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Characterisation of secondary metabolites in saffron from central Italy (Cascia, Umbria)

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ABSTRACT

Saffron's quality depends on the concentration of secondary metabolites, such as crocins, picrocrocin and safranal. The aim of this research was to evaluate the influence of drying conditions on the secondary metabolite contents of saffron produced in the area of Cascia, in central Italy. Different aliquots of the same saffron sample were subjected to various dehydration conditions and analysed by UV–Vis spectrophotometry to determine crocins, picrocrocin and safranal.. Safranal was also analysed by high resolution gas chromatography, while the crocins and picrocrocin were determined by high-performance liquid chromatography with diode array and mass spectrometric detectors.

The results of chromatographic analyses showed that the samples dried in the milder conditions had the lowest content of secondary metabolites. Moreover the sample dried at 60 °C for 55 min presented the highest contents of *trans*-crocin-4 and picrocrocin, while safranal was most represented in saffron dried at 55 °C for 95 min.

1. Introduction

Saffron is one of the oldest and most expensive spices in the world. It is obtained from the red dried stigmas of flowers of *Crocus sativus* L., a member of the *Iridaceae* family. The quality of saffron, which is determined by its taste, aroma and colour, depends on the concentration of the biologically active secondary metabolites (Carmona et al., 2006a). The major bioactive compounds are crocins, picrocrocin, and safranal. The crocins, a family of red-coloured and water-soluble carotenoids, are glycosyl esters of crocetin with different sugar moieties, such as glucose, gentiobiose, neapolitanose or triglucose; they can be present in saffron in *cis* and *trans* isomeric forms (Carmona, Zalacain, Sanchez, Novella, & Alonso, 2006b). Picrocrocin, a monoterpene glycoside, is the main bitter principle of saffron. Moreover it is a precursor of safranal, the major volatile compound responsible for the aroma (Lage & Cantrell, 2009). During the drying process, the β-glucosidase hydrolyses picrocrocin to give the aglycone, 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC), which is transformed by dehydration to safranal, a terpene aldehyde (Lozano, Delgado, Gomez, Rubio, & Iborra, 2000).

A dehydration postharvesting treatment, necessary to convert *C. sativus* L. stigmas into saffron spice, brings about the physical, biochemical and chemical changes, necessary to achieve the desired attributes of saffron (Carmona et al., 2005). The drying process differs from country to country (Ordoudi & Tsimidou, 2004) and the different conditions of drying and aging affect the saffron constituents (Carmona et al., 2005). This process also plays an important role not only in the preservation of saffron, but it is a critical step in the enzymatic release of safranal from picrocrocin (Del Campo et al., 2010).

In recent years, there has been a growing concern in order to guarantee and to defend the quality of saffron historically produced in specific regions. Among the methods used for saffron characterisation, currently recommended by the International Standardisation Organisation (ISO/TS 3632, 2003), is UV–Vis spectrophotometry. Unfortunately, this method is non-specific and unable to adequately discriminate between genuine and adulterated saffron, and thus unable to provide a quality category on the international market (Zougagh, Ríos, & Valcarcel, 2005). For the quantitative analysis of picrocrocin and crocetin Corti, Mazzei, Ferri, Franchi, and Dreassi (1996) developed a new high performance thin layer chromatographic method. A rapid and non-destructive technique to determine safranal and HTCC contents was performed by Lozano et al. (2000) using supercritical carbon dioxide extraction combined with high-resolution gas chromatography (HRGC) and reverse-phase high-performance liquid chromatography (RP-HPLC). For HPLC analysis, a DAD (diode array detector) was commonly used (Lage & Cantrell, 2009), sometimes in combination with electrospray ionisation (ESI) mass spectrometry (MS) (Lech, Witowska-Jarosz, & Jarosz, 2009). Sánchez, Carmona, Del Campo,

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and Alonso (2009) developed a solid-phase extraction procedure coupled with RP-HPLC-DAD analysis. Koulakiotis, Pittenauer, Halabalaki, Tsarbopoulos, and Allmaier (2012) have also evaluated different mass spectrometric desorption/ionisation techniques, in combination with tandem MS for the analysis of crocins and picrocrocin.

The aim of the present research was to characterize the secondary metabolites of saffron produced in Cascia, in central Italy. The modification of secondary metabolite contents as a result of different dehydration conditions was also evaluated. Initially, the secondary metabolites were characterized by UV–Vis spectrophotometric analysis, then chromatographic techniques were applied, among which HRGC with a flame ionisation detector (FID) to analyze safranal and HPLC-DAD-MS to analyze the crocins and picrocrocin.

2. Materials and methods

2.1. Samples and chemicals

Fresh stigmas of *C. sativus* L., harvested in the year 2011, from Cascia in the Umbria region (Italy), were obtained from a local producer, with the guarantee of their origin. The stigmas were immediately stored at 4 °C in a dark place until arrival at the laboratory, where they were divided into nine aliquots and stored at 4 °C in the absence of light until their analysis.

