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Mass spectrometric characterization of flavonoids in extracts from *Passiflora incarnata*

Andrea Raffaelli[a,*], Gloriano Moneti[b], Valentina Mercati[c], Emilio Toja[c]

[a]*Centro di Studio del CNR per le Macromolecole Stereordinate ed Otticamente Attive, Dipartimento di Chimica e Chimica Industriale, Università di Pisa, Via Risorgimento 35, I-56126 Pisa, Italy*

[b]*Centro Interdipartimentale di Servizi di Spettrometria di Massa, Università di Firenze, Viale G.B. Morgagni 65, I-50134 Firenze, Italy*

[c]*ABOCA S.a.S. Azienda Agraria, Loc. Aboca 20, I-52037 Sansepolcro (Arezzo), Italy*

Abstract

Passiflora incarnata is a medicinal plant widely used for its sedative properties. The type of active compounds responsible for the sedative effects is not known yet, despite the fact that flavonoids are its most abundant active compounds. We present here a mass spectrometry study by ionspray ionization of extracts from *Passiflora incarnata*. The use of on-line HPLC–tandem mass spectrometry allowed the characterization of most of the flavonoids present. The interpretation of $[M-H]^-$ product ion spectra permitted the identification of several isomeric flavonoids containing different sugar moieties on the same aglycone. Some minor components were partially characterized. Their possible structures have been postulated on the basis of their $[M-H]^-$ product ion spectra. © 1997 Elsevier Science B.V.

Keywords: *Passiflora incarnata*; Flavonoids

1. Introduction

Passiflora incarnata (Passion flower) has been known for centuries for its sedative activity and is still widely used for this indication in many pharmaceutical preparations, mainly in combination with other drugs. A summary of the pharmacological properties of this medicinal plant has been recently published [1]. The main constituents of *Passiflora incarnata* are C-glycosyl flavones [2] based on apigenin and luteolin, while harman alkaloids, previously indicated as the active principles, are found only in trace amounts [3]. Within the framework of a research project started at Aboca on 'Active Principles from Medicinal Plants' we were interested in defining the complete flavonoid pattern of a multi-fraction dry extract prepared [4] from *Passiflora incarnata* cultivated in the central regions of Italy. This fraction has been analyzed by different high-performance liquid chromatographic (HPLC) approaches for the qualitative and quantitative characterization of flavonoids.

Although flavonoids have been previously identified and characterized several times, the results have been contradictory [5]. We decided, therefore, to analyze the dry extracts using HPLC. An HPLC protocol capable of giving the largest number of chromatographic peaks in the flavonoid region has been published recently by other authors [6]. The use of this method, after a flash-chromatography concentration step of the flavonoid fraction, allowed us to isolate two components not previously identified in the Rehwald paper and three flavonoids having a known structure but which were not commercially

*Corresponding author.

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available: schaftoside, isoschaftoside and isoorientin-2″-O-glucopyranoside. Due to the low amounts of the unknown substances isolated, mass spectrometry (MS) appears to be the ideal technique for the identification of their structure.

A literature survey on the use of MS for the characterization of flavonoids revealed only a few applications in this field. A review has been published by Harborne [7]. The identification of C-glycosyl flavonoids of *Passiflora incarnata* has been reported by Qimin et al. [8] using liquid secondary ions mass spectrometry (L-SIMS) in combination with collisionally activated dissociation (CAD) of the quasi-molecular ions $[M-H]^-$ and $[M+H]^+$ and B/E linked scanning. This paper describes the identification of four major flavonoids from *Passiflora incarnata*, i.e. schaftoside, isoschaftoside, isoorientin-2″-O-glucopyranoside and isovitexin-2″-O-glucopyranoside. The same technique was used also by Li et al. [9,10] for the characterization and differentiation of di-glycosyl flavonoids. Gluchoff-Fiasson et al. used fast atom bombardment (FAB) MS for the identification of complex structures [11]. The most detailed investigation has been reported by Becchi and Fraisse [12]. The negative ion FAB ionization, also coupled with MIKES (mass-analyzed ion kinetic energy spectra) collision techniques, allowed characterization of 23 different flavonoids, classification criteria to distinguish di-C-glycosyl, di-O-glycosyl and C-O-glycosyl flavonoids and some other useful structural information without derivatization. As far as the use of LC–MS is concerned, a recent review [13] illustrates the use of thermospray (TS) LC–MS in phytochemical analysis.

