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Determination of saffron (*Crocus sativus* L.) components in crude plant extract using high-performance liquid chromatography–UV–visible photodiode-array detection–mass spectrometry☆

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Abstract

The determination of saffron components in crude plant extracts by high-performance liquid chromatography –UV–visible photodiode-array detection on-line with mass spectrometry is described. The method is shown to be suitable for the determination of picrocrocin, the glycosidic precursor of safranal, safranal and flavonoids; it is the technique of choice for the analysis of crocetin glycosides (crocins) carrying one up to five glucoses and differentiation of their *trans* and *cis* isomers.

1. Introduction

We reported previously [1] an investigation of the separation of picrocrocin, *cis–trans*-crocins and safranal of saffron (*Crocus sativus* L.). Those studies were carried out using a methanolic extract of the dried saffron intact stigma by reversed-phase high-performance liquid chromatography (HPLC) with UV–visible photodiode-array (PDA) detection, using a gradient solvent system.

The major components of saffron are *cis*- and *trans*-crocins, which are glucosyl esters of 8,8′-diapocarotene-8,8′-dioic acid (crocetin), one of the few families of carotenoids that are freely soluble in water. It contains also safranal, which is a monoterpene aldehyde, and picrocrocin, which is a glycosidic precursor of safranal [1–13].

All these compounds are the responsible agents for the multiple organoleptic properties of saffron, widely used as a condiment and food additive; it has very powerful colouring and taste properties at it contains highly water-soluble glycosides and the odour properties of safranal could be present or formed during storage and cooking of food preparations.

Glycosidic carotenoids of saffron, as all glycosides, are in general thermally labile and not

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☆ Second report on analysis of saffron components; for the first report, see Ref. [1].

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volatile compounds and carotenoid aglycones are photochemically sensitive compounds. Investigation of their mass spectra requires fast and soft ionization techniques if information on the molecular masses or sugar sequences is desired. The development of new LC–MS interfaces now allows mass spectrometric analysis to be coupled on-line with analytical HPLC-separation with UV–Vis detection.

Among the different techniques of combined LC–MS, thermospray (TS) and more recently electrospray (ES) interfaces have gained popularity and have been widely used for the analysis of plant crude extracts [14–18].

We describe here the analysis of crude saffron extract using HPLC–UV–Vis diode-array detection (DAD) coupled on-line with MS. The advantages of ES over TS ionization in HPLC–MS analysis and the capability of determining glycosidic carotenoids carrying 1–5 glucoses and isomeric differentiation are also discussed. HPLC–TS- or ES-MS provide analytical information which, combined with the conventional HPLC–UV–Vis-DAD analytical data may be very useful for the characterization of unknown compounds [17–19].

2. Experimental

2.1. Plant materials

Stigmata of pure red Greek saffron were kindly supplied by the Cooperative of Saffron, Krokos Kozanis.

2.2. Sample preparation

For the determination of saffron components, 5 ml of methanol and 5 ml of water were added to 100 intact stigmata of saffron (200 mg) and macerated for about 2 h in the dark at ambient temperature with occasional stirring [1,2,8]. The crude methanolic extract was analysed by HPLC–UV–Vis-DAD–MS using both TS and ES interfaces.

2.3. Equipment and conditions

Mass chromatography

The HPLC system consisted of a Hewlett-Packard (Palo Alto, CA, USA) HP-1090 Series liquid chromatograph with a DR5 binary solvent delivery system and variable-volume autoinjector. A UV–Vis diode-array detector monitored the elution profile at multiple wavelengths. A Hewlett-Packard ODS Hypersil 799160D-552 (5 μm) stainless-steel column (100 × 2.1 mm I.D.) was used. The mobile phase was a linear gradient from 10% to 100% methanol in water solution containing 1% acetic acid in 60 min. The solvent flow-rate was 0.5 ml/min and the sample injection volume was 5 μl. Detection was performed simultaneously at 190–600 nm.

Mass spectrometry

The mass spectrometer was a Hewlett-Packard Model 5989A, equipped with a hyperbolic quadrupole mass analyser, a high-mass option, high-energy dynode (HED) detector multiplier detector and a differentially pumped vacuum system with diffusion pumps. The voltage applied to the electron multiplier was 2300 V and that to the HED 9000 V. The quadrupole temperature was held at 100°C. The ionization mode was positive. The mass spectrometer scanned from m/z 130 to 1200. An HP mass spectra DOS ChemStation system (G1023B) was used as an analytical workstation.

Thermospray interface. The HP 5989 A mass spectrometer was equipped with an HP thermospray interface. The thermospray probe temperature was set at 100°C and time programmed to 90°C in 60 min, following the column linear gradient elution profile. The source was maintained at 280°C and the aerosol temperature was varied from 260 to 280°C (beginning–end of gradient). The measurements were performed in the filament-on, acquisition mode. To avoid any alteration of the chromatographic conditions, a postcolumn addition of a 0.2 *M* ammonium acetate was effected with an HP 1050 isocratic

pump, giving a final concentration of about 0.1 *M* buffer after dilution with the column effluent; this provided the volatile buffer for the ion evaporization ionization process. The TS mass spectra look like chemical ionization spectra and fragmentation is related to the vaporization temperature.

