this study was obtained by the extraction of 0.3 g of ground gardenia using 6 mL of ethanol:borate buffer at pH 9.0 (50:50 v/v) and a 5-fold dilution before injecting the sample in the LC system (in order to avoid matrix interferences). As it can be observed in **Figure 1,** which shows the MS spectra obtained for geniposide, different MS fragments and adducts (Na^+ and NH_4^+) were obtained for geniposide under ESI+, whereas when the ESI- mode was employed the most abundant ion corresponded to the adduct $[M+HCOO]^-$ (433.1384 *m/z*) and no fragmentation was observed. Taking into account that in ESI- mode geniposide ionized satisfactorily without fragmentation, and that the S/N ratio was much better (4 times better), the negative (ESI-) mode was chosen to carry out the detection of geniposide by MS. One aspect that should be taken into consideration is the possibility of forming different ion adducts by modifying the composition of the mobile phase what can significantly influence the

ionization. Therefore, besides 0.1 % formic acid, the effect of adding 0.1 M ammonium acetate or 0.1 M ammonium formate to the mobile phase was investigated in negative ionization mode (ESI-). When formic acid or ammonium formate were added to the mobile phase, the most abundant ion of geniposide was the adduct $[M+HCOO]^-$. On the contrary, when ammonium acetate was employed, the most abundant ion corresponded to deprotonated molecular ions $[M-H]^-$. In any case, the highest S/N ratio for geniposide was reached when formic acid was added to the mobile phase.

In order to further increase the S/N values obtained, a complete optimization of ESI- parameters was carried out.. Thus, those parameters depending on mobile phase flow rate and composition (nebulizer pressure (1.7, 2.4, and 3.1 bar), drying gas flow-rate (8, 9, and 10 L/min), sheath gas flow-rate (5.5, 6.5, 7.5, and 12 L/min), and capillary voltage (2000, 2500, 3000, and 3500 V)), those limited by analyte thermal stability (drying gas temperature (200, 250, 300, and 350 ºC) and sheath gas temperature (250, 300, 350, and 400 ºC)), and those that only depended on analyte (nozzle voltage (0, 500, 1000, and 1500 V) and fragmentator voltage (125, 150, 175, and 225 V)) were optimized. To do that, an authentic saffron sample adulterated with 5% of gardenia extract was employed. The optimized ESI parameters obtained using the above mentioned mobile-phase at a flow-rate of 0.4 mL/min were: nebulizer pressure, 1.7 bar; drying gas flow-rate, 10 L/min; sheath gas flow-rate, 7.5 L/min; capillary voltage, 3500 V; drying gas temperature, 350 ºC, and sheath gas temperature, 350 ºC. Optimal nozzle and fragmentator voltages were 500 and 150 V, respectively.

Under the optimized ESI conditions, a saturation of the MS signal for geniposide standard at low concentration (around 10 ng/mL) was observed. Taking into account that the geniposide content in gardenia extracts can be as high as 73 mg/g (7.3%) (Tsai et al., 2002), the

extraction protocol was optimized to avoid MS saturation. Thus, 25 mL instead of 6 mL of ethanol:borate buffer at pH 9.0 (50:50 v/v) were used as dilution solvent, and different dilutions (5, 10, and 50-fold) were tested on the supernatant fraction after extraction and centrifugation for saffron samples as well as gardenia extracts. From the results obtained, the use of a 50-fold dilution enabled to overcome MS geniposide saturation (even when gardenia extracts were analyzed) and to obtain a signal intensity with enough sensitivity to determine geniposide.

Once the ESI polarity, the composition of the mobile phases, the ESI- parameters, and the extraction protocol were established, the results obtained with a C18 column were compared with those obtained with a CN column of similar characteristics. **Figure 2** shows the extracted ion chromatograms (EICs) obtained by the extraction of the signal corresponding to the adduct $[M+HCOO]^-$ (433.1384 *m/z*) for geniposide standard at 10 ng/mL using as extraction window ± 50 ppm. By comparing the chromatographic profiles depicted in **Figure 2**, it can be seen that the use of a C18 column gave rise to a better ionization of geniposide possibly due to a longer retention time and therefore to a higher percentage of organic solvent present in the mobile phase. A significant loss of geniposide signal was observed when an authentic saffron sample (extracted using the above described initial extractant conditions used for the gardenia powder) spiked with 10 ng/mL geniposide was analyzed with the cyano column, probably due to the existence of matrix interferences. For that reason, the C18 column was chosen for further studies. Finally, in order to reduce the analysis time, the possibility of using an isocratic elution instead of a gradient elution was investigated with the C18 column. As shown in **Figure 3**, it was possible to short the analysis time from 7.2 to 1.4 min without a significant loss of geniposide signal when an isocratic elution of water:acetonitrile (85:15 v/v) containing 0.1 % formic acid at a flow rate of 0.4 mL/min was employed.

3.2 Validation of the LC-MS method.

To demonstrate the method suitability for routine detection of saffron adulteration with gardenia (using geniposide as adulteration maker), the analytical characteristics of the developed LC-MS methodology for the determination of geniposide were evaluated (see **Table 1**).

Linearity was established from five calibration levels ranging from 0.8 to 8 μg/mL using a commercially available geniposide standard. Satisfactory results were obtained in terms of linearity with a correlation coefficient higher than 0.99, and with the confidence interval at 95 % for intercept including the zero value. In addition, an ANOVA test enabled to confirm that experimental data fit properly to a linear model (p-value > 0.05).

The comparison of the confidence intervals for the slopes obtained by the external standard and the standard additions calibration methods was used to investigate the existence of possible matrix interferences. Calibration by the standard additions method was carried out using two types of samples, authentic saffron and adulterated saffron with 50% of gardenia extract, both spiked with known and increasing amounts of geniposide in the range 0-6 μg/mL. The comparison of the confidence intervals obtained for the slope of each calibration straight line showed that there were no statistically significant differences between the slopes (confidence level of 95 %).

LOD and LOQ were determined based on the signal to noise ratio calculated as the concentration yielding an S/N ratio of 3 and 10, respectively. Geniposide LOD and LOQ obtained were 10 ng/mL and 30 ng/mL, respectively, which allow the detection of 41.7 μg of geniposide per gram of sample analysed, i.e up to 0.004 %, showing the high sensitivity of the

developed methodology for geniposide determination. **Figure 3** confirms experimentally the LOD obtained for geniposide (10 ng/mL).

The accuracy of the developed analytical method was assessed by evaluating the recovery obtained for geniposide when an authentic saffron sample was spiked with geniposide standard at 1 μg/mL. As shown in **Table 1** the average of the recovery values obtained was 89 ± 14 %.

Finally, precision was evaluated considering the instrumental repeatability and the intermediate precision. Instrumental repeatability was determined from three consecutive injections of geniposide standard solutions at two concentration levels (0.8 and 8 μg/mL) and an authentic saffron sample adulterated with percentages of 10 and 90 % of gardenia extract. As it can be observed in **Table 1**, RSD values were lower than 1.5 % with respect to peak areas. Intermediate precision was obtained by injecting (in triplicate) three replicates, during two consecutive days, of geniposide standard solutions at two concentration levels (0.8 and 8 μg/mL) and an authentic saffron sample adulterated with percentages of 10 and 90 % of gardenia extract. RSD values lower than 1.8 % for standard solutions and 2.9 % (with respect to peak areas) for saffron samples were obtained.

3.3 Application of the developed LC-MS methodology to the analysis of saffron samples

The developed LC-MS method was applied to the analysis of eighteen saffron samples (eight authentic saffron samples and ten saffron samples suspected of being adulterated) all of them extracted following the protocol described in section 2.2. Geniposide was not found in any of the different authentic saffron samples analyzed, as it was expected. Regarding the saffron samples suspected of being adulterated, geniposide was not detected in seven of the ten suspicious samples tested. On the contrary, geniposide was detected in three of the studied

samples. **Figure 4** shows the EICs at 433.1384 *m/z* obtained for the three saffron samples (samples 1-3) adulterated with gardenia and a suspicious sample not adulterated with gardenia (sample 4, as an example of EIC from samples without geniposide). The data obtained in these analyses (see Table 2) clearly demonstrated that the new adulteration method by gardenia has reached the European saffron market. The amounts of geniposide determined in these samples by the LC-MS method developed were 5.90 ± 0.01, 13.2 ± 0.2, and 15.30 ± 0.01 mg/g of sample analyzed

4. Conclusions

The LC-MS methodology developed in this work enables the sensitive and unequivocal determination of geniposide as marker of saffron adulteration with gardenia extracts. Even though different studies previously reported in the literature carried out the analysis of geniposide in gardenia, this is the first time that an analytical method is developed for the determination of geniposide in saffron samples as an adulteration marker with gardenia extracts.