We present here mass spectral results obtained by ionspray ionization (ISI) of extracts from *Passiflora*

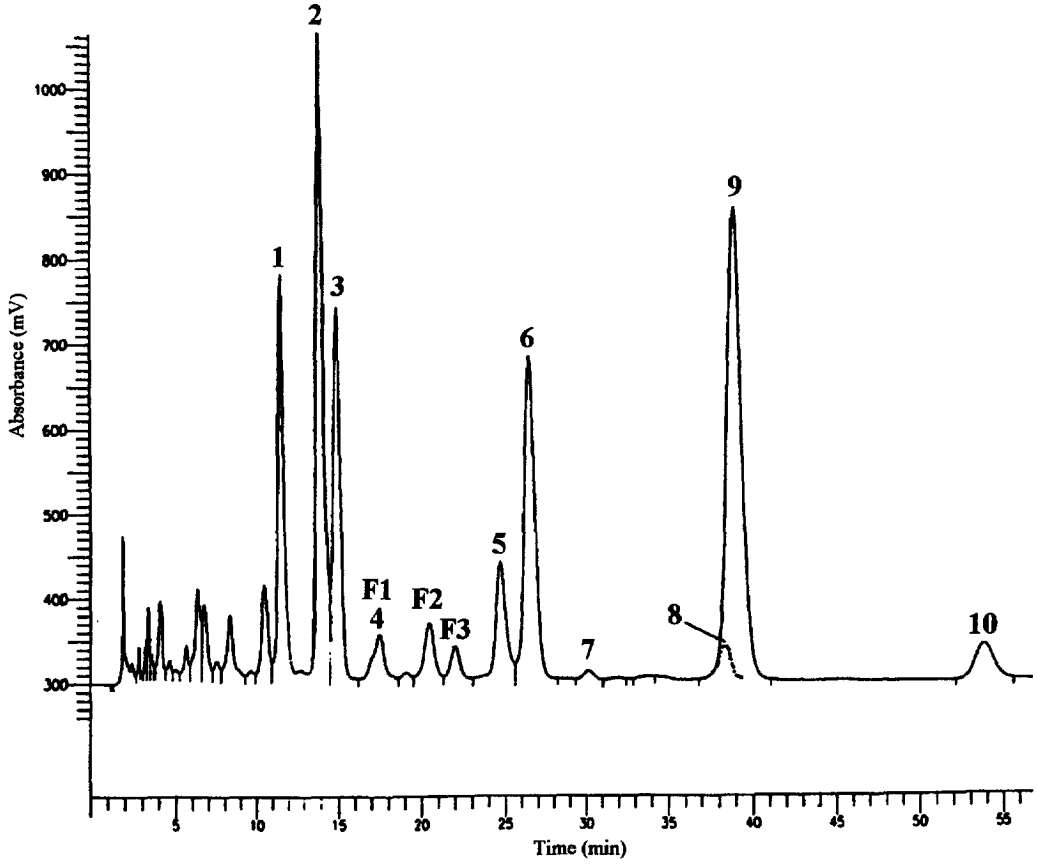
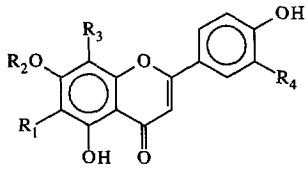


Fig. 1. HPLC–UV chromatogram of *Passiflora incarnata* flavonoid extracts.

