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Qualitative Profile and Quantitative Determination of Flavonoids from *Crocus sativus* L. Petals by LC-MS/MS

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From the methanolic extract of *Crocus sativus* petals nine known flavonoids have been isolated and identified, including glycosidic derivatives of quercetin and kaempferol as major compounds (**1-2**), and their methoxylated and acetylated derivatives. Additionally, LC-ESI-MS qualitative and LC-ESI-MS/MS quantitative studies of the major compounds of the methanolic extract were performed. The high content of glycosylated flavonoids could give value to *C. sativus* petals, which are a waste product in the production of the spice saffron.

Keywords: *Crocus sativus*, LC-ESI-MS, LC-MS/MS, quercetin, kaempferol.

Saffron, the dried stigmas of *Crocus sativus* L. (Iridaceae), is a very expensive spice, and is used as a herbal medicine, for food coloring and as a flavoring agent in different parts of the world [1a]. Saffron originally grew in India, Iran, Europe and other countries, and it has been successfully cultivated in different countries, including Europe. The most important European production areas are Sardinia and Abbruzzo (Italy), Castile-la Mancha (Spain) and western Macedonia (Greece). For saffron, the flowers are cultivated to produce the stigmas. After harvesting, the flowers are subjected to a delicate treatment which will give the saffron spice. This procedure is performed the same day of harvest. One of the most traditional procedures is the separation of petals from stigmas. A large amount of petals is discarded for obtaining a small amount of stigmas.

Earlier investigation reported the isolation of carotenoids, crocins, monoterpenoids and flavonoids from the stigma, leaves, petals and pollen of *C. sativus* [1b,1c].

Considering the large amount of petals that are waste products in this production procedure, we have undertaken a study to recover chemical compounds from this matrix. Only one previous paper concerning petals is reported in the literature, oriented towards the biological activity of some new phenols isolated from this part of the flowers [2a].

Flavonoids are polyphenolic compounds with antioxidant properties [2b,2c]. Several studies have shown that a high intake of flavonoids has been correlated to a decrease in heart disease; in addition, biological effects of this class of compounds have been described in several *in vivo* and *in vitro* studies [3a-3d]. These compounds are largely used for chemotaxonomic surveys of plant genera and families because of their almost ubiquitous presence in vascular plants and of their structural variety.

A phytochemical study was undertaken with the aim of identifying and determining quantitatively the major compounds in the petals. In this study flavonoid compounds were isolated; the major compounds were glycosidic derivatives of quercetin and kaempferol, including their methoxylated and acetylated derivatives (**1-9**).

The study provided a method to define the flavonoids fingerprint by LC-ESI- IT MS/MS (liquid chromatography electrospray tandem mass spectrometry with ion trap analyser), with full scan acquisition in data dependent scan mode, and a method to quantify the content of the major compounds by LC-ESI- TQ

MS/MS (liquid chromatography electrospray tandem mass spectrometry with triple quadrupole analyser) by using a MRM (multiple reaction monitoring) mode. The quantitative method, performed by using internal and external standards, was validated in agreement with EMEA note guidance on validation of analytical methods [4].

By using tandem in time mass spectrometry it was possible to reveal the compounds on the basis of their specific fragmentation. The specific fragmentation pattern was used for developing the selective MS/MS method for the two major compounds (**1**, **2**). The use of tandem mass spectrometry in quantification of secondary metabolites from plants has led to sensitive, selective and robust methods for quality control and standardisation of plant extracts [5a-5c].

Phytochemical investigation of the methanolic extract of *C. sativus* led to the isolation of flavonoid compounds **1-9**. The compounds, identified by comparing their NMR data with those reported in the literature, were quercetin-3,7-di-*O*-β-D-glucopyranoside (**1**) [6], kaempferol-3,7-di-*O*-β-D-glucopyranoside (**2**) [2a], isorhamnetin-3,7-di-*O*-β-D-glucopyranoside (**3**) [7], kaempferol-3-*O*-β-D-glucopyranoside (**4**) [2a], quercetin-3-*O*-β-D-glucopyranoside (**5**) [8], isorhamnetin-3-*O*-β-D-glucopyranoside (**6**) [9], kaempferol-7-*O*-β-D-glucopyranoside (**7**) [2a], kaempferol-3-*O*-β-D-(2-*O*-β-D-glucosyl) glucopyranoside (**8**) [2a], and kaempferol-3-*O*-β-D-(2-*O*-β-D-6-O-acetylglucosyl) glucopyranoside (**9**) [2a]. The high content of glycosylated flavonoids could give value to *C. sativus* petals, which are a waste product in the production of saffron spice.