The developed LC-MS methodology requires the use of a C18 column and an isocratic elution (water:acetonitrile (85:15 v/v) containing 0.1% formic acid). No matrix effects were observed and good results were obtained with respect to instrumental repeatability (RSD < 1.5 % for peak area), intermediate precision (RSD < 3.0 % for peak area) and LOD (41.7 µg of geniposide per gram of sample analysed, i.e up to 0.004 %). The analysis of eighteen commercial samples of saffron using this methodology showed the suitability of the method for routine analysis because of its simplicity, sensitivity, accuracy and reproducibility. Geniposide was not detected in the eight authentic saffron samples analyzed but it was detected in three of the ten saffron samples suspected of being adulterated confirming the adulteration of these saffron

samples with gardenia extracts. The fact that the three saffron samples adulterated with gardenia were in powder form showed that adulteration mainly occurs when saffron is in this form since gardenia extract can be more easily hidden.

Acknowledgements

Authors thank financial support from the Spanish Ministry of Science and Innovation (project CTQ2009-09022), the Comunidad Autónoma of Madrid (Spain) and the European funding from FEDER program (project S2013/ABI-3028, AVANSECAL-CM), and the University of Alcalá (project UAH2011/EXP-020). M.G.D. thanks the Ministry of Science and Innovation for his predoctoral contract (BES-2010-033465) and M.C.P. thanks the University of Alcalá for her postdoctoral contract and the Ministry of Economy and Competitiveness (Spain) for her "Ramón y Cajal" research contract (RYC-2013-12688). Authors also thank the kind gift of saffron and gardenia samples by Carmencita Company.

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Figure captions

Figure 1. MS spectra of geniposide in a gardenia extract analysed by LC-MS with A) ESI+ and B) ESI-. LC conditions: Ascentis Express C18 column; flow rate, 0.4 mL/min; injected volume, 5 µL; temperature 40 ºC; mobile phases, water containing 0.1 % formic acid (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B); elution gradient: 5-17.5 % B in 10 min, 17.5-5 % B in 1 min, and 5 % B for 10 min in order to re-equilibrate the column at the initial conditions. MS conditions for both ESI+ and ESI-: capillary voltage, 3000 V; nozzle voltage, 0 V; drying gas conditions, 10 L/min and 300ºC; nebulizer pressure, 1.7 bar; sheath gas conditions, 6.5 L/min and 300ºC; fragmentator, 150 V; skimmer, 60 V; octapole voltage, 750 V.

Figure 2. LC-MS extracted ion chromatogram of geniposide from geniposide standard and from an authentic saffron sample adulterated with geniposide in the Ascentis Express C18 (A and B) and cyano (C and D) columns. LC conditions as in **Figure 1.** MS conditions: capillary voltage, 3500 V; nozzle voltage, 500 V; drying gas conditions, 10 L/min and 350 ºC; nebulizer pressure, 1.7 bar; sheath gas of jet stream, 7.5 L/min and 350 ºC; fragmentator, 150 V; skimmer, 60 V; octapole voltage, 750 V. Peak identification: (1) geniposide.

Figure 3. Comparison of LC-MS profile obtained for the extracted ion chromatogram of geniposide from an authentic saffron sample adulterated with 10 ng/mL of geniposide standard in a C18 column under (A) gradient elution and (B) isocratic elution. LC conditions for gradient elution as in **Figure 1**. LC conditions for isocratic elution: water:acetonitrile (85:15 v/v) containing 0.1 % formic acid for 5 min; flow rate, 0.4 mL/min; injected volume, 5 µL; temperature 40 ºC. MS conditions as in **Figure 2.**

Figure 4. LC-MS extracted ion chromatogram for geniposide in three different saffron samples adulterated with gardenia extract (A, B, and C) and a suspicious saffron sample not adulterated with gardenia extract (D). LC conditions as in **Figure 3** under isocratic elution. MS conditions as in **Figure 2**.

Figure 1.

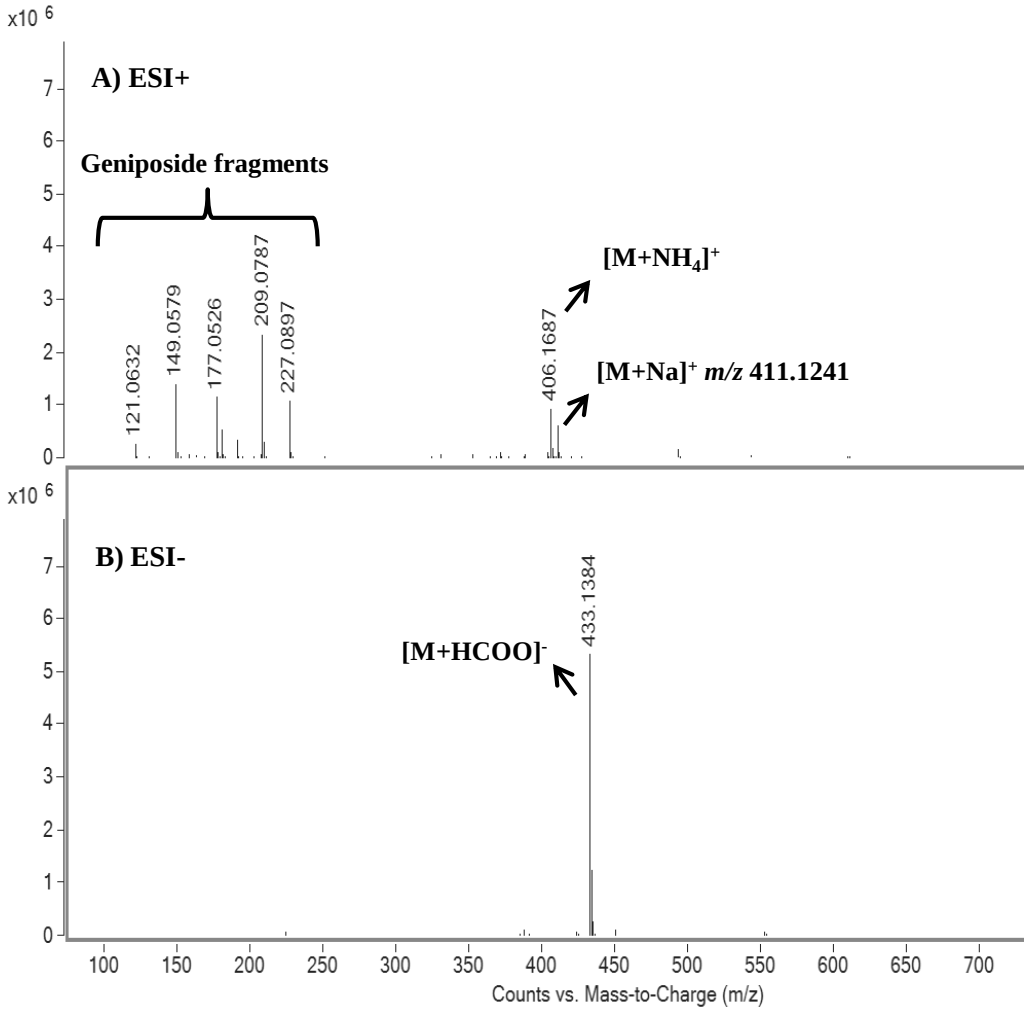

Figure 2.

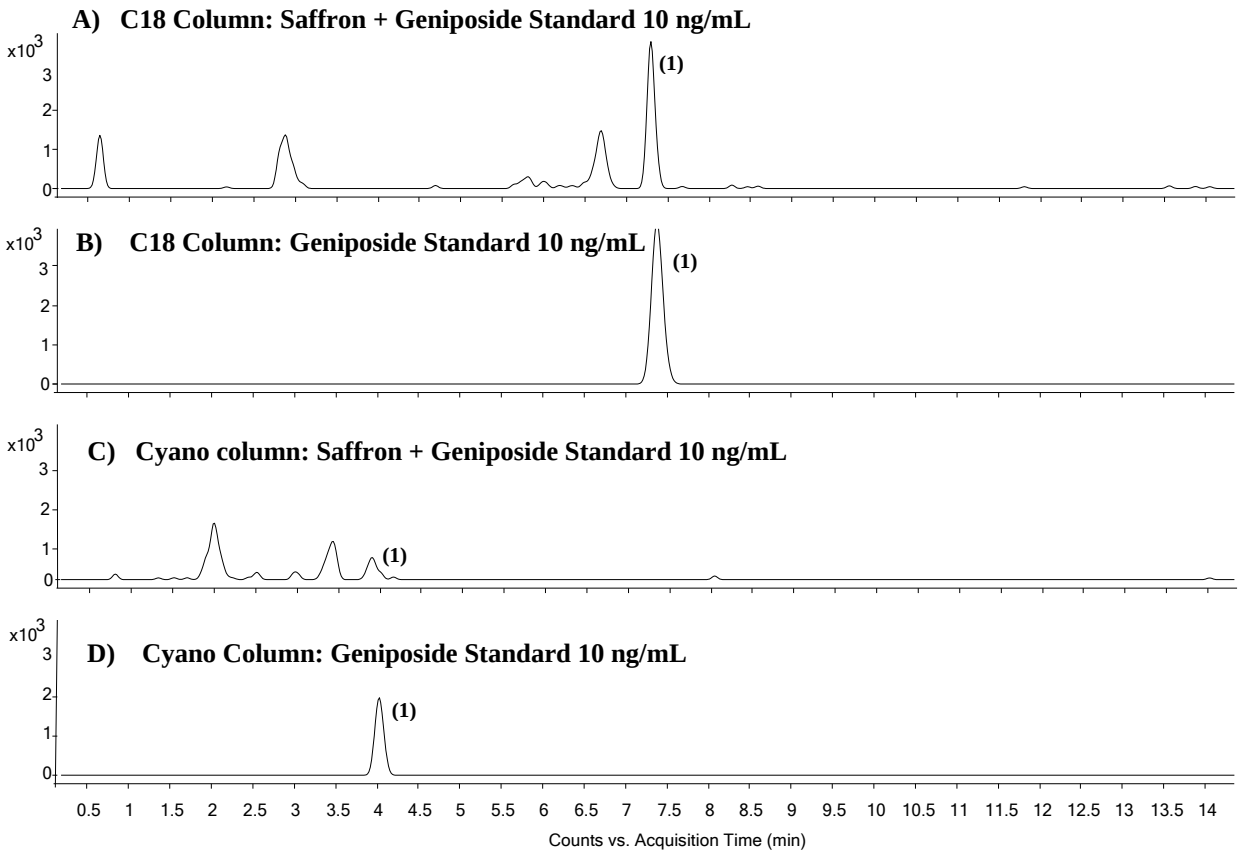

Figure 3.

