Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002.pca.740

Identification and Quantification of Components in Extracts of *Uncaria tomentosa* by HPLC-ES/MS → testing method owned by

P. Montoro,[1]* V. Carbone,[2] J. de Dioz Zuniga Quiroz,[3] F. De Simone[1] and C. Pizza[1]

[1]Dipartimento di Scienze Farmaceutiche, Facoltá di Farmacia, Universitá di Salerno, Via Ponte don Melillo, 84084 Fisciano, Italy
[2]Centro di Spettrometria di Massa Proteomica e Biomolecolare, Istituto di Scienze dell'Alimentazione-C.N.R., Via Roma 52a-c, 83100 Avellino, Italy
[3]Parque Caceres, 86, CP Libre, Lima, Peru

The two main classes of secondary metabolites, alkaloids and quinovic acid glycosides, of *Uncaria tomentosa* (Willd.) DC. (Rubiaceae), a Peruvian plant commonly known as 'uña de gato', have been analysed. Separation of the alkaloidal fraction was achieved using a solid phase extraction method based on cationic exchange, and an analytical method employing HPLC-ES/MS has been developed. Quantitative data for commercial wild bark, cultivated bark and leaves are reported. The analysis of quinovic acid glycosides was performed directly on the crude extract using both a fast analytical method based on flow injection ES/MS, and a more complete analytical technique using HPLC-MS. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: HPLC-MS; ES-MS; flow injection assay; indole alkaloids; quinovic acid glycosides; *Uncaria tomentosa*.

INTRODUCTION

The bark of *Uncaria tomentosa* (Willd.) DC. (Rubiaceae), a Peruvian plant commonly known as 'uña de gato', is widely used in local medicine (Wagner *et al.*, 1984; Keplinger *et al.*, 1999) for the treatment of cancer (Rizzi *et al.*, 1993), arthritis, gastritis and some epidemic diseases (Keplinger *et al.*, 1999). Phytochemical studies led to the isolation of 17 alkaloids (Seki *et al.*, 1993; Laus *et al.*, 1997) which exhibited an immuno-stimulant activity (Lamaire *et al.*, 1999), and several quinovic acid glycosides showing anti-inflammatory activity, which co-occurred with their respective aglycones (Aquino *et al.*, 1990, 1991).

The analysis of the alkaloids of *U. tomentosa* by HPLC and capillary electrophoresis has been reported in a number of papers (Stuppner and Sturm, 1992; Stuppner *et al.*, 1992; Ganzera *et al.*, 2001), but the only study involving the HPLC-MS analysis of these alkaloids did not lead to an identification of the constituents (Lopez Avila and Benedicto, 1992). The alkaloids of this plant have a spiro oxindole structure (Fig. 1) which can undergo isomerisation by a retro-type Mannich reaction such that the composition of the equilibrium mixture depends on whether an acidic or a basic solvent is employed (Laus and Keplinger, 1994; Laus *et al.*, 1996). In order to minimise problems of isomerisation during the isolation of alkaloids in the present study, extraction with an accelerated solvent extractor and purification using an ion-exchange cartridge were performed. Analysis of the alkaloid fraction was initially carried out by HPLC with photodiode array detection (PAD) followed by HPLC coupled with electrospray ionisation (ES)/MS operated in the positive ion mode: HPLC-MS has previously been proposed as a technique of choice for the analysis of these alkaloids (Verpoorte and Niessen, 1994).

In addition to alkaloids, a number of studies have reported the presence of quinovic acid glycosides and polyhydroxylated triterpenes in *U. tomentosa*, and these latter compounds have a primary role in the activity of the plant, and of its extracts, in the inflammatory process (Aquino *et al.*, 1991). In the present paper, crude extracts of the plant were analysed using ES/MS and ES/MS/MS with acquisition in the negative ion mode in order to characterise the quinovic acid glycosides. Subsequently, an analytical method was developed using HPLC-ES/MS in order to obtain qualitative and quantitative information about these metabolites.

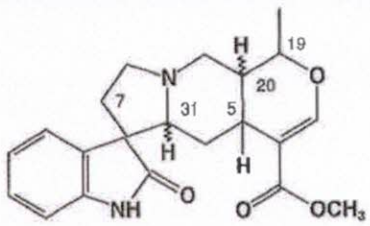
Owing to the various problems involved in collecting material from *U. tomentosa* growing wild in the rain forest, cultivation of this plant is of particular ecological interest. For this reason it is important that the content of secondary metabolites in both wild and cultivated plants be evaluated. Furthermore, it would also be of value to establish if the bioactive metabolites present in the bark of the plant are also present in the leaves. If this were to be the case, appropriate material could be collected for medicinal purposes without significantly damaging the plant.

In the present work, crude extracts of bark and leaf material of wild and cultivated examples of *U. tomentosa* were investigated by HPLC-ES/MS in order to develop and optimise simple and rapid techniques to determine both the alkaloids and the quinovic acid glycosides

* Correspondence to: P. Montoro, Dipartimento di Scienze Farmaceutiche, Facoltá di Farmacia, Universitá di Salerno, Via Ponte don Melillo, 84084 Fisciano, Italy.
Email: pmontoro@unisa.it

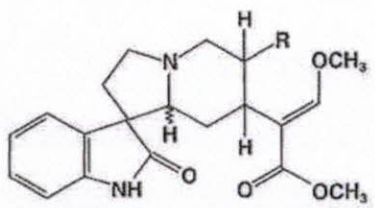
Received 23 August 2002

Alkaloids – pentacyclic (molecular weight 368)



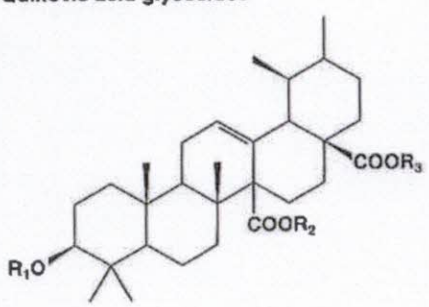
(1)	Pteropodine	3*S*, 7*R*, 15*S*, 19*S*, 20*S*
(2)	Isopteropodine	3*S*, 7*S*, 15*S*, 19*S*, 20*S*
(3)	Speciophylline	3*R*, 7*S*, 15*S*, 19*S*, 20*S*
(4)	Uncarine F	3*R*, 7*R*, 15*S*, 19*S*, 20*S*
(5)	Mytraphylline	3*R*, 7*R*, 15*S*, 19*S*, 20*R*
(6)	Isomytraphylline	3*S*, 7*S*, 15*S*, 19*S*, 20*R*

Alkaloids – tetracyclic (molecular weight 384)



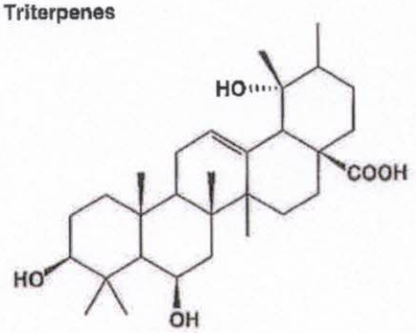
(7)	Ryncophylline	3*S*, 7*R*, 15*S*, 20*R*	R = ethyl
(8)	Isoryncophylline	3*S*, 7*S*, 15*S*, 20*R*	R = ethyl
(9)	Corynoxeine	3*S*, 7*R*, 15*S*, 20*R*	R = vinyl
(10)	Isocorynoxeine	3*S*, 7*S*, 15*S*, 20*R*	R = vinyl

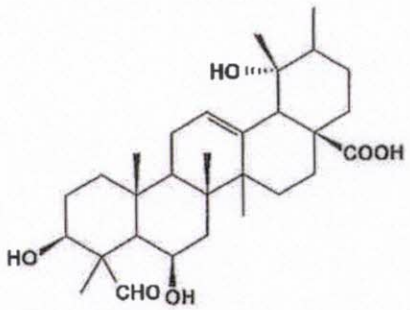
Quinovic acid glycosides



	R_1	R_2	R_3
(11)	Glu-Fuc	Glu	H
(12)	Glu-Fuc	H	Glu
(13)	Fuc	H	Glu
(14)	Rha	H	Glu
(15)	Glu-Glu	H	H
(16)	H	H	Glu-Glu

Triterpenes





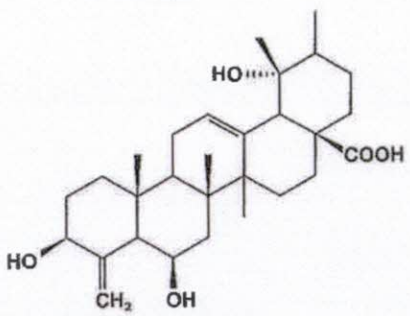


Figure 1. Structures of the major alkaloids, quinovic acid glycosides and triterpenes reported in *Uncaria tomentosa*.

present therein for the purposes of quality control of collected material.

EXPERIMENTAL

Reagents and standards. Ammonium acetate, ammonium hydroxide, hydrochloric acid, sulphuric acid, ethyl acetate and ethanol were purchased from Carlo Erba (Milan, Italy). HPLC-grade methanol, acetonitrile and acetic acid were from J.T. Baker (Baker Mallinckrodt, Phillipsburg, NJ, USA). HPLC-grade water (18 mΩ) was prepared using a Millipore (Bedford, MA, USA) Milli-Q purification system. Standards of pure alkaloids were isolated by liquid–liquid extraction followed by HPLC separation and their structures were confirmed by NMR [Bruker (Spectroscopin, Fallanden, Switzerland) model DRX-600 spectrometer operating at 599.19 MHz with UXNMR software package: samples dissolved in deutero-methanol]. Standards of pure quinovic acid glycosides and triterpenes were available from our previous studies (Cerri *et al.*, 1988; Aquino *et al.*, 1989, 1990, 1991, 1997) and their structures were elucidated by NMR. Tryptophol, used as internal standard (IS), was purchased from Sigma (St. Louis, MO, USA). A standard stock solution of isopteropodine (1 mg/mL) was prepared by dissolving the compound in methanol, and four solutions containing, respectively, 0.5, 1.5, 3 and 5 μg/mL of the alkaloid (as external standard) and 4 μg/mL of tryptophol (as IS) were prepared in methanol for use in method development.

Plant material and preparation of samples. Powdered commercial extract of *Uncaria tomentosa* from wild bark was supplied by Quimica Suiza (Lima, Peru). Bark and leaf samples were collected from plants cultivated in Peru, and voucher samples are deposited at the Department of Pharmaceutical Science, University of Salerno, Italy (reference numbers 7–8). In order to isolate alkaloid standards on a preparative scale, powdered commercial extract of *U. tomentosa* (100 mg) was dissolved 0.1 M sulphuric acid (4 mL) and extracted with ethyl acetate (3 × 10 mL). The aqueous phase was rendered alkaline with ammonia and extracted with ethyl acetate (10 mL). The organic phase containing the alkaloids was evaporated to dryness.

For analytical purposes, cultivated bark and leaves (12.5 g) were extracted for 7 min with methanol (40 mL) using a Dionex Corporation (Sunnyvale, CA, USA) accelerated solvent extraction system operated at a pressure of 10 atm. An aliquot (500 mg) of each dried crude extract was dissolved in methanol (10 mL) and analysed directly by ES/MS and HPLC-ES/MS to determine quinovic acid. For the HPLC-MS analyses of the alkaloids, an aliquot (100 μL) of the methanolic solution of each extract was added to IS, acidified with 10 μL of hydrochloric acid and deposited on an Accell (Waters, Milford, MA, USA) cationic exchange solid phase extraction (SPE) cartridge which had been preconditioned with methanol and water. The column was eluted with ethanol:water (1:2) and the eluent evaporated under reduced pressure. When required, the sample was reconstituted with methanol (100 μL) and analysed by HPLC-MS.

ES/MS analysis. ES/MS analyses were performed using a Finnigan (Thermo Finnigan, San José, CA, USA) LCQ Deca ion trap instrument equipped with Xcalibur software. Samples of isolated compounds were dissolved in methanol to obtain 1 μg/mL solutions and infused into the ES ionisation source using a syringe pump at a flow rate of 5 μL/min. For the analysis of alkaloids, the instrument was operated in the positive ion mode with a capillary voltage of 5 V, a spray voltage of 5 kV, and a tube lens offset of –10 V. For the analysis of quinovic acid glycosides, the instrument was operated in the negative ion mode with a capillary voltage of 43 V, a spray voltage of 5 kV, and a tube lens offset of 30 V. In each case the capillary temperature was 220°C, and data were acquired in the MS1 and MS/MS scanning modes. Bark and leaf extracts were analysed by direct infusion at a flow rate of 10 μL/min. Samples were dissolved in methanol (1 mg/mL) and centrifuged before injection.

HPLC-PAD analysis. Initially, the extract was analysed using an Agilent (Palo Alto, CA, USA) 1100 series system consisting of a G-1312 binary pump, a G-1328A Rheodyne injector (20 μL loop), a G-1322A degasser and a G-1315A photodiode array detector. Alkaloids were analysed using a Merck (Darmstadt, Germany) Lichrosorb RP-C18 column (250 × 4 mm i.d.; particle size 5 μm) and a mobile phase of 30 mM ammonium acetate (pH 5) as eluent A and methanol:acetonitrile (1:1) as eluent B. Elution was performed by means of a linear gradient from 60:40 (A:B) to 30:70 over 30 min at a flow rate of 1 mL/min. For preparative analysis, a Waters μ-Bondapack semi-preparative column (300 × 7.8 mm i.d; particle size 5 μm) was employed with the same gradient program and a flow rate of 2 mL/min. In each case the column effluent was monitored at 245 nm. Quinovic acid glycosides were separated using a Waters Symmetry C_{18} column (150 × 2.1 mm i.d.; particle size 5 μm) and a mobile phase of 30 mM ammonium acetate (pH 5) as eluent A and methanol as eluent B. Elution was initially isocratic with 50:50 (A:B) for 5 min, followed by a linear gradient from 50:50 to 30:70 in 50 min, and from 30:70 to 5:95 in 15 min at a flow rate of 0.3 mL/min. The column effluent was monitored in the range 206–400 nm with the acquisition of full spectra.

HPLC-ES/MS analysis. Extracts were analysed by HPLC-ES/MS 'on-line' using a Thermo Finnigan Spectra System HPLC coupled to an LCQ Deca ion trap. The chromatographic and spectroscopic conditions used were as described above. For the analysis of alkaloids the flow was subject to a split of 1:9 before introduction into the ion source, whilst for the analysis of glycosides the flow was directly injected into the electrospray ion source. MS spectra were acquired and elaborated using the software provided by the manufacturer.

Calibration, quantification and statistical analysis. Calibration curves for each of the alkaloid standards were constructed over the concentration range of 0.5–5 μg/mL with four different concentration levels and triplicate injections at each level. Ratios of the peak areas of the isopteropodine standard (at each concentration) to those of tryptophol (as IS) were calculated and plotted against the corresponding standard concentration using weighted linear regression to generate standard curves.

RESULTS AND DISCUSSION

HPLC-ES/MS analyses of alkaloids

The major alkaloids of *Uncaria tomentosa* are represented by six stereoisomers of pentacyclic alkaloids, namely, pteropodine (**1**), isopteropodine (**2**), speciophylline (**3**), uncarine F (**4**), mytraphylline (**5**) and isomytraphylline (**6**), and two major tetracyclic alkaloids identified as ryncophylline (**7**) and isoryncophylline (**8**) which co-occurred with two minor tetracyclic alkaloids, corynoxeine (**9**) and isocorynoxeine (**10**) (Fig. 1). In order to optimise the extraction method and HPLC conditions for the determination of these alkaloids, a preliminary HPLC-UV analysis was carried out on a fraction obtained by liquid–liquid extraction of a commercial extract. The same alkaloid fraction was also used in a semi-preparative HPLC step to isolate pure standards of the alkaloids for later quantitative analyses: such compounds were identified by ^{1}H-NMR by comparison with data reported in literature (Toure *et al.*, 1992; Seki *et al.*, 1993).

A good separation of alkaloids was obtained using the gradient elution procedure reported in the Experimental section with solvents buffered at pH 5. Comparison of the chromatograms obtained using two different procedures for the isolation of the alkaloids, namely, liquid–liquid extraction and cationic exchange SPE, showed that the latter procedure was more selective and resulted in less extensive isomerisation of the spiro structures. Figure 2(a) shows the HPLC-ES/MS analysis of the alkaloids obtained by this extraction method. The relative ratios of alkaloids obtained in the present study were very similar to the results obtained using a supercritical fluid extraction (Lopez Avila and Benedicto, 1992); this was probably because of the low rate of isomerisation associated with the techniques employed in these studies.

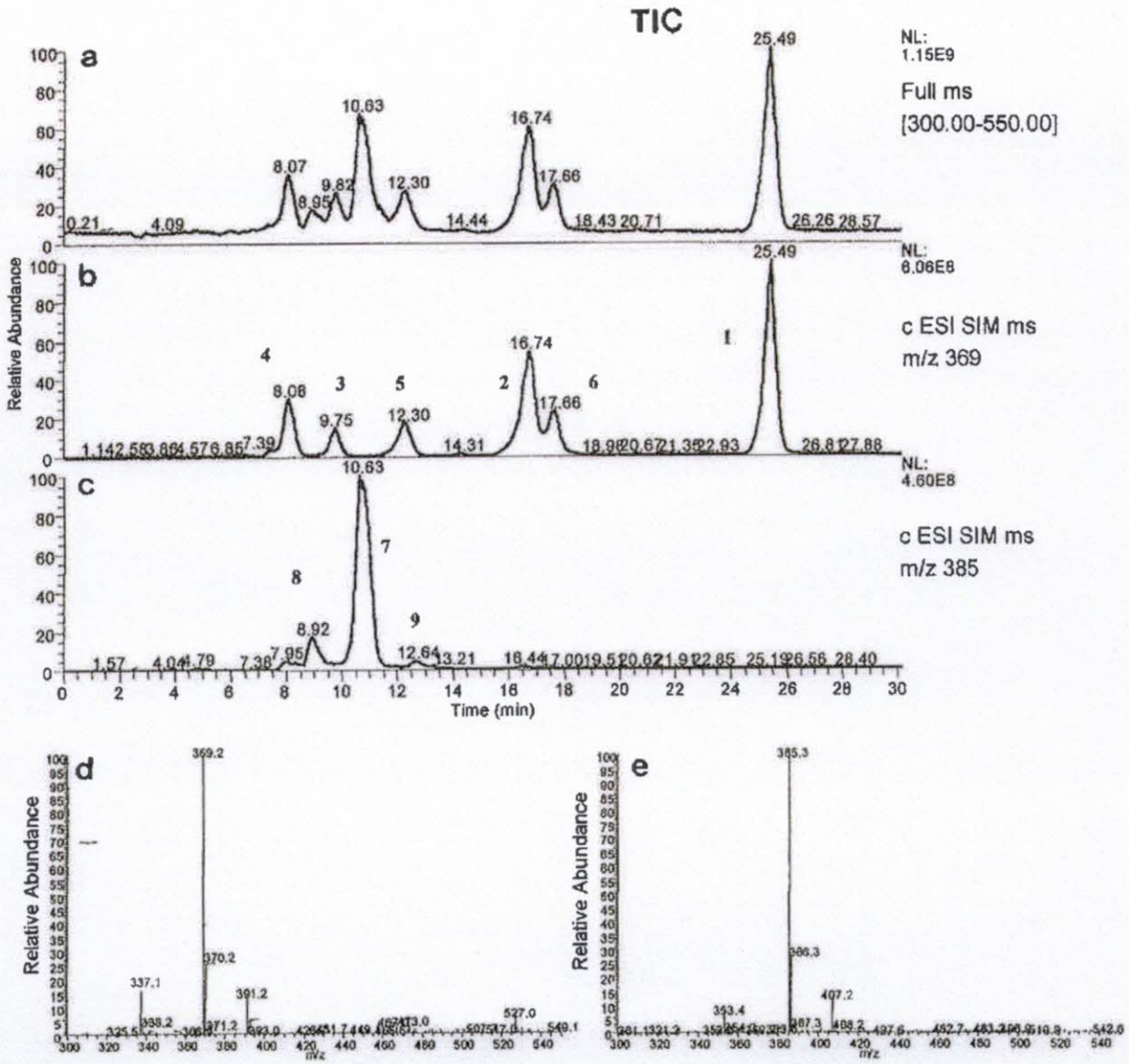


Figure 2. Typical HPLC-ES/MS chromatogram of the alkaloids in wild bark of *Uncaria tomentosa* showing: (a) the total ion current profile (*m/z* range 300–550); and (b) and (c) the HPLC-SIMM/MS at *m/z* values of 369 and 385, respectively. The ES/MS shown in (d) and (e) are, respectively, of a pentacyclic alkaloid and of a tetracyclic alkaloid. Key to peak identity: **1**, pteropodine; **2**, isopteropodine; **3**, speciophylline; **4**, uncarine F; **5**, mytraphylline; **6**, isomytraphylline; **7**, ryncophylline; **8**, isoryncophylline; and **9**, corynoxeine. (For chromatographic protocols see the Experimental section.)

The ES/MS analysis of the pentacyclic alkaloids gave a pseudomolecular ion $[M + H]^+$ at *m/z* 369, whilst the tetracyclic alkaloