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Identification of the flavonoid fraction in saffron spice by LC/DAD/MS/MS: Comparative study of samples from different geographical origins

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Abstract

The flavonoid fraction in saffron spice has been analysed, for the first time, by LC-DAD-MS/MS ESI and five kaempferol derivatives have been found. Compounds such as kaempferol-3-sophoroside, kaempferol-3-sophoroside-7-glucoside and kaempferol-3,7,4′-triglucoside were tentatively identified, whereas other compounds, such as kaempferol tetrahexoside and kaempferol-3-dihexoside were detected. Saffron samples from different geographical origins were clearly separated by their kaempferol 3-sophoroside contents that were able to explain 100% of the variance when a discriminant test was carried out.
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Keywords: Kaempferol-3-sophoroside-7-glucoside; Kaempferol-3,7,4′-triglucoside; Kaempferol-3-sophoroside; LC-DAD MS/MS; Saffron spice

1. Introduction

Saffron spice is made up of dried stigmas of *Crocus sativus* L., which is cultivated in different countries, such as Greece, India, Iran, Morocco and Spain. Nowadays, it is appreciated by consumers as a colorant for foodstuffs as well as for its aromatic and flavouring properties. Yet, in ancient times, its use as a drug in folk medicine was even more remarkable. Its extracts and tinctures have been used as an antispasmodic, eupeptic, gingival sedative, anticatarrhal, nerve sedative, carminative, diaphoretic, expectorant, stimulant, stomachic, aphrodisiac and emenagogue (Basker & Negbi, 1983; Sampathu, Shivashankar, & Lewis, 1984). In recent decades, biological and medical properties of this spice and its constituents have again focussed scientific attention. It has been proposed that saffron is effective against arteriosclerosis, while reducing cholesterol levels in the blood (Gainer & Jones, 1975; Miller, Willett, Moss, Miller, & Belinka, 1982). Many in vivo tests on tumors in rats, as well as in vitro trials on established cellular lines, have been carried out (Escribano, Alonso, Coca-Prados, & Fernández, 1996; Escribano et al., 2000; Jagedeeswaran, Thirunavukkarasu, Gunasekaran, Ramamurty, & Sakthisekaran, 2000; Konoshima et al., 1998; Morjani, Tarantilis, Polissiu, & Manfait, 1990). In vitro cell toxicity tests proved that saffron stigma extracts inhibited the growth and synthesis of nucleic acids in tumor cells, while normal cells were less susceptible and even completely unsusceptible (Abdullaev & Frenkel, 1992; Nair, Pannikar, & Panikkar, 1991). It was verified that concentrations which induced inhibition of 50% of tumor cell growth were only slightly higher than those for all-*trans* retinoic acid but without its secondary effects (Tarantilis et al., 1992; Tarantilis, Tsoupras, & Polissiou, 1995). In addition, its lower toxicity compared to retinoic acid (Martín, Goh, & Neff,

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2002), plus the absence of cases of sensitization to saffron ingestion (Moneret-Vautrin, Morisset, Lemerdy, Croizier, & Kanny, 2002), open up the possible extension of its pharmacological use. In fact, saffron constituents have been proposed as alternative antitumor agents which, alone or in combination with other chemical substances, could achieve a certain relevance in future treatment of some cancers (Abdullaev, 2002; Winterhalter & Straubinger, 2000).

The compounds considered to be pharmacologically active in saffron are the bitter principles and the pigment derivatives from the carotenoid crocetin (Ríos, Recio, Giner, & Manez, 1996). In addition to picrocrocin, that is to say 4-(β-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde, the major compound responsible for saffron bitterness, other compounds with this organoleptic property have been characterized in saffron spice. These are related to picrocrocin and flavonoids (Straubinger, Bau, Eckestein, Fink, & Winterhalter, 1998; Straubinger, Jezussek, Waibel, & Winterhalter, 1997). Flavonoids have many functions in the biochemistry, physiology and ecology of plants, and they are important in both human and animal nutrition (Forkmann & Martens, 2001). The antioxidant activity of flavonoids towards free radicals and reactive oxygen species, plus their potential oestregenic and anticancer activity, draw attention to their health-protecting role in human and animal foods (Harborne & Williams, 2000). Some of these health properties may be due to the flavonoid content, as has been reported for anticonceptive and anti-inflammatory effects (Hosseinzadeh, Karimi, Khaleghpanah, & Niapoor, 2003).

The first identification of a flavonoid in saffron spice (by mass spectrometry) was by Tarantilis et al. (1995), proposing a kaempferol structure with a disaccharide moiety. Straubinger et al. (1997) identified kaempferol 7-*O*-glucopyranosyl-3-*O*-sophoroside and kaempferol 7-*O*-sophoroside by NMR and MS after counter-current preparative chromatography. Taking this determination into account, the same authors considered that the identification of a new flavonoid named kaempferol 3-*O*-gentiobioside carried out by Lozano, Castellar, Simancas, and Iborra (1999) was not correct (Winterhalter & Straubinger, 2000). Moreover, other flavonoids may be found in saffron spice, as they have already been described in other *Crocus* species (Nørbæk & Kondo, 1999).

The purpose of this study was to identify the flavonoid fraction in saffron spice by LC-DAD/MS/MS ESI. Differences in the flavonoid contents of samples from various geographical origins were also studied in order to know whether they could be used as biomarkers for the determination of saffron origin.

2. Materials and methods

2.1. Plant material and standards

A total of 12 saffron spice samples produced in different countries (3 from Iran, 3 from Greece, 3 from Morocco and 3 from Spain) were analysed in triplicate. The reduced number of samples was due to the fact that they were obtained directly from the producers with the guarantee of their origin and lack of adulteration. All samples were of Category I according to ISO/TS 3632 Normative (2003).

For standards, kaempferol-3-*O*-sophoroside-7-*O*-glucoside and kaempferol-3-*O*-sophoroside, from the collection of the Research group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC (Murcia, Spain), were used. Both standards came from cauliflower (*Brassica oleracea* L. var. *botrytis*) (Llorach, Gil-Izquierdo, Ferreres, & Tomas-Barberán, 2003).

2.2. Extraction and isolation of the flavonoid fraction

A solution of 200 mg of powdered saffron in 200 ml of water was stirred for 1 h at room temperature in the dark. A C_{18} solid-phase cartridge (Waters, Milford, MA, USA), was used for the isolation and concentration step which had been previously conditioned with acetonitrile (2 ml) followed by water (5 ml), acetonitrile (2 ml) and water (5 ml). Four ml of the saffron extract were added to the SPE cartridge and washed with water (15 ml) and further eluted with 10 ml of a solution of acetonitrile 10% in order to elute the flavonoids. The water–acetonitrile extract was taken to dryness and redissolved in 1 ml of water:methanol (1:1 v/v) for chromatographic analysis.

2.3. Acid hydrolysis

This was carried out by adding HCl (1 N) up to pH 0.1 to the flavonoid saffron fraction obtained, as described in Section 2.2, which was heated for 30 min at 80 °C. The extract was then taken to dryness and redissolved in 1 ml of water:methanol (1:1 v/v) for chromatographic analysis.

2.4. LC-DAD/MS/MS ESI

Analysis was achieved with a LichroCART column (250 nm × 4 mm, RP-18, 5 μm particle size, Lichrospher 100 stationary phase, Merck, Darmstadt, Germany), protected with a LichroCART guard column (4 mm × 4 mm, RP-18, 5 μm particle size, Lichrospher 100 stationary phase, Merck, Darmstadt, Germany). The mobile phase consisted of two solvents: A, water–acetic acid (1%) and methanol (B), while a linear gradient, starting with 20% B, was installed to reach 50% B at 35 min and 80% B at 37 min. The flow rate was 1 ml/min, and the injection volume 40 μl. Spectral data from all peaks were accumulated in the range of 240–400 nm and chromatograms were recorded at 355 nm since, in a previous screening with chromatograms at 280 and 355 nm, the spectra of the obtained peaks showed that 355 nm was the suitable wavelength.

The LC/DAD/MS/MS analysis was carried out in an Agilent 1100 chromatograph equipped with a diode array detector and mass detector in series (Agilent Technologies,

Waldbronn, Germany). The HPLC was controlled by Chemstation software (Agilent, v.08.03). The mass detector was an ion trap spectrometer equipped with an electrospray ionization interface and controlled by LCMSD software (Agilent, v.4.1). The ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebuliser pressure was 65 psi and the nitrogen flow rate was 11 ml/min. The full scan mass covered the range from m/z 200 up to 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as a collision gas, with voltage ramping cycles from 0.3 up to 2 V. All mass spectrometry data were recorded in the negative ion mode. Total ion chromatograms were recorded as alternating automatic events: full scan mass spectra (MS) and MS/MS of the pseudomolecular ion, and MS^n ($n =$ up to 3) in order to fragment the major ions obtained in every step. Table 1 shows most frequent ions which characterize the fragmentation of these flavonoid *O*-glycosides. Other ions were found but they have not been included owing to their low significance for the MS behaviour ions.

3. Results and discussion

3.1. Identification of flavonoids from saffron spice

The chromatogram profile of the flavonoid saffron extract is shown in Fig. 1. Of flavonoid family, only flavonol compounds were found. Five compounds were tentatively identified by combination of the UV and mass spectra by HPLC-DAD-ESI-MS/MS, while the peak at 12.5 min observed in the chromatogram corresponds to picrocrocin (m/z 353 and 185). After acid hydrolysis, all the flavonoids gave kaempferol as an aglycone. The MS spectra of the major compounds (**2** and **5**) showed deprotonated molecular ions at m/z 771 and 609 and ions at m/z 285 corresponding to a deprotonated aglycone. Therefore, they were kaempferol derivatives with three and two

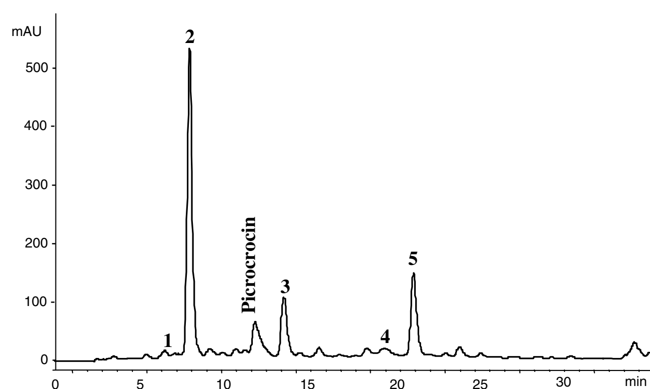

Fig. 1. HPLC profile of the flavonoid fraction from saffron extracts recorded at 355 nm: **1**, kaempferol tetrahexoside; **2**, kaempferol-3-sophoroside-7-glucoside; **3**, kaempferol-3,7,4′-triglucoside; **4**, kaempferol-3-dihexoside; **5**, kaempferol-3-sophoroside.

Table 1
Rt, UV, −MS: $[M-H]^-$, −MS2$[M-H]^-$ and −MS3$[(M-H)\rightarrow(M-H-162)]^-$ data for kaempferol glycoside derivatives in saffron

Compounds[a]	Rt (min)	UV (nm)	*[M − H]⁻ (m/z)	*-MS2[M − H]⁻ (m/z) (%)			*-MS3[(M − H) → (M − H−162)]⁻ (m/z) (%)	
				Kaempferol tetrahexoside				
1	6.3	265, 319sh, 349	933	Fragmentation pattern is not clearly defined				
				Kaempferol trihexosides				
				−162			−(162 + 18)	−(162 × 2)
2	7.7	265,321sh, 348	771	609(100)			429(20)	285(100)
3	13.8	267,295sh,333	771	609(100)			429(100)	285(63)
				Kaempferol dihexosides				
				−162	−(162 + 18)	−(162 × 2)		
4	18.2	265, 319sh, 349	609	447(37)		285(100)		
5	20.9	265, 320sh, 348	609		429(53)	285(100)		

* The fragmentation ions shown are the most significant ones.
[a] **1**, kaempferol tetrahexoside; **2**, kaempferol-3-sophoroside-7-glucoside; **3**, kaempferol-3,7,4′-triglucoside; **4**, kaempferol-3-dihexoside; **5**, kaempferol-3-sophoroside.

hexoses, respectively (Table 1). Their fragmentation patterns coincided with two standards, the -3-sophoroside-7-glucoside and the -3-sophoroside of kaempferol, respectively (Ferreres, Llorach, & Gil-Izquierdo, 2004). All these data confirm the structures reported previously by Straubinger et al. (1997) for the main saffron flavonoids.