Table 1
Flavonoids identified in the extracts from *Passiflora incarnata*



Peak no.	Flavonoid	R_1	R_2	R_3	R_4	M_r[a]
1	Vicenin-2	Glc	H	Glc	H	594
2	Schaftoside	Glc	H	Ara	H	564
3	Isoschaftoside	Ara	H	Glc	H	564
4	Isoorientin-2″-O-β-glucopyranoside	Soph	H	H	OH	610
5	Isoorientin	Glc	H	H	OH	448
6	Isovitexin-2″-O-β-glucopyranoside	Soph	H	H	H	594
7	Swertisin	Glc	Me	H	H	446
8	Orientin	H	H	Glc	OH	448
9	Isovitexin	Glc	H	H	H	432
10	Vitexin	H	H	Glc	H	432

[a] M_r=molecular mass.
Glc: β-D-glucopyranosyl; ara: α-L-arabinopyranosyl; soph: sophorose.

incarnata for the identification and characterization of flavonoids. The main goals of the present investigation were the evaluation of the potentials of on-line HPLC–ISI-MS and HPLC–ISI-MS–MS for the characterization of flavonoids, the confirmation of the structure of the known flavonoids and the identification of the structure of the two unknown components isolated from extracts of *Passiflora incarnata*.

2. Experimental

2.1. Mass spectrometry

The ISI mass spectra and ISI $[M-H]^-$ product ion spectra were taken on a Perkin–Elmer (PE) Sciex API III plus triple quadrupole mass spectrometer (Sciex, Thornhill, Canada) equipped with an articulated ISI interface. The operative parameters were set as follows: ionspray voltage: 5.5 kV (−4 kV in negative ion mode); orifice voltage: 60 V (−60 V in negative ion mode); scan range: 150–700 u, scan speed 2.89 s; resolution >1 u. ISI-MS–MS product ions were produced by CAD of selected precursor ions in the collision cell of the PE Sciex API III plus and mass analyzed using the second analyzer of the instrument under the same experimental conditions as above. Additional experimental conditions for MS–MS included collision energy: 30 eV; collision gas thickness (CGT): $240 \cdot 10^{13}$ molecules/cm^2 (CGT is equal to the collision gas density times the length of the collision cell; it gives a better information on the collision conditions with respect to the gas pressure); scan range: as necessary for the precursor ion selected.

2.2. HPLC separations

On-line HPLC separations were performed using a Perkin–Elmer Series 200 dual solvent delivery system (Perkin–Elmer, Norwalk, CT, USA) equipped with a 7125 Rheodyne injector with a 20-μl loop (Rheodyne, Cotati, CT, USA). We used a 25 cm×4.6 mm C_{18} LiChrospher column in isocratic conditions with a quaternary water–tetrahydrofuran–isopropanol–acetonitrile (88:8:1.6:2.4) mobile phase containing 0.07% formic acid, at a flow-rate of 1 ml min^{-1}, following the protocol described by Rehwald et al. [6]. The UV detection for on-line HPLC–MS was performed on a Perkin–Elmer LC 295 variable-wavelength detector set at 340 nm. The flow was split 40:1 after the UV detector so that a flow of 25

$\mu l\ min^{-1}$ was directed into the ISI source of the mass spectrometer.

The split of the F3 peak into two peaks was obtained under the following chromatographic conditions: column C_{18} LiChrospher 25 cm×10 mm; isocratic elution water–acetonitrile (90:10) for 10 min, followed by a gradient from 10% to 40% acetonitrile in 30 min, then back to initial conditions in 5 min; flow-rate 3 $ml\ min^{-1}$.

3. Results and discussion

The off-line UV chromatogram obtained is reported in Fig. 1. The numbered peaks (1–10) indicate the known flavonoids (Table 1), whereas the peaks labelled F1, F2 and F3 indicate the three fractions isolated in the Aboca laboratories, F2 and F3 being the two unknown components.

The on-line coupling with ISI-MS did not degrade the HPLC separation. The HPLC–ISI-MS analysis was performed both in positive ion and negative ion mode. Fig. 2 shows the UV, the positive ion total ion current (TIC) and the negative ion TIC chromatograms. The negative ion mode provided better sensitivity and the interpretation of the spectra was found to be easier, as every component gave a single ion, the $[M-H]^-$ quasi-molecular ion, under these conditions. Under positive ion conditions, we very often observed ammonium and/or sodium adducts in addition to the $[M+H]^+$ ion. For this reason we will discuss mainly the negative ion ISI spectra. The

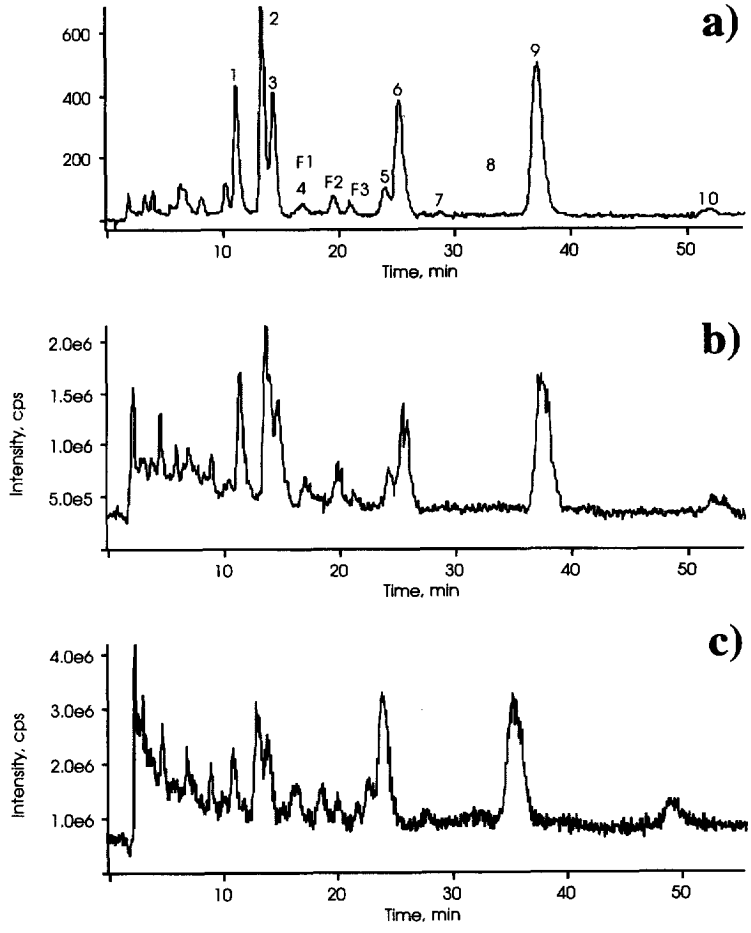


Fig. 2. (a) On-line HPLC–UV, (b) positive ion TIC and (c) negative ion TIC chromatograms of *Passiflora incarnata* flavonoid extracts.

HPLC–MS analysis confirmed the molecular mass of the different components, so that a more reliable assignment could be done. The lack of structural information, typical of ISI, a soft ionization technique which usually does not provide any fragmentation, can be overridden by the use of CAD and MS–MS. We also obtained the best results in negative ion mode in this case. The fragmentation of the $[M-H]^-$ proceeded very readily, whereas under positive ion conditions sometimes the protonated molecule signal was too low for MS–MS measurements, and the sodium adducts did not give useful fragmentation. The on-line HPLC–ISI-MS–MS analysis provided a good confirmation of the assignments of the HPLC peaks. A couple of examples are shown in Fig. 3. The molecular mass is correct for all the peaks of the known compounds and the fragmentation obtained by HPLC–MS–MS is in agreement with their structures as well.