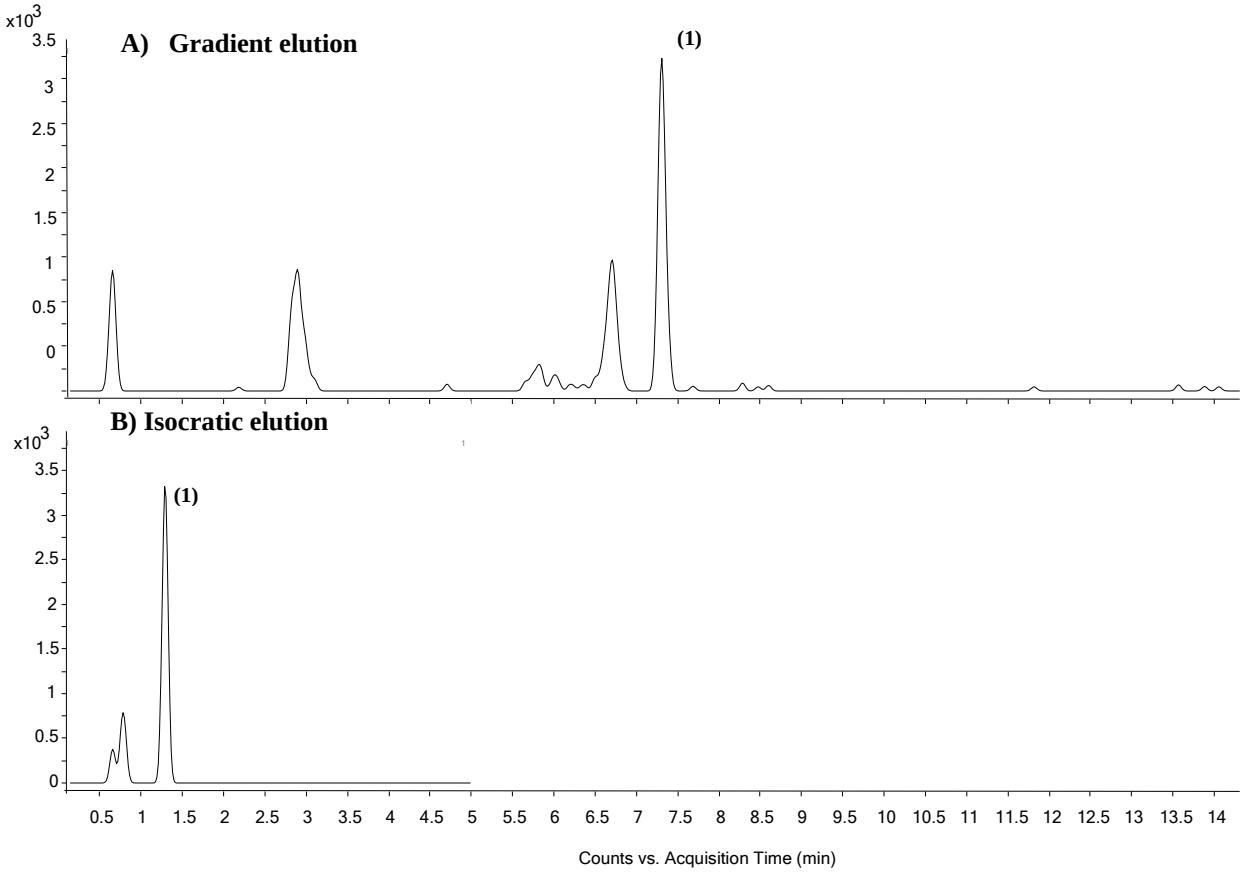

Figure 4.

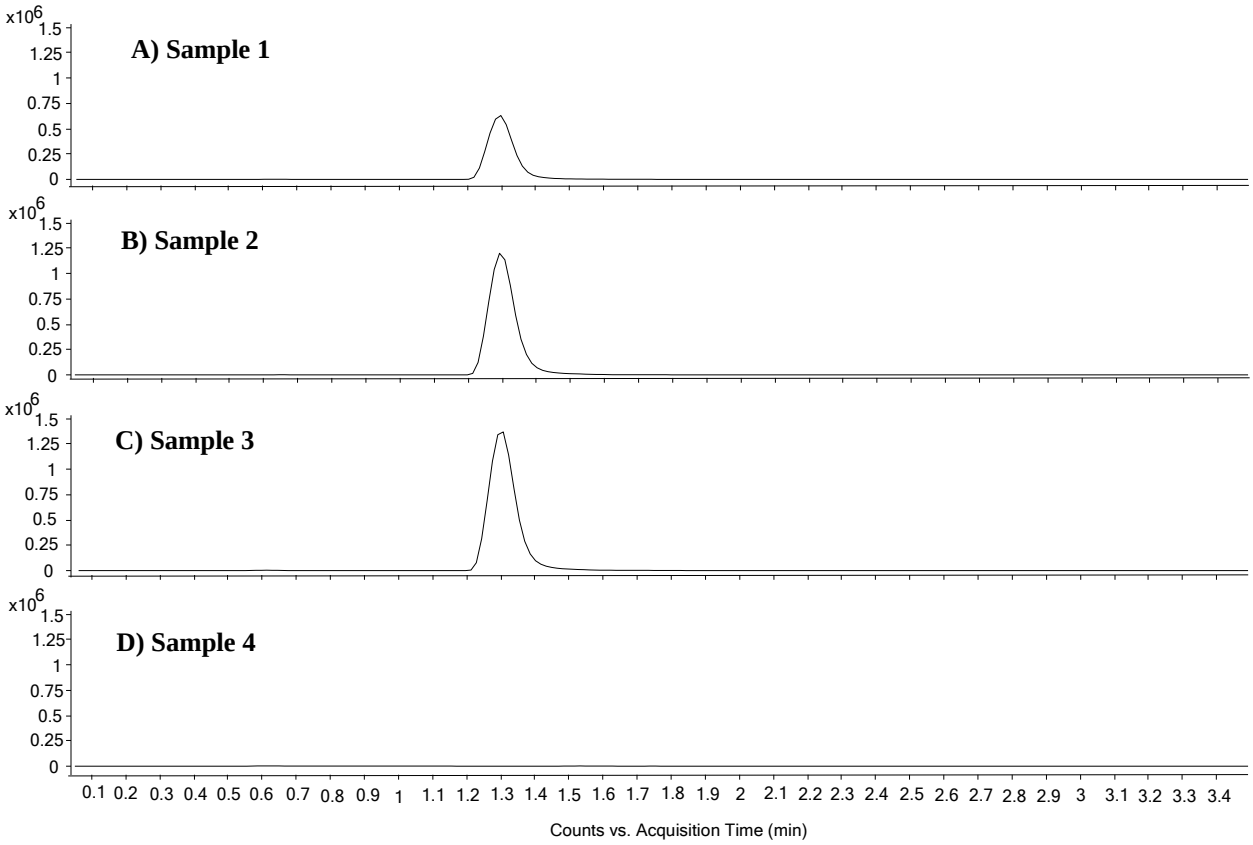

Table 1. Analytical characteristics of the developed LC/MS method for the determination of geniposide.

Linearity		
Linear range [a]	0.8-8 µg/mL	
Slope ± $t \cdot s_b$	$(1.91 \pm 0.21) \times 10^6$	
Intercept ± $t \cdot s_a$	$(6.7 \pm 9.5) \times 10^5$	
R^2	0.997	
p-value of ANOVA [b]	0.078	
Matrix Interferences [c]	*Sample*	*Confidence interval (Slope ± $t \cdot s_b$)*
	Standard	$(1.91 \pm 0.21) \times 10^6$
	Authentic saffron	$(2.06 \pm 0.21) \times 10^6$
	Saffron/Gardenia (50:50)	$(2.06 \pm 0.22) \times 10^6$
LOD [d]	10 ng/mL (41.7 µg/g extract)	
LOQ [e]	30 ng/mL (138.9 µg/g extract)	
Accuracy [f]	*Added concentration*	(*% average*$\pm t_{(n-1)} * s/n^{1/2}$)
Recovery	1 µg/mL	89 ± 14
Precision	*Concentration level*	RSD (%)
Repeatability [g] (n = 3)	0.8 µg/mL of standard	1.5
	8 µg/mL of standard	0.7
	90% of gardenia extract in saffron	0.8
	10% of gardenia extract in saffron	0.6
Intermediate precision [h] (n = 6)	0.8 µg/mL of standard	1.8
	8 µg/mL of standard	1.6
	90% of gardenia extract in saffron	0.9
	10% of gardenia extract in saffron	2.9

[a] Five standard solutions at different concentration levels were injected in triplicate for 3 consecutive days.
[b] p-value for ANOVA to confirm that experimental data fit properly to linear models.
[c] Comparison of slopes corresponding to the standard addition and the external standard calibration methods.
[d] LOD calculated as the concentration yielding an S/N ratio of 3
[e] LOQ calculated as the concentration yielding an S/N ratio of 10
[f] Accuracy was evaluated as the recovery obtained for geniposide when three different samples solutions of authentic saffron were spiked with geniposide standard (1 µg/mL) and injected in duplicate.
[g] Repeatability was determined from three consecutive injections of geniposide standard solutions at two concentration levels and an authentic saffron sample adulterated with percentages of 10 and 90 % of gardenia extract.
[h] Intermediate precision was calculated by using the mean value obtained each day for three replicates (injected in triplicate during two consecutive days) of geniposide standard solutions at two concentration levels and an authentic saffron sample adulterated with percentages of 10 and 90 % of gardenia extract.

Table 2. Geniposide content in the saffron samples adulterated with gardenia.

	Geniposide (mg/g)
Sample 1	5.90 ± 0.01
Sample 2	13.2 ± 0.2
Sample 3	15.30 ± 0.01