Another flavonoid observed with a relevant relative abundance was compound **3**. Its deprotonated molecular ion at *m/z* 771 indicated that it was an isomer of compound **2**, but its MS fragmentation pattern, as well as its UV spectrum (Table 1), differed from those of compound **2**. In the MS2$[M-H]^-$ fragmentation, it was observed that, in both compounds, the only ion that appeared was at *m/z* 609, produced by a hexose loss from the deprotonated molecular ions and indicating the occurrence of a monohexoside linked directly to a phenolic hydroxyl (Ferreres et al., 2004). However, the fragmentation MS3$[(M-H) \rightarrow (M-H-162)]^-$ was different (Table 1, Fig. 2), and while in compound **2** the characteristic fragmentation of a sophoroside was observed, with a base peak corresponding to the deprotonated aglycone (*m/z* 285), in compound **3**, the base peak is the ion at *m/z* 429, produced by the loss of 180 m.u. (162 + 18), with a relative abundance for the ion at *m/z* 285 of 60%. This showed that, in **3**, both sugars were not in the form of a disaccharide, but they were linked to different phenolic hydroxyls and, consequently compound **3** should be tentatively identified as kaempferol-3,7,4′-triglucoside. Such structure was in accordance with the UV spectrum observed (Fig. 3), since the maximum at 333 nm and its low absorbance supported the idea that the hydroxyl at 4′ was substituted. The chromatographic behaviours of compound **2** and **3** were difficult to explain due to the fact that the hydroxyl substitution in position 4′ might modify its chromatographic mobility, as was experimentally observed.

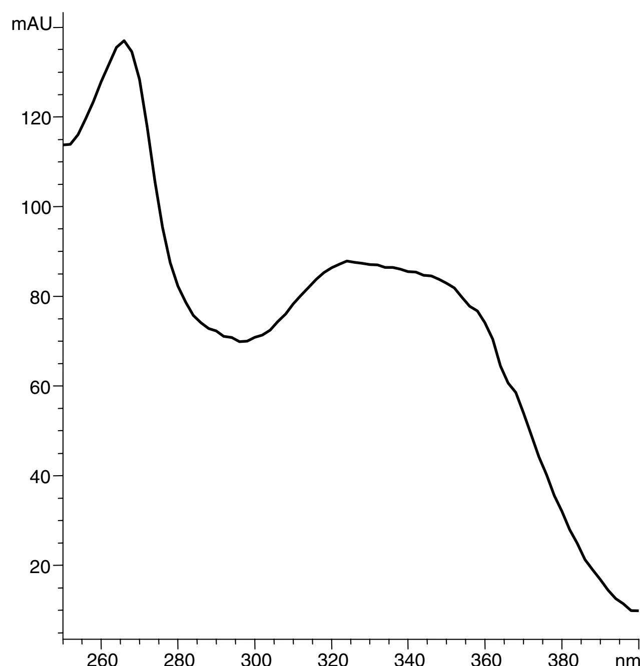

Fig. 3. UV spectrum for compound **3**: kaempferol-3,7,4′-triglucoside.

In addition to these three flavonoids, two other minor ones were observed in trace amounts (**1** and **4**). Both showed a characteristic UV spectrum of kaempferol derivatives substituted, at least, at position 3 (Table 1). In the MS analysis of compound **1,** a deprotonated molecular ion at *m/z* 933 and an ion at *m/z* 285, corresponding to the aglycone, were observed. Even so, its fragmentation pattern was not well defined and a specific structure was not assigned, although the mass coincided with that of a kaempferol tetrahexoside. Compound **4** (Table 1) was an isomer of **5** with a very similar MS. The fragmentation of

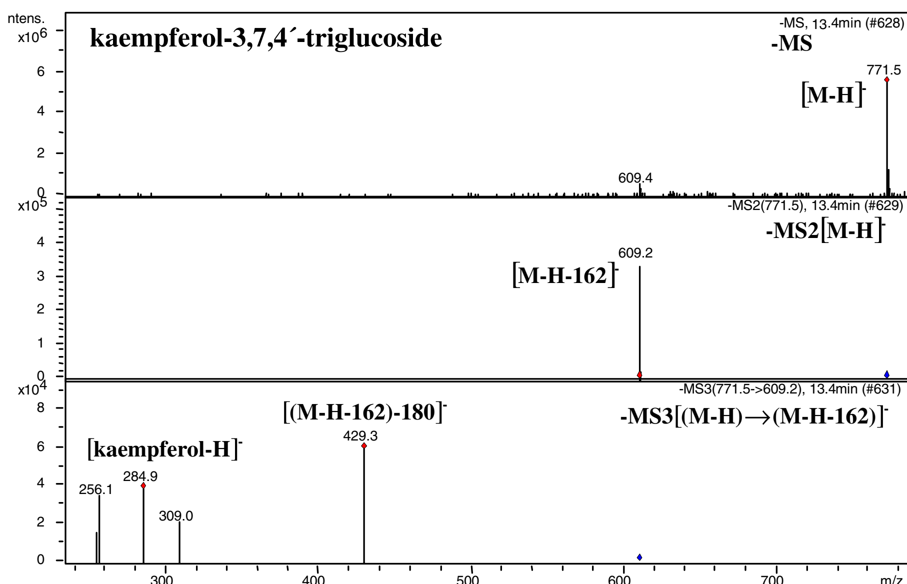

Fig. 2. $-MS^n$ analysis of kaempferol-3,7,4′-triglucoside (**3**).

these compounds revealed a base peak at m/z 285, indicating that both sugar moieties corresponded to a disaccharide, and therefore they were linked to a single phenolic hydroxyl group. UV–Vis spectra showed that the hydroxyl group at the 3 position was blocked in both compounds. Besides, in compound **5**, an ion at m/z 429 was observed, with loss of $162 + 18$ m.u. from the molecular ion, and in compound **4** the ion m/z 447 (loss of 162 m.u. from the molecular ion) was similar to the mentioned signal m/z of 429, confirming the isomer's tentative identification, as kaempferol-3-dihexoside.

3.2. Saffron origin discrimination

No qualitative differences in relation to the flavonoid fraction were observed when samples from different geographical origins were analysed. This could be due to the poor genetic variability between cultivars. It is proposed that, saffron, being a sterile plant, all available vegetal material contains the same genetic information (Chichiriccó, 1987, 1989; Grilli & Chichiricco, 1991). Besides, heterogeneous edaphoclimatic conditions and postharvesting treatments, necessary to convert *C. sativus* stigmas into saffron spice (Carmona et al., 2005), generate a different flavonoid profile. While the content of compounds **1** and **4** did not offer valuable information because the presence of both of them was in trace amounts, the rest of the compounds quantified led us to establish that Spanish saffron was the one with the highest flavonoid content (Table 2).

Saffron samples were clearly separated by their kaempferol 3-sophoroside contents (compound **5**), which was able to explain 100% of the variance when a discriminant test was carried out with the geographical origin functioning as the differentiating variable. Although the number of samples is reduced, the flavonoid fraction was shown as a reliable tool for origin discrimination. Some other efforts have been carried out with other saffron constituents (Carmona et al., 2005; Semiond et al., 1996), although no analytical tool is available for quality control laboratories, at an international level, to certify saffron origin. Saffron price depends greatly on origin, with fraudulent interchanges in the international market frequently being produced: the cheapest saffron, coming from Iran, is sold as if it had come from other traditional areas, such as Spain, Greece or Italy, where saffron quality is considerably better.

This study confirms the idea expressed by Tarantilis et al. (1995) that more flavonoids should be found in *C. sativus* L. spice than the ones already characterized. The fact that exactly the same flavonoids have been found in samples coming from various countries suggested that its different content is the result of different edaphoclimatic conditions and postharvesting treatments. The main difficulty to overcome, in any future approach, is obtaining certified samples from different origins with detailed dehydration conditions, as has occurred in this study. Finally, it remains to be seen whether some of the traditionally recognized pharmacological properties of saffron could be attributed to the flavonoids identified at the concentration detected.

Table 2
Flavonoid contents for saffron samples of differing origin

Saffron	Compound **2**	Compound **3**	Compound **5**
	Content[a]	Content[a]	Content[a]
Spain	2.58 (37.4)^{b}	1.09 (15.5)^{b}	3.12 (47.1)^{b}
Greece	2.24 (63.2)^{a,b}	0.73 (20.4)^{a,b}	0.61 (16.4)^{a}
Iran	1.47 (50.9)^{a}	0.59 (19.7)^{a}	1.07 (29.5)^{a}
Morocco	1.91 (48.7)^{a,b}	0.88 (22.4)^{a,b}	1.24 (28.8)^{a}

Compound **2**, kaempferol-3-sophoroside-7-glucoside; Compound **3**, kaempferol-3,7,4′-triglucoside; Compound **5**, kaempferol-3-sophoroside.

[a] Flavonoid content expressed as equivalent mg of rutin/g of saffron (relative content expressed as % of the total fraction); Different superscript letters between columns indicate significant differences ($p < 0.05$).

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