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Uncovering a challenging case of adulterated commercial saffron

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ABSTRACT

The analytical approach presented uncovers the type of adulteration of a commercial product labeled as "saffron", sold packed in powder form in a major consuming country. Simple colorimetric and spectrometric tests included in the ISO 3632 trade standard indicated only that it was not "pure saffron". The TLC and HPLC methods recommended in the same standard for the detection of artificial colorants were not applicable due to limited sample amount available. Since it could not be precluded that substances other than artificial colorants have been used, deeper investigation through metabolic fingerprinting was necessary to uncover chemical composition of the sample. The multistep workflow that exploited chromatographic (HPLC) and spectroscopic (UV–Vis, mid-infrared (FT-IR), and nuclear magnetic resonance (NMR)) data from in-house databases uncovered a "tailor-made" case of 100% substitution of saffron by a mixture of exogenous chemical compounds in such a way that the commercial product would approximately mimic not only the appearance of saffron but also its UV–Vis spectrum and specific absorbance values. The findings indicated a sophisticated practice, including total substitution of saffron constituents by tartrazine and sunset yellow along with propane-1,2-diol, propan-2-ol and acylglycerols, probably as emulsifier agents. Interestingly, the perpetrators avoided the use of toxic compounds. To our knowledge such a type of fraud has not been elucidated so far.

1. Introduction

The recent years, authorities show a growing concern regarding the frequency and character of food fraud incidents in the global market. Scientists along with authorities develop databases using evidence from media records and academic references for target foods and prioritize them in terms of vulnerability toward fraudulent practices (United States Pharmacopeial Convention, 2016). Most indexed references present methods for laboratory made adulterated samples and for a priori known type and level of fraud (Tsimidou, Ordoudi, Nenadis, & Mourtzinos, 2016). However, some of the real adulteration cases examined by authorities present a complexity and sophistication that cannot be effectively addressed using recommended or official forensic tools. Furthermore, they may raise concerns not only about the economic impact of these practices but also for the extent of toxicological implications to consumers (de Lange, 2013).

Saffron, a spice produced from the red stigmas of the flowers of *Crocus sativus* L. is an added-value agricultural product due to its coloring, flavoring and biological properties (Carmona, Zalacain, & Alonso, 2006; Kyriakoudi, Ordoudi, Roldan-Medina, & Tsimidou, 2015), highly appreciated not only by the consumers but also by the food and pharmaceutical industry. It commands a high price in the market (up to 20,000€/kg) partially because of the manual labor-intensive production/processing and partially of the limited quantities produced worldwide. These characteristics explain why it is reported to be a rather profitable target for counterfeits (Everstine, Spink, & Kennedy, 2013). Stigmas (whole or cut) or powdered saffron are traded in different quality categories (ISO 3632-1, 2011) in bulk or packaged. Misclassification is, hence, a frequent

Abbreviations: CSEs, Crocetin sugar esters; 1,2 DAG, diacylglycerol; DMSO, dimethylsulfoxide; FT-IR, Fourier Transform Infrared Spectroscopy; g, gravitational force; HMBC, Heteronuclear multiple-bond correlation; HR-NMR, High Resolution Nuclear Magnetic Resonance; HSQC, Heteronuclear single quantum coherence; ISO, International Standardization Organisation; 1MAG, monoacylglycerol; 2P, propane-2- ol; PDO, Protected Designation of Origin; PD, propane-1,2-diol; RP-HPLC-DAD, Reversed Phase; High performance liquid chromatography, diode array detector; rpm, revolutions per minute; SY, sunset yellow; T, tartrazine; TA, triacetin; TLC, Thin Layer Chromatography; TOCSY, Total correlation spectroscopy; *trans*-4-GG crocetin ester, *trans* di-(β-D-gentiobiosyl) ester of crocetin; UV–Vis, Ultraviolet–visible spectroscopy.

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fraudulent practice. Different types of adulterants or substitutes have been reported so far. The use of other parts of the *C. sativus* flower (styles, stamens) is a typical example of auto-adulteration (Soffritti et al., 2016). Total substitution by vegetal parts of plants of similar appearance (e.g. *Carthamus tinctorius* L. petals) is another frequent type of fraud (Sanchez, Maggi, Carmona, & Alonso, 2011). More recently the use of new generation bio-adulterants of pigment composition similar to that of saffron (e.g. fruits of *Gardenia jasminoides* Ellis) gained the interest of researchers to develop advanced detection tools (Carmona, Zalacain, Sánchez, Novella, & Alonso, 2006; Guijarro-Díez, Castro-Puyana, Crego, & Marina, 2017; Paredi et al., 2016; Petrakis & Polissiou, 2017; Petrakis, Cagliani, Polissiou, & Consonni, 2015; Sabatino et al., 2011). Last but not least, the addition of synthetic dyes seems to be the intertemporal means of saffron adulteration recognized in the respective ISO technical standard (ISO 3632-1, 2011). This practice aims at mimicking the appearance of the authentic product or increasing absorbance of aqueous extracts around 440 nm (Ordoudi & Tsimidou, 2011; Sanchez et al., 2011; Zalacain et al., 2005). In the frame of the recently closed COST Action FA1101 "Saffron-OMICS" (COST Action FA 1101, 2015), a large number of researchers joined their efforts to develop new, high-throughput and effective "omics" tools for the control of saffron authenticity and quality (Consonni, Ordoudi, Cagliani, Tsiangali, & Tsimidou, 2016; Nenadis, Heenan, Tsimidou, & Ruth, 2015; Ordoudi et al., 2015; Ordoudi, de los Mozos Pascual, & Tsimidou, 2014; Paredi et al., 2016; Petrakis et al., 2015; Petrakis, Cagliani, Tarantilis, Polissiou, & Consonni, 2017; Rubert, Lacina, Zachariasova, & Hajslova, 2016; Soffritti et al., 2016; Villa, Costa, Meira, Oliveira, & Mafra, 2016).