In order to realise a qualitative analysis for the flavonoid derivatives in *C. sativus* extracts, MS experiments were performed by using an LC-MS system equipped with an ESI source and an Ion Trap analyser. Positive ion electrospray LC/MS analysis, total ion current (TIC) profile and reconstructed ion chromatograms (RICs) of extract are shown in Figure 1. Flavonoid derivatives were identified by comparing retention times and *m/z* values in the total ion current chromatogram with those of the selected standards, obtained in the isolation step. Reconstructed ion chromatograms were obtained for each value of *m/z* observed for the standard compounds (*m/z* 627, **1**; *m/z* 611, **2**; *m/z* 641, **3**; *m/z* 449, **4**, **7**; *m/z* 465, **5**; *m/z* 479, **6**; and *m/z* 653, **9**) in order to improve the separation and identification of single compounds. The chromatographic profile obtained in Total Ion Current revealed two very major compounds, respectively **1** and **2**, in *C. sativus* extracts, whereas the other compounds were present in lesser amounts. Quantitative analysis was focused on compounds **1** and **2**, which could potentially be recovered from discarded petals as economic secondary products.

In order to obtain an accurate quantitative determination of compounds **1-2**, a quantitative LC-MS/MS method was developed. Since the sugar loss is the most representative fragmentation for glycosidic flavonoids, ESI-MS/MS analyses were recorded for the two major compounds by using an LC-MS equipped with an ESI source and a triple quadrupole analyser. Analyses were performed by direct introduction and both the spectra showed the characteristic fragment resulting from sugar loss. Thus an MRM method was developed. Transition from the specific pseudomolecular ion $[M+H]^+$ of each compound to the corresponding aglycon ion $[A+H]^+$ was selected to monitor the flavone glycosides in *C. sativus* using as internal standard (I.S.), rutin (*m/z* 611) (I.S.).

Compound **1**: precursor ion *m/z* 627.0, product ion *m/z* 303.0, collision energy 30%; compound **2**: precursor ion *m/z* 611.0, product ion *m/z* 287.0, collision energy 30%; I.S. precursor ion *m/z* 611.0, product ion *m/z* 303.0, collision energy 30%.

The MRM analyses of *C. sativus* methanolic extract, spiked with I.S., contained the peaks corresponding to the compounds under investigation, with appreciable intensity for quantitative purposes. Validation of the method was realised in agreement with EMEA note guidance on validation of analytical methods [4].

Validation of the LC/MS/MS method included intra and inter-day precision and accuracy studies on three days. Accuracy and precisions were calculated by analysing five samples of each extract (MeOH and water). Standard deviations calculated in this assay were < 7% for the two compounds under investigation. Specificity is usually reported as the non interference with other substances detected in the region of interest; the present method, developed by using a characteristic fragmentation of flavone glycosides **1**, **2**, was specific with no other peak interfering in the MS/MS detection mode.

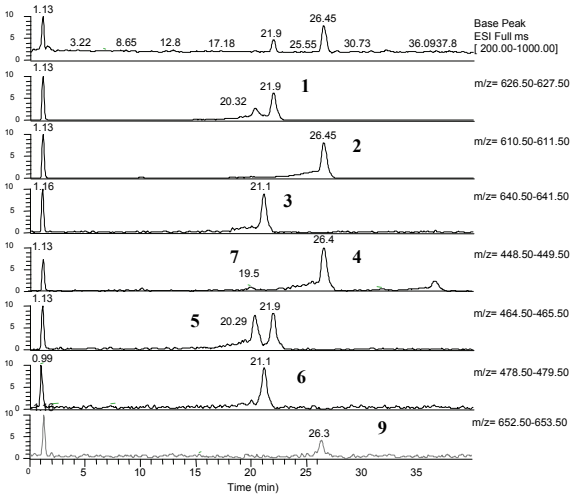

Figure 1. HPLC-MS qualitative analysis

Table 1: Quantitative results and quantification data.

n	mg/g MeOH ext.	mg/g water ext.	mg/g petals	Calibration equation	r^2
1	41.8±6.2	8.1±0.2	27.6±6.0	y = 0.183x-0.74	0.998
2	31.1±4.7	5.5±0.1	20.2±4.6	y = 0.094x-0.39	0.997

The calibration graphs, obtained by plotting area ratio between external and internal standards versus the known concentration of each compound, were linear in the range of 1-100 μg mL^{-1} for all compounds. Correlation values (r^2) are reported in Table 1.

Five aliquots of methanol and water extracts, respectively, obtained from *C. sativus* were analysed in order to quantify the flavonoid contents. Table 1 reports the quantitative data for compounds **1-2**, regression of calibration curves, and quantitative values. Quantitative analyses results confirmed that waste petals of *C. sativus* can represent an interesting source of such phenolic compounds, with respect to the high content showed.

Experimental

Reagent and standards: Standards of pure compounds **1-2** were isolated in our laboratory and their structures were elucidated by NMR spectroscopy (Bruker DRX-600). Each standard was dissolved in methanol. HPLC grade methanol (MeOH), acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Merck (Merck KGaA, Darmstadt, Germany). HPLC grade water (18mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA). The reagents used for the extractions, of analytical grade, were purchased from Carlo Erba (Rodano, Italy). Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

Equipment: Semi-preparative HPLC was performed using an Agilent 1100 series chromatograph, equipped with a G-1312 binary pump, a G-1328A rheodyne injector and a G-1365B multiple wave detector. The column was an RP C18 column μ-bondapak 300 mm x 7.6 mm (Waters, Milford, MA). HPLC–ESI-MS analysis was performed using a Thermo Finnigan Spectra System HPLC coupled with an LCQ Deca ion trap. Chromatography was performed on an RP C18 column Symmetry Shield (Waters, Milford, MA). HPLC–ES-MS/MS for quantitative analysis was performed on a 1100 HPLC system (Agilent, Palo Alto, CA) coupled with a triple quadrupole instrument [API2000 (Applied Biosystems, Foster City, CA, USA)]. The instrument was used in the tandem MS mode, with multiple reaction monitoring (MRM).