Electrospray interface. A Hewlett-Packard Model 59987A electrospray interface was used. The needle was grounded and charging occured by keeping the cylindrical electrode at about −3 kV, the end plate at −3.5 kV and the capillary at −4.5 kV. The potential difference between the skimmer and the end of the capillary was varied between 150 and 300 V. The other lenses were held at potentials such as to obtain the maximum signal intensity. Hot high-purity nitrogen was used for desolvation. The HPLC flow was split 1:150 and the resulting flow into the electrospray source was about 3 μl/min.

3. Results and discussion

Fig. 1 shows the structures and molecular formulae of the most abundant compounds identified in saffron extract. Fig. 2 demonstrates the reversed-phase HPLC separation at three wavelengths (250, 308 and 440 nm) and Fig. 3 the total ion current chromatogram and selected-ion chromatograms, which corresponded well with the HPLC–UV–Vis trace. The number of absorption bands and the general aspects of their UV spectra allowed the assignment of the peaks on the chromatogram in three families of compounds: (a) picrocrocin and derivatives of picrocrocin, (b) flavonoid derivatives and (c) crocins.

3.1. UV–visible spectra

Saffron components present characteristic UV–Vis spectra. These spectra are easily recorded during the HPLC separation of the plant extract, on-line with the aid of a diode-array detector.

Picrocrocin and derivatives of picrocrocin

Picrocrocin [4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde] is the saffron bitter agent. The UV spectrum of picrocrocin (Fig. 4) exhibits a characteristic broad absorption band at 250 nm due to the α,β-unsaturated cycloaldehyde in the molecule. Oxidation of the aldehyde group gives the acid form of picrocrocin and an absorption band of the α,β-unsaturated cyclic acid at 246 nm appears in the UV spectrum. Picrocrocin aglycone dehydration gives safranal (2,6,6-trimethyl-3-cyclohexadiene-1-carboxaldehyde) with a characteristic UV absorption band at 310 nm (Fig. 4). Oxidative decarboxylation of picrocrocin gives the precursor isophorone. Isophorone (3,5,5-trimethyl-2-cyclohexen-1-one) has been described as a constituent of the volatile part of saffron [4,13]. Picrocrocin, picrocrocin aglycone, isophorone glycoside and safranal are present in the crude saffron extract.

Flavonoid derivatives

The presence of flavonoids in saffron has not previously been described. Nevertheless, polyphenols (including flavonoids) have long been recognized as one of the largest and most widespread classes of plant constituents, occurring throughout the higher and lower plants [20]. They occur as aglycones but very often as mono- and diglycosides in the methanolic plant extracts. Flavonoids derive from 2-phenyl-γ-benzopyrone; the characteristic UV spectra depend considerably on the number and substitution pattern of the hydroxy groups in the flavonoid nucleus; the location of free hydroxy groups and structure elucidation of flavonoids can be established with the aid of classical shift reagents that induce shifts of the absorption maxima, as has been extensively described [20]. Kaempferol diglycoside was identified in the crude extract on the basis of UV and MS data (Fig. 5).

Crocins

Crocin derivatives show characteristic UV–Vis spectra of the carotenoid moiety in the molecule. Crocins are glucosyl esters of crocetin, the cen-

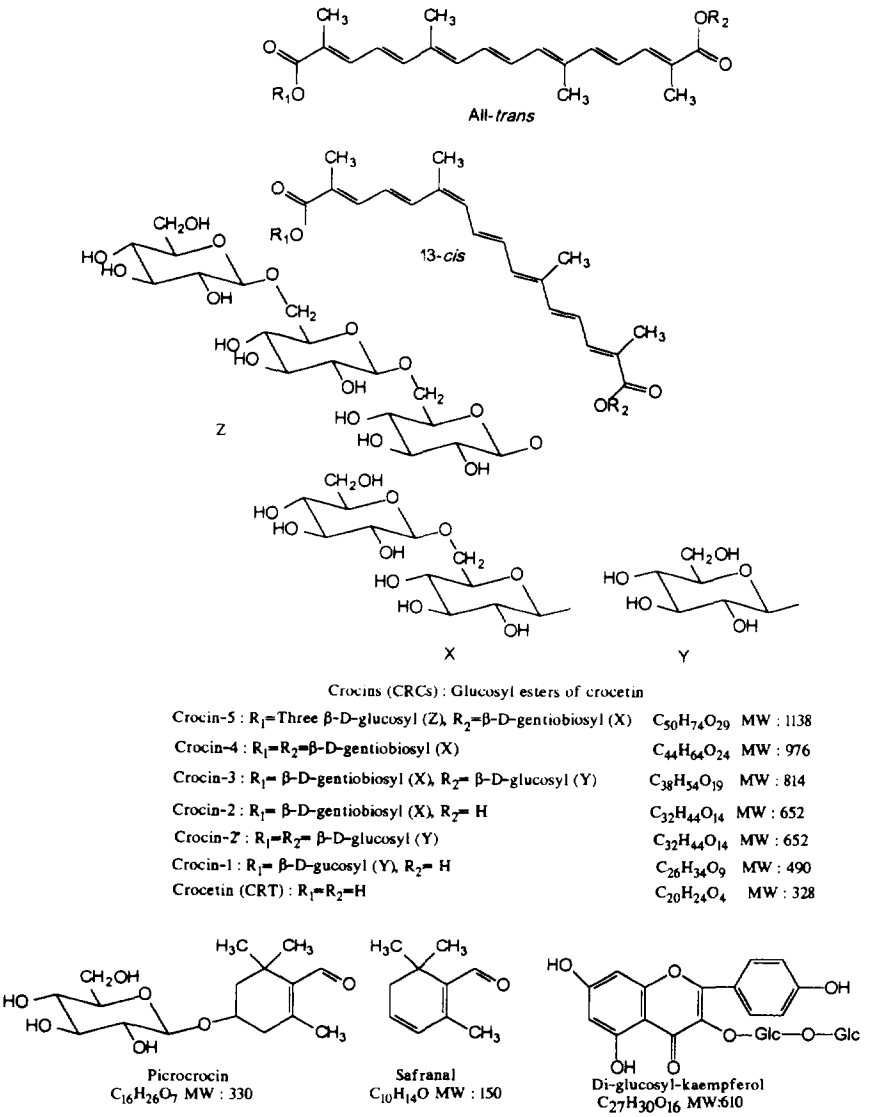

Fig. 1. Structures of saffron components. MW = Molecular mass.

tral unit consisting of seven conjugated double bonds and four side-chain methyl groups. The end-groups are esterified with one glucose (Glc), two glucoses (gentiobiose, Gnt = β-D-Glc-1→6-β-D-Glc) and three glucoses (β-D-Glc-1→6-β-D-Glc-1→6-β-D-Glc), and they can be the same or different. Structures of the crocins are shown in Fig. 1.

Carotenoids have characteristic spectra in the visible region with double peaks between 400 and 500 nm. In the UV region between 320 and 340 nm, an absorption peak referred to as the *cis* peak is apparent for *cis* isomers, with the intensity of this peak varying with the position and number of *cis* double bonds [1,21–24].

The UV–Vis spectra of all-*trans* glycosidic carotenoids of saffron show two absorption bands (Fig. 6). The first, at 256 nm, corresponds to glucosyl ester bonds of crocins, and the second, between 400 and 500 nm, with λ_{max} at 437 nm, is characteristic of all-*trans*-carotenoids. The *cis* isomers show three absorption bands.

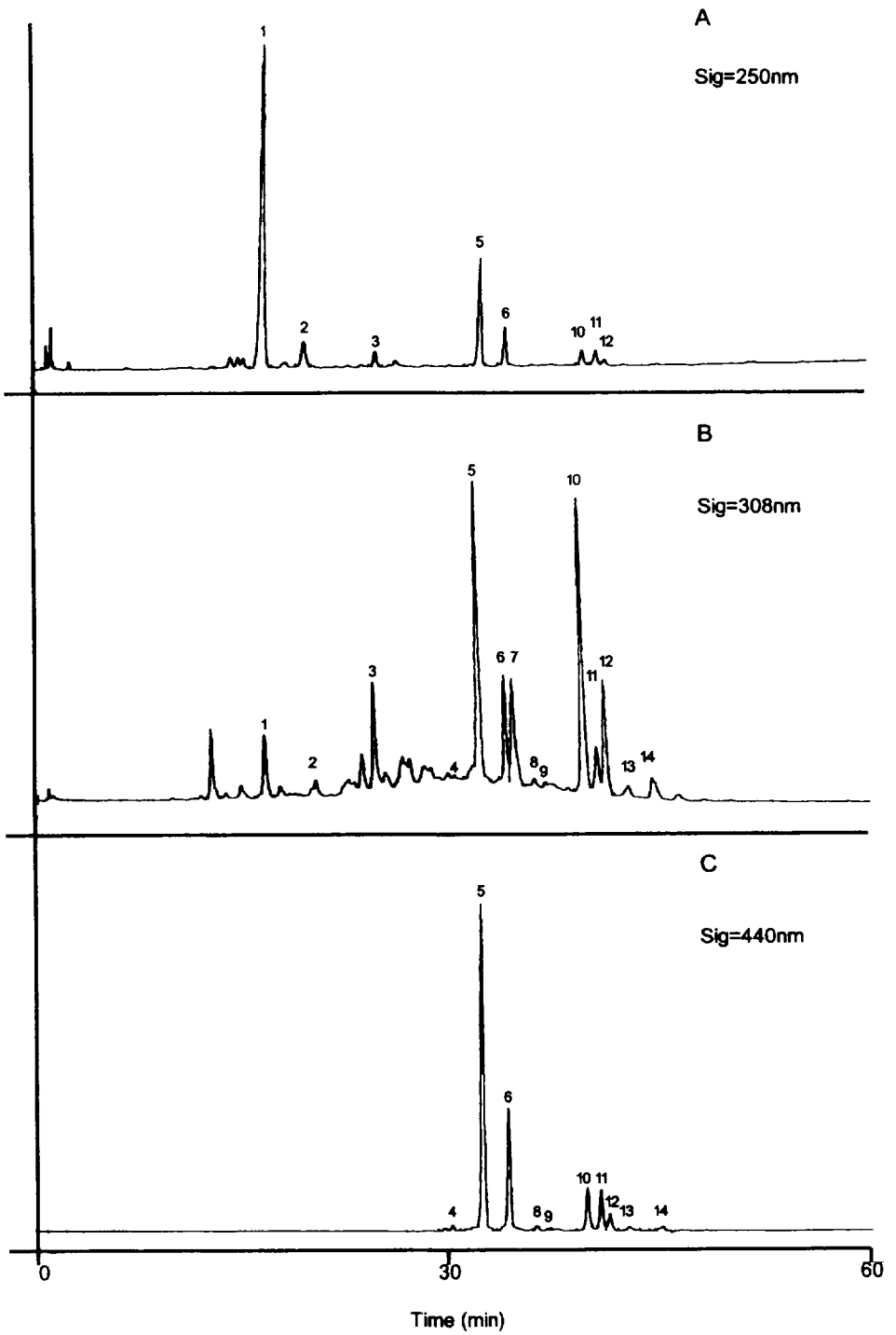

Fig. 2. Chromatographic signals for a methanol–water (50:50) extract of saffron recorded at (A) 250, (B) 308 and (C) 440 nm. Peaks: 1 = picrocrocin; 2 = picrocrocin acid form; 3 = kaempferol diglycoside; 4 = *trans*-crocin-5 and 9 = *cis*-crocin-5; 5 = *trans*-crocin-4 and 10 = *cis*-crocin-4; 6 = *trans*-crocin-3 and 12 = *cis*-crocin-3; 8 = *trans*-crocin-2′ and 14 = *cis*-crocin-2; 11 = *trans*-crocin-2 (*trans* gentiobiosyl ester of crocetin); 13 = *cis*-crocin-1; 7 = safranal.

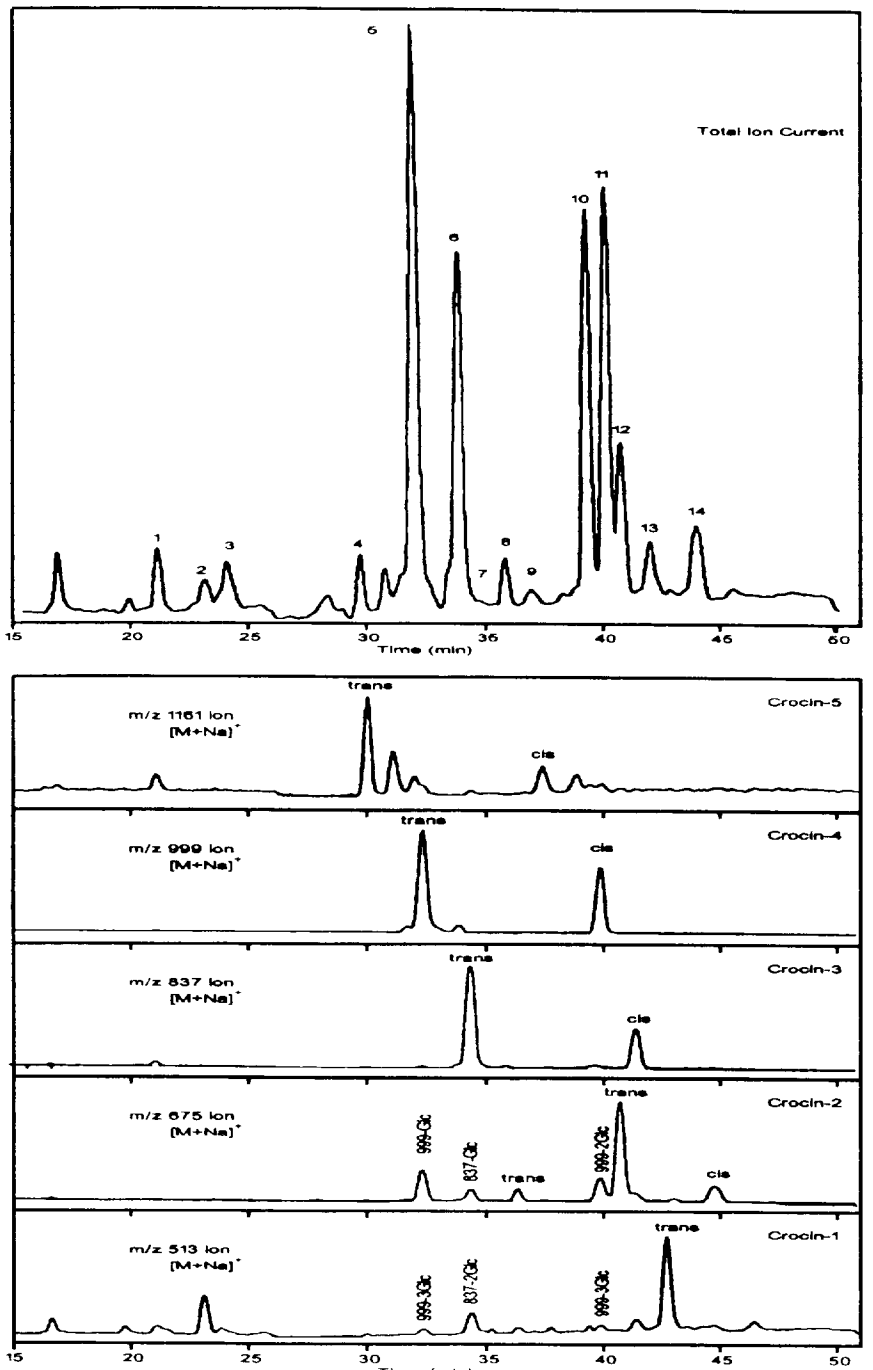

Fig. 3. Total ion chromatogram and selected-ion chromatograms for the HPLC–ES-MS analysis of a methanol–water (50:50) extract of saffron.

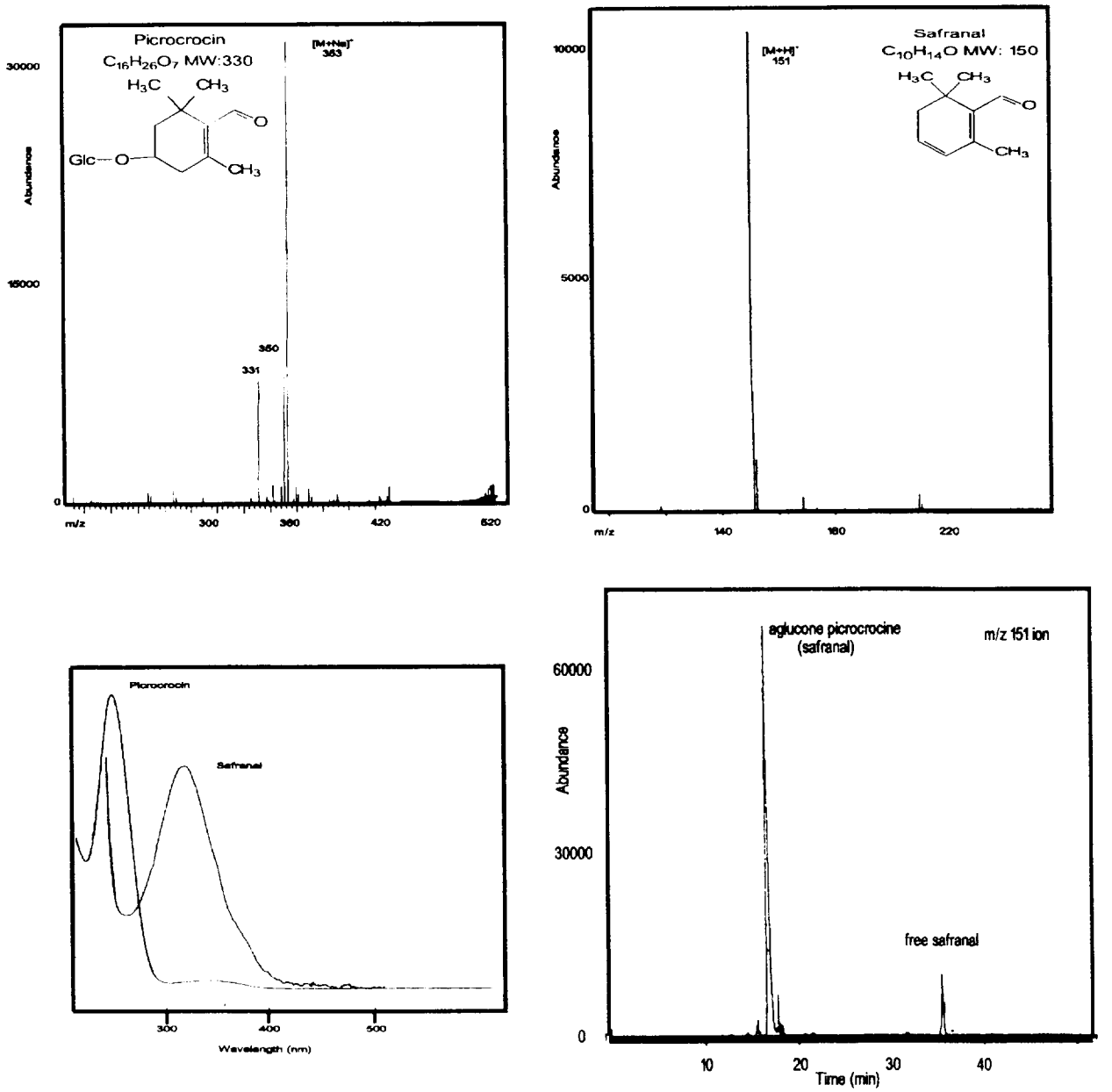

Fig. 4. Structure, mass spectra, UV–visible spectra and selected-ion of chromatogram picrocrocin and safranal.

The first, at 260 nm, corresponds to glucosyl ester bonds of crocins, the second, at 325 nm, is attributed mainly to the presence of *cis* double bonds in the polyene conjugated system of crocins, with a medium intensity of absorption which is characteristic of the 13-*cis*-carotenoids, and the third band, between 400 and 500 nm, with λ_{max} at 435 nm, is characteristic of all-*trans*-

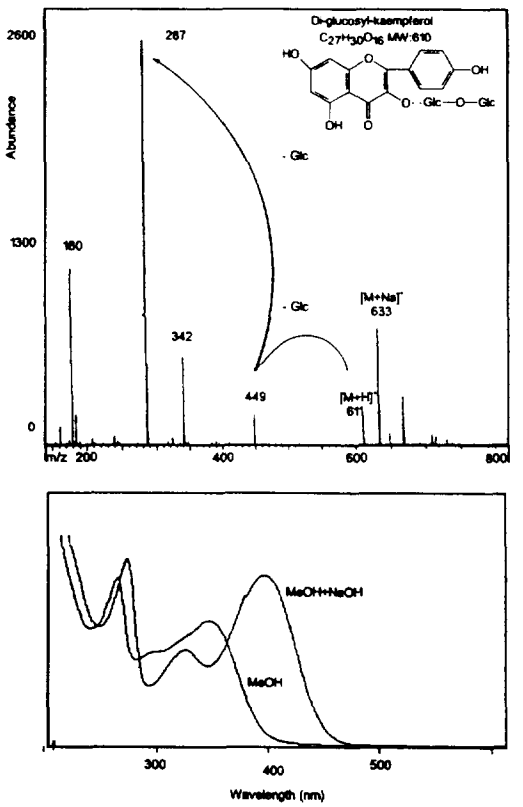

Fig. 5. Structure, mass spectrum and UV–visible spectra of kaempferol diglycoside.

carotenoids [1,7,21–24]. The *trans–cis* isomers of crocins were identified with the aid of the PDA detector.

3.2. Liquid chromatography–mass spectrometric analysis

In order to obtain more information on the molecular masses, sugar number and sequence of all the saffron compounds, LC–MS analysis of the crude extract was carried out. LC–TS-MS was initially used under the same LC conditions as for UV–Vis analysis. The ammoniun acetate buffer needed for ion evaporation ionization in the thermospray ionization process was added postcolumn to avoid any modification of the chromatographic elution profile. As glycosides are thermolabile compounds, the ability to observe the molecular ions depends on the vaporizer and source temperatures, set at 100 and 280°C, respectively.

Picrocrocin and derivatives of picrocrocin

The mass spectrum of picrocrocin (Figs. 2 and 3, peak 1) in the extract displayed strong $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ quasi-molecular ions at m/z 331, 348 and 353 (Fig. 4); other adducts ions due to the ammonium acetate buffer were also present together with the $[A+H]^+$ and $[A-H_2O+H]^+$ fragment ions due to the aglycone part of the molecule. Picrocrocin acid form (Figs. 2 and 3, peak 2) showed quasi-molecular ions at m/z 369 $[M+Na]^+$ and 364 $[M+NH_4]^+$. Quasi-molecular ions $[M+NH_4]^+$ and $[M+Na]^+$ at m/z 510 and 515, corresponding to the diglycoside of picrocrocin aglycone, were present in the crude extract in trace amounts. Free picrocrocin aglycone and safranal (Figs. 2 and 3, peaks 1 and 7) exhibited strong $[M+H]^+$ and $[M+NH_4]^+$ quasi-molecular ions at m/z 151 and 168, respectively (Fig. 4).

Flavonoid derivatives

The mass spectrum of a flavonoid identified as kaempferol diglycoside (Figs. 2 and 3, peak 3) showed quasi-molecular ions at m/z 611 $[M+H]^+$ and 633 $[M+Na]^+$. Elimination of the terminal glucosyl moiety was observed, generating peaks at m/z 449 and 287, followed by the loss of a second glucosyl unit ($[A+H]^+$ aglycone); another fragment ion was also present in the mass spectrum at m/z 342 ([Glc–Glc] moiety) (Fig. 5). The presence of other flavonoids in small amounts could not be excluded; investigation of their structures is in progress.

Crocins

Crocins-5 [crocetin tri(β-D-glucosyl)-(β-D-gentiobiosyl)ester], crocetin esterified with three glucoses at the one end and one gentiobiose at the other, *trans* and *cis* isomers (Figs. 2 and 3, peaks 4 and 9, respectively), displayed an ion at m/z 1161, $[M+Na]^+$ (molecular mass 1138). Additional signals could be observed at m/z 837, 797 and 592, corresponding to $[(M+Na)-Gnt]^+$, $[M-Gnt]^+$ and $[M+2Na]^+/2$, respectively.

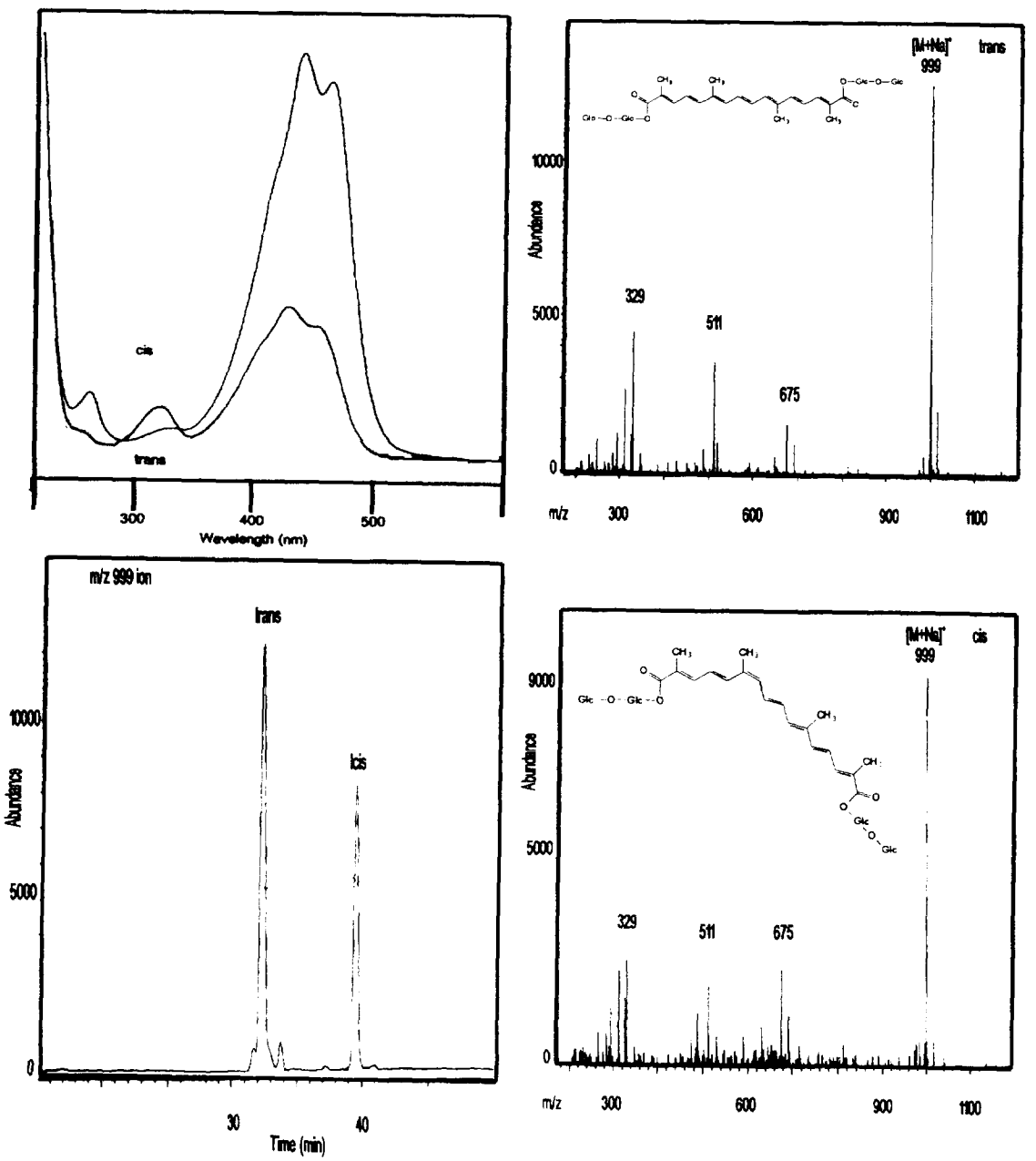

Fig. 6. Mass spectra, UV–visible spectra and selected-ion chromatogram of *cis*- and *trans*-crocin-4 [crocetin di(β-D-gentiobiosyl)ester].

Crocins-4 [crocetin di(β-D-gentiobiosyl) ester], crocetin esterified with one gentiobiose at the each end, *trans* and *cis* isomers (Figs. 2 and 3, peaks 5 and 10, respectively), displayed an ion at m/z 999 $[M+Na]^+$ (molecular mass 976). Elimination of terminal gentiobiosyl groups was observed, generating peaks at m/z 675 $[(M+Na+H)-Gnt]^+$ and 329 $[\{(M+Na+H)-Gnt\}-Gnt]^+$. An additional signal could be observed at m/z 511 $[M+2Na]^+/2$ (Fig. 6).

Crocins-4 are the major components of the colouring power of saffron.

Crocins-3 [crocetin (β-D-glucosyl)-(β-D-gentiobiosyl)ester], crocetin esterified with one gentiobiose at one end and one glucose at the other, *trans* and *cis* isomers (Figs. 2 and 3, peaks 6 and 12, respectively), displayed an ion at m/z 837 $[M + Na]^+$ (molecular mass 814). Additional signals were observed at m/z 675 $[(M + Na + H) - Glc]^+$ and 329 $[\{(M + Na + H) - Glc\} - Gnt)]^+$.

Crocins-2′ [crocetin di-(β-D-glucosyl)ester], crocetin esterified with two glucoses, one glucose at each end, *trans* and *cis* isomers (Figs. 2 and 3, peaks 8 and 14, respectively), displayed an ion at m/z 675 $[M + Na]^+$ (molecular mass 652).

Crocin-2 [crocetin (β-D-gentiobiosyl)ester], crocetin esterified with one gentiobiose at one end and a free carboxylic group at the other (Figs. 2 and 3, peak 11), displayed an ion at m/z 675 $[M + Na]^+$ (molecular mass 652). A loss of one glucose was identified at m/z 513 $[(M + Na + H) - Glc]^+$ and a loss of second glucose at m/z 349 $[\{(M + Na - H) - Glc\} - Glc]^+$.

Crocin-1 [crocetin (β-D-glucosyl)ester], crocetin esterified with one glucose at one end and a free carboxylic group at the other, was found in the *trans* configuration (Figs. 2 and 3, peak 13), and displayed an ion at m/z 513 ($[M + Na]^+$ (molecular mass 490). An additional signal was observed at m/z 328 $[(M + H) - Glc]^+$.

Mono- and diglycosides of crocetin exhibited high $[M + Na]^+$, $[M + NH_4]^+$ quasi-molecular ions and fragments corresponding to the glucose part or to the carotenoid moiety in the molecule. As the molar absorptivities of crocins were of the same order of magnitude, poor MS responses were observed for tri- and tetraglycosides of crocetin; even more in the TS mass spectrum the quasi-molecular ions were weak or absent. Fragment ions attributed to the sugar and aglycone moieties were very important. Although TS-MS permits an unambiguous molecular mass determination of mono- and glycosides, the determination of the molecular mass of the parent ions of tri- and higher dilycosides is difficult or impossible.

Other "softer" ionization techniques are required to record higher molecular mass ions of these thermally unstable and non-volatile compounds. The newly developed electrospray mass spectrometric liquid interface was selected. Electrospray is a softer ionization technique; ionization occurs from the liquid phase through ion evaporation of highly charged micron-sized droplets and no heat is needed. The main adducts ions are $[M + nH]^{n+}$ and sodiated species. No molecular mass limitations exist; even more due to the formation of multiply charged ions. Large molecules can now be analysed using traditional mass spectrometers. However, restrictions are placed on the conductivity and flow-rate of the mobile phase.

The saffron extract was also analysed under the same conditions by HPLC–UV–Vis-ES-MS. It is worth noting that the results obtained by this technique were similar or complementary to those obtained using TS-MS.

Similarities were observed for the small molecules (safranal, picrocrocin derivatives, flavonoids). Protonated and sodiated species are in general the major ions in the spectra and fragmentation of the molecular ions is limited.

Complementary results were obtained in the area where the TS-MS technique failed in molecular ion determination. Tri- and tetracrocetin glycosides were easily analysed by ES-MS. The major ions in the spectra were $[M + Na]^+$ at m/z 837 and 999 for the tri- and tetraglycosides, respectively, and low-intensity potassium adducts were also present. Few fragments were observed in the spectra, useful structural information can be obtained by varying the CAP EXIT potential (V_{cap}) of the electrospray interface. Characteristic fragment ions can be formed, useful for structure elucidation studies. Ions at m/z 329 corresponding to the sodiated aglycone moiety and ions due to the loss of sugar units from the molecular ions were present. As the molecular mass increases, doubly charged species could also be formed. Minor amounts of crocetin pentaglucosylates were detected using this technique for the first time in saffron (Figs. 2 and 3, peaks 4 and 9, respectively). The quasi-molecular ion $[M + Na]^+$ at m/z 1161 corresponded with fragment ions at m/z 999, 837 and 675 due

to the loss of one, two and three sugar units, respectively; the doubly charged ion $[M + 2Na]^{2+}$ at m/z 592 confirmed the molecular mass of the crocetin pentaglycoside. As the number of sugar units increased in the crocin derivatives, the number of isomers due to the different attachment positions of the glucose unit in the molecule also increased. Selected ion monitoring of the ion m/z at 1161 ($[M + Na]^+$ demonstrated that there are at least four isomers for each *trans* and *cis* isomer of crocetin pentaglycoside. Isomers for the other crocetin glycosides were observed in small amounts.

All crocin derivatives occur as pairs of *cis–trans* isomers, except crocin-1, the *cis* isomer having a longer retention time in the reversed phase column [7,24]. It is worth noting that *trans*-crocetin diglycoside (gentiobiosyl ester), with one free carboxylic group (Figs. 2 and 3, peak 11), eluted between tetra- and tri-sugar crocin (*cis* derivative, Figs. 2 and 3, peaks 10 and 12, respectively). This could be explained by the effect of the presence of the 13-*cis* double bond in the carotenoid skeleton, the molecular spatial conformation exposing the lipophilic chain more than the *all-trans* unit to outside influences.

4. Conclusions

HPLC–UV–visible photodiode-array detection–mass spectrometry has been described for the separation and determination of saffron components. This method is suitable for the separation and determination of crocetin glycosides, safranal and picrocrocin (glycoside of safranal) in crude saffron extract, even in presence of other products such as flavonoids. The UV–visible and the mass spectra recorded online provide information about the identification of *trans* and *cis* isomers, molecular masses and the sequence of glucoses. TS ionization is suitable for the determination of glycosidic carotenoids carrying up to three glucoses. The advantage of ES over TS ionization in HPLC–MS is due to the capability to determine glycosidic carotenoids carrying up to five glucoses. The combination of LC–UV–Vis–MS data with crystal structure analysis. Fourier transform-infrared and Raman [25] and ^1H, ^{13}C NMR data provides useful information in identifying unknown compounds of saffron. Further work along these lines is in progress.

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