This study presents a multidisciplinary analytical approach to uncover the type of adulteration of a commercial product labeled as "saffron", which was bought packed in powder form in a major saffron consuming country. Simple colorimetric and spectrometric tests included in the ISO 3632 standard indicated only that it was not "pure saffron". The TLC and HPLC methods recommended in the same standard for the detection of artificial colorants were not applicable because of the limited sample amount (<1 g) available. Since it could not be precluded that substances other than artificial colorants have been used, we extended our previous efforts using metabolic fingerprinting approaches to examine more thoroughly the composition and whether the economic fraud could involve chemical hazards. In-house spectroscopic databases at LFCT (UV–Vis, FT-IR) and ISMAC (NMR) for saffron and various other materials reported as its potential adulterants, were explored for this aim.

2. Materials and methods

2.1. Saffron samples

The commercial product labeled as saffron (in Arabic) and packed in an opaque, yellow-colored plastic bottle was purchased at a herbal shop in the Kingdom of Saudi Arabia in 2013. It was in powder form, and of about 1 g in quantity. After its delivery to LFCT, it was stored at room temperature in the dark till analysis. Saffron samples obtained from Saffron-OMICS stakeholders who guaranteed authenticity, were used as reference materials.

2.2. Standards, reagents and solvents

Trans-crocetin di-(β-D-gentiobiosyl) ester (*trans*-4-GG crocetin ester) was laboratory-isolated by semi-preparative reversed phase-high performance liquid chromatography (RP-HPLC) and checked for purity (97%) as previously described in detail by (Kyriakoudi & Tsimidou, 2015). Diphenylamine (p.a.) was from Schering-Kahlbaum A.G. (Berlin, Germany). HPLC grade acetonitrile, methanol (Chem-Lab, Zedelgen, Belgium) and acetic acid (Fluka Chemie, Buchs, Switzerland) were used. Ultra-high purity water was produced using a SG Ultra Clear Basic UV system (SG Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel, Germany). Deuterated dimethylsulfoxide (DMSO-d_6, 99.96 atom% D) and chloroform ($CHCl_3$-d_1, 99.96 atom % D) were purchased from Euriso-Top (Saclay, France). All other reagents used were of analytical grade.

2.3. Extraction of crocetin sugar esters and picrocrocin

Methanol-water extracts of ground stigmas or powder were prepared according to the ISO 3632-2 method (ISO 3632-2, 2010) with slight modifications. Briefly, 0.1 g is mixed with methanol-water (1:1, v/v) in a 200-mL volumetric flask. Crocetin sugar esters (CSEs) and picrocrocin are extracted by rigorous magnetic agitation 1000 rpm for 1 h at room temperature (25 °C) away from direct sunlight. Prior to analysis, an aliquot from the extract is diluted (1:10) with methanol-water (1:1, v/v) and the corresponding solutions are filtered through RC-55 filter (13 mm i. d., 0.45 μm pore size).

2.4. Colorimetric identification test and UV–Vis spectrophotometric examination according to ISO 3632-2 trade standard (ISO 3632-2, 2010)

The colorimetric test was performed by gradually adding the test sample (0.2 g) to a non-colored diphenylamine solution (0.1 g of diphenylamine, 20 mL of sulfuric acid and 4 mL water) and observation of the color formation; change from blue to reddish brown indicates that the sample is pure saffron while no change of the blue color shows the presence of nitrates.

The UV–Vis spectra of the extracts were recorded in the region 200–600 nm with a spectrophotometer (Shimadzu UV 1601, Kyoto, Japan) equipped with quartz cells (1 cm × 1 cm × 4 cm). The spectra were processed with the aid of UVPC 1601 (Personal Spectroscopy Software, v.3.9, Shimadzu) software facilities. The $E^{1\%}\lambda_{max}$ values were calculated according to the equation $E^{1\%}_{\lambda max} = (D \times 100)/(m \times (100 - H))$, with D as the absorbance value, m as the mass of the test portion (g), H as the moisture and volatile content of the sample (%, w/w), λ_{max} for crocetin sugar esters, 440 nm, λ_{max} for picrocrocin, 257 nm and λ_{max} for safranal, 330 nm. The 2nd derivative spectra were calculated with a Δλ value of 10. Spectral processing tools were also used and measurements of each extract were obtained in triplicate. The above-mentioned conditions of analysis were the same as those that had been already applied in the course of the COST FA1101 Action, to aqueous extract of authentic saffron (belonging to ISO categories I-III) as well as to aqueous solutions of 13 synthetic colorants (allura red AC, amaranth, erythrosine, orange II, ponceau 4R, quinoline yellow, red 2G, rocelline, sudan I, sunset yellow, tartrazine, yellow 2G and carminic acid). The zero-order and 2nd derivative spectra of all these extracts and solutions are available in LFCT and the database is continuously updated.

2.5. Liquid chromatographic analysis

Crocetin sugar esters and picrocrocin were determined by high performance liquid chromatography (HPLC) (Kyriakoudi, Chrysanthou, Mantzouridou, & Tsimidou, 2012). The HPLC system consisted of a pump, model P4000 (Thermo Separation Products, San Jose, CA, USA), a Midas autosampler (Spark, Emmen, The Netherlands) and a UV 6000 LP diode array detector (DAD) (Thermo Separation Products, San Jose, CA, USA). Separation was carried out