The on-line coupling with ISI-MS also established that the F3 peak contains two co-eluting components: one having a molecular mass of 564 and the other with a molecular mass of 624, as shown in Fig. 4. In the negative ion spectra of this fraction it was possible to detect two peaks at m/z 563 and 623 (Fig. 4a). The positive ion spectrum confirmed the presence of two components: four ions at m/z 565 and 625, attributable to the $[M+H]^+$ ions and at m/z 587 and 647, corresponding to the respective sodium adducts (Fig. 4b). The extracted ion chromatogram (XIC) of the positive ions at m/z 565 and 625 provided an additional proof, as the two ions have slightly different elution times (Fig. 4c and d). The use of the different HPLC conditions listed in the experimental part permitted further separation of this peak into two components F3a and F3b, as shown in Fig. 5.

As far as the identification of the F2 and F3a components is concerned, an examination of their $[M-H]^-$ product ion spectra indicated that F2 corresponds to a structural isomer of schaftoside and F3a to a structural isomer of isoschaftoside, as

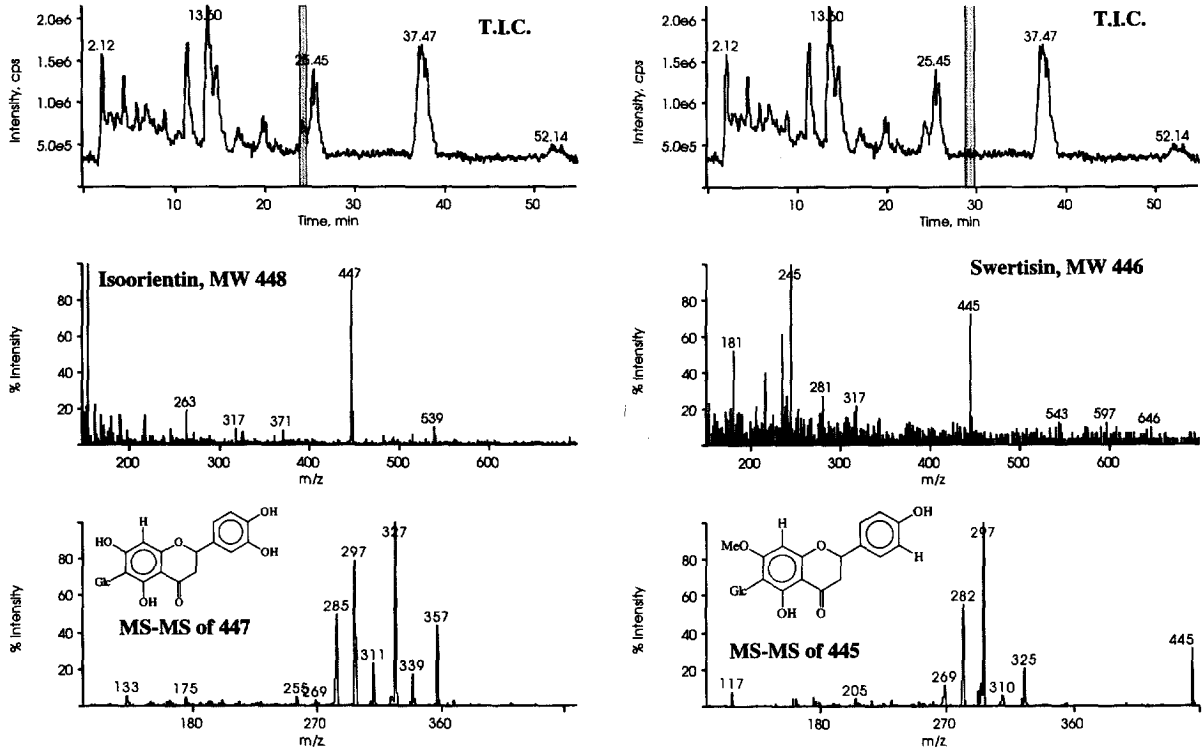


Fig. 3. Identification and MS–MS characterization of two known components (isoorientin and swertisin). MW=Molecular mass.

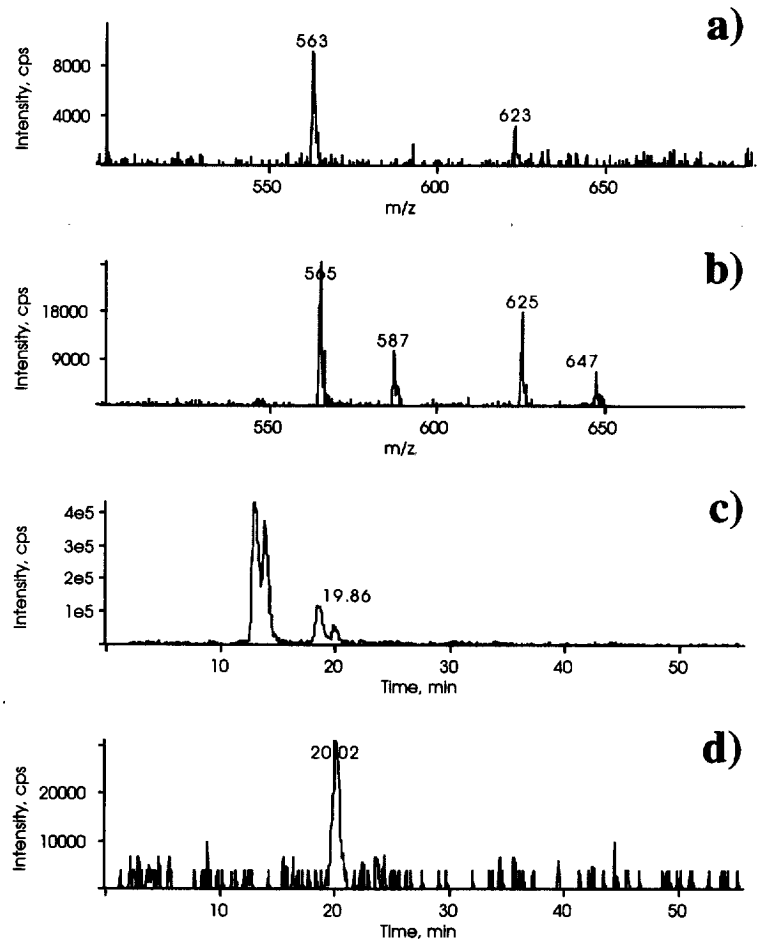


Fig. 4. (a) Negative ion ISI mass spectrum (b) positive ion mass spectrum and (c) XIC chromatograms of the *m/z* 565 and (d) 625 ions relative to the F3 peak.

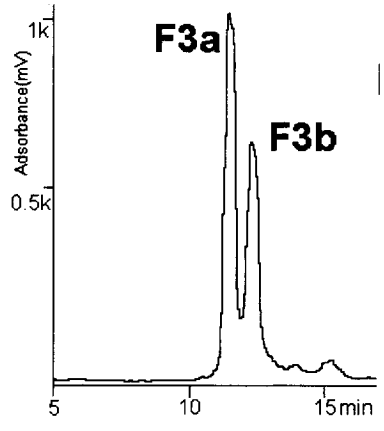


Fig. 5. Separation of F3 into two components F3a and F3b by modified chromatographic conditions.

clearly indicated by the comparison of the $[M-H]^-$ product ion spectra shown in Figs. 6 and 7. No differentiation was found between the $[M-H]^-$ product ion spectra of schaftoside and F2 and of isoschaftoside and F3a, under several different collision energy conditions (5–30 eV with a 5 eV step size). This suggests that F2 and F3a are indeed structural isomers of schaftoside and isoschaftoside, respectively, and hence they have the same aglycon (apigenin), being 6,8-di-C-glycosyl apigenins. F2 bears a hexose in position 6 and a pentose in position 8, the same as in schaftoside, whereas in F3a the positions of hexose and pentose are inverted, as in

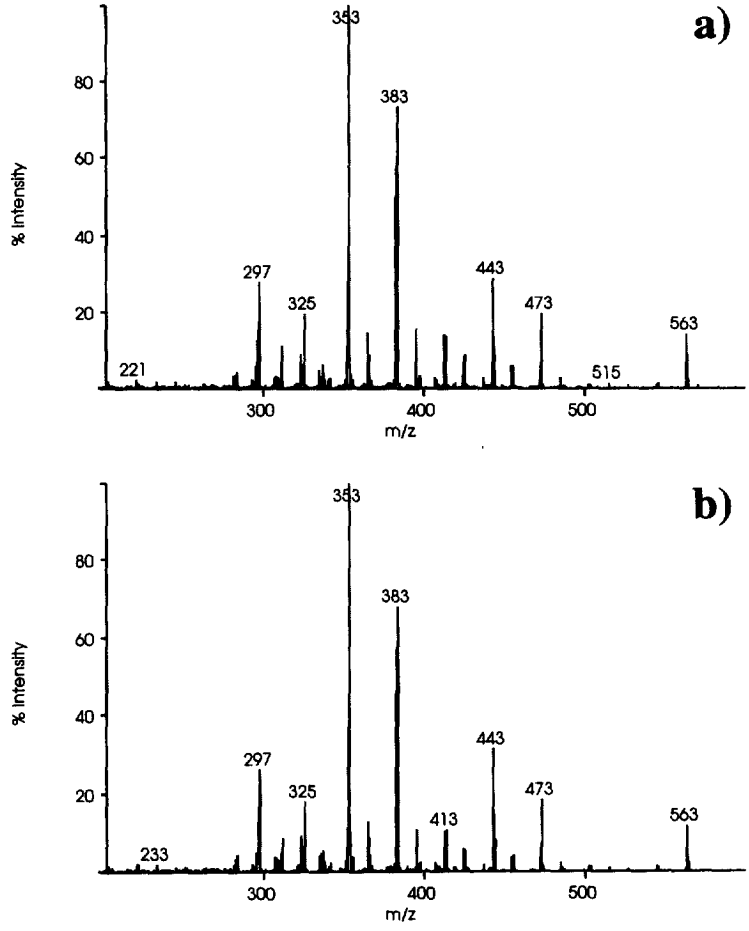


Fig. 6. $[M-H]^-$ product ion (parent ion: m/z 563) spectra at 30 eV of (a) schaftoside and (b) F2.

isoschaftoside. On the basis of these considerations, the possible structures postulated for F2 and F3a are summarized in Table 2.

The novel component F3b, whose presence was detected by mass spectrometry, has a molecular mass of 624. No structural characterization has been performed yet because of its very small amount.

4. Conclusions

The results obtained in the present investigation demonstrate very clearly the potential of on-line HPLC–ISI-MS for the identification and characterization of flavonoids in medicinal plant extracts or, more generally, of polar compounds present in vegetal extracts. The structure of all flavonoids previously identified has been confirmed by HPLC–ISI-MS and HPLC–ISI-MS–MS analysis.

On-line analysis permitted the identification of a new component, F3b, whose molecular mass is 624, which was not previously detected by simple HPLC. This finding points out, once again, the great utility of a 'third dimension tool' for the detection of co-eluting components.

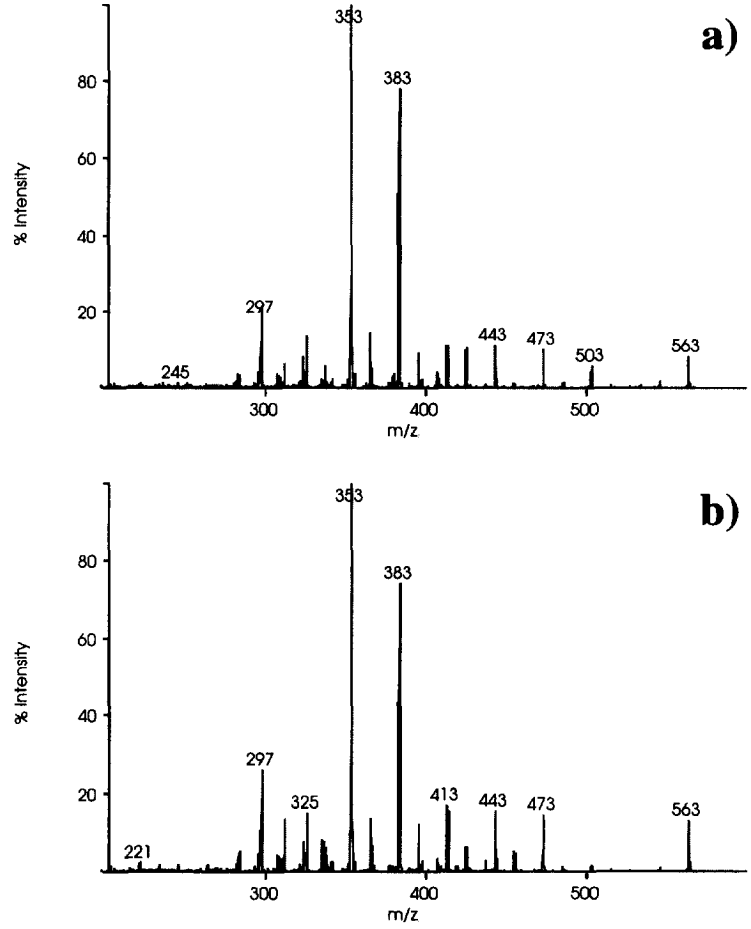


Fig. 7. $[M-H]^-$ product ion (parent ion: m/z 563) spectra at 30 eV of (a) isoschaftoside and (b) F3a.

The $[M-H]^-$ product ion analysis of the isolated F2 and F3a components has been performed and shows that they correspond to structural isomers of schaftoside and isoschaftoside, respectively. On the basis of their product ion spectra four possible structures have been postulated for F2 and F3a. Further work is now in progress to collect more of the isolated components so that nuclear magnetic resonance (NMR) and possibly X-ray analysis of these novel flavonoids can be performed so as to definitively determine their structures. It is noteworthy, however, that we could obtain significant information without any need to separate the different components, simply by a direct analysis of the complex extract's mixture. The separation of components is necessary to provide a detailed structure elucidation and, of course, for activity tests of the single flavonoids.

Acknowledgments

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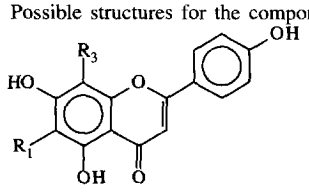
Table 2
Possible structures for the components F2 and F3a



Component	Possible structure	R_1	R_3
F2	Vicenin-3	Glc	Xyl
F2	Neoschaftoside	Glc	β-Ara
F2	Isocorimboside	Gal	α-Ara
F2	Neoisocorimboside	Gal	β-Ara
F3a	Vicenin-1	Xyl	Glc
F3a	Neoisoschaftoside	β-Ara	Glc
F3a	Corimboside	α-Ara	Gal
F3a	Neocorimboside	β-Ara	Gal

Glc: β-D-glucopyranosyl; gal: β-D-galactopyranosyl; α-ara: α-L-arabinopyranosyl; β-ara: β-L-arabinopyranosyl; xyl: β-xylopyranosyl.

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