LC-ESI-MS and LC-ESI-MS/MS analysis: The mass spectrometer was operated in the positive ion mode under the following conditions: capillary voltage 3 V, spray voltage 5 kV, tube lens offset 40 V, capillary temperature 260°C, and sheath gas (nitrogen) flow rate 60 arbitrary units. Data were acquired in the MS^1 scanning mode with scan ranges of 200 – 1000 *m/z*: the maximum injection time was 50 ms, and the number of microscans was 3. In order to tune the LCQ for flavonoids, the voltages on the lenses were optimised using the TunePlus function of the Xcalibur software in the positive ion mode whilst infusing a standard solution of quercetin (1 μg mL^{-1} in methanol) at a flow rate of 3 μL min^{-1}. For qualitative LC-ESI-MS analysis, a gradient elution was performed on a RP C18 column Symmetry shield (Waters, Milford, MA), 2mm x 150 mm, by using a mobile phase A represented by water acidified with trifluoroacetic acid (0.05%) and a mobile phase B represented by water: acetonitrile 50:50 acidified with trifluoroacetic acid (0.05%). The gradient started from 20% of eluent B, to achieve the 33% of solvent B in 18 min. After another 12 min the percentage of B became 40%, and remained at this value for 10 min, then became 50%. The flow (250 μL min^{-1}) generated by chromatographic separation was directly injected into the electrospray ion source. MS were acquired and elaborated using the software provided by the manufacturer.

For quantitative LC-ESI-MS/MS a gradient elution was performed by using a mobile phase A represented by water acidified with trifluoroacetic acid (0.05%) and a mobile phase B represented by acetonitrile acidified with trifluoroacetic acid

(0.05%). The gradient started from 5% of eluent B, remained isocratic for 5 minutes, to achieve the 80% of solvent B in 15 min. The flow (250 μL min^{-1}) generated by chromatographic separation was directly injected into the electrospray ion source.

The mass spectrometer was operated in the positive ion mode under the following conditions: declustering potential 200 eV, focusing potential 155 eV, entrance potential 10 eV, collision energy 30 eV, and collision cell exit potential 15 eV, ion spray voltage 5000, temperature 250°C. The instrument was used in the tandem MS mode with multiple reaction monitoring (MRM). For all flavonoids analyzed, the selected fragmentation reaction was the loss of the glycoside moiety.

Plant material: Petals of *C. sativus*, discarded by production companies of saffron spice, were collected in Sardinia (Italy) in November 2004.

Extraction and isolation: Dried and powdered petals (23 g) of *Crocus sativus* were extracted for 3 days, 3 times, at room temperature with methanol to give 9.7 g of crude methanolic extract. This was extracted for one day with water. The filtered extract was lyophilized to give 3 g of crude extract. Part of the methanolic extract (3.7 g) was fractionated initially on a 100 cm×5.0 cm Sephadex LH-20 column, using CH_3OH as mobile phase, and 56 fractions (10 mL each) were obtained. Fractions 28-29 (24.5 mg) (**a**), 19-20 (250 mg) (**b**), 31-33 (31.4 mg) (**c**) and 36-40 (40.4 mg) (**d**) were chromatographed by HPLC-UV. The mobile phase was a linear gradient of water/acetonitrile (50:50) with trifluoroacetic acid 0.1% (solvent B) in water acidified with trifluoroacetic acid 0.1% (solvent A), at a flow rate of 2.000 mL min^{-1}. From sample **a**, compounds **1** (3.3 mg, t_R=26.8), **2** (2.9 mg, t_R=29.7) and **3** (1 mg, t_R=28.7) were obtained; from sample **c**, compounds **4** (1.6 mg, t_R=36.4) and **6** (0.9 mg, t_R=37.8); and from sample **d**, compounds **5** (0.6 mg, t_R=32) and **7** (1.1 mg, t_R=36.9) using the following gradient: 0 min, 10% B, 0-5 min, 10-20% B, 5-25 min, 20-40% B; 25-40 min, 40% B; 40-50 min, 40-70% B, 50-60 min, 70-100% B.

From sample **b**, compounds **8** (5.6 mg, t_R=29.8) and **9** (1.2 mg, t_R=37.9) were obtained using the following gradient: 0 min, 20% B; 0-18 min, 20-33% B; 18-30 min, 33-40% B; 30-40 min, 40% B; 40-45 min, 40-85% B; and 45-55 min, 85-100%.

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