Safranal (⩾88%, CAS No. 116-26-7) was from SAFC supply Solutions (St. Louis, MO, USA). Crocin (CAS No. 42553-65-1) was purchased from Sigma (St. Louis, MO, USA). Water, acetonitrile, hexane and ethyl acetate for HPLC were purchased from Carlo Erba (Milan, Italy). Analytical grade chloroform and methanol were obtained from Sigma–Aldrich.

2.2. Saffron dehydration process

The nine aliquots were dehydrated in a hot air oven at different conditions of temperature and time, as reported in Table A. Approximately 130 mg of stigmas were placed on weighing dish which had been previously dried.

2.3. Extraction of saffron metabolites

For the spectrophotometric and HPLC determinations, each sample of the dehydrated stigmas (50 mg) was ground with a potter using double distilled water (100 ml) for 10 min at room temperature. Then each aqueous extract was filtered through a 0.45 μm nylon syringe filter (Corning Incorporated, Corning, Germany). The solution was used for HPLC analysis, while for the spectrometric analysis an aliquot (1 ml) was diluted with double distilled water to 10 ml.

For the HRGC determination, each sample of dehydrated stigmas was extracted using a mixture of methanol/ethyl acetate (70:30, v/v) with the same ratio indicated above. The solution was then filtered through a 0.45 μm PTFE (polytetrafluoroethylene) syringe filter (MDI Membrane Technologies, Advanced Microdevices Ltd, Ambala Cantt, India).

Table A
Conditions for stigma dehydration.

Samples	Temperature (°C)	Time (min)
1	50	45
2	50	60
3	50	75
4	55	60
5	55	80
6	55	95
7	60	55
8	60	65
9	60	75

2.4. Spectrophotometric analysis

The nine saffron samples were analysed using a Jasco 7850 UV–vis spectrophotometer (Jasco Inc., Easton, MD, USA). The absorbance at 257, 330 and 440 nm of the 1% aqueous solutions of saffron was evaluated using a 1 cm pathway quartz cell. The reference solution was double distilled water. For the measurement of the spectrophotometric indexes, moisture and volatile contents were evaluated by using the saffron in filaments. After weighing, the samples were introduced uncovered in an oven set at 103 °C for 16 h. The moisture and volatile matter content is expressed as a percentage of the initial sample using the following relation: [(initial mass − final mass)/initial mass] × 100. All analyses were carried out in duplicate.

2.5. HRGC-FID analysis

A Perkin-Elmer Autosystem gas-chromatograph, equipped with split/splitless injector and FID was used. The separation was obtained using a HP1-ms capillary column (30 m × 0.25 mm i.d., 0.25 μm f.t., from Agilent Technologies Inc., USA). The chromatograms were acquired and processed using Turbochrom Navigator 6.1.1.0.0:K20 integration software (Perkin–Elmer Instruments, Norwalk, Connecticut).

The injector and detector temperatures were set at 250 and 300 °C, respectively. The oven temperature was 50 °C, held for 3 min and raised to 180 °C at 3 °C/min, then increased to 250 °C at 15 °C/min and held for 5 min. Carrier gas (He) flow rate was 1 ml/min; the injection volume was 1 μl with a split ratio of 1:70.

Safranal in the saffron samples was identified by comparison of the retention time with that of the standard compound. Repeated injections of standard solutions were carried out to test the analytical precision. The relative standard deviations were less than 4.6%, considering both the intra-day precision, calculated on four repeated injections, and the inter-day precision, evaluated over four days. A calibration line was built using the safranal standard and the least square method was used to calculate the regression equation. The limit of detection (LOD) and the limit of quantification (LOQ) were estimated according to the standard deviation response and slope method (Dolan, 2009).

2.6. HPLC-DAD-MS analysis

HPLC analyses were performed using a Shimadzu GT-154 system equipped with a Thermo Spectra Series pump, a Gemini C18 column (5 μm particle size, 100 × 2 mm i.d., Phenomenex, Chemtek Analytica, Bologna, Italy), a Spectra System UV6000LP DAD and a Finnigan LC Aqua quadrupole MS detector (Finnigann, Manchester, UK) with ESI. Detection was performed on-line using the DAD between λ_{200nm} and λ_{700nm}. MS experiments were carried out both in negative and positive modes with a scan range *m/z* 100–1200 and with a scan rate of 1.3 scans/s. The spray capillary voltage was 3000 V, the cone voltage was 30 V and the ionisation capillary temperature was 250 °C. The nebulizer pressure was 50 psi and the nitrogen flow rate was 12 l/min.

The determinations were performed according to a modified version of the method described by Hadizadeh, Mohajeri, and Seifi (2010). The solvents were water (A) and acetonitrile (B). The samples were analysed by gradient elution at a flow rate of 0.8 ml/min: 90% A for 5 min; 90% A to 20% A for 20 min; 20% A for 5 min. The chromatograms were acquired and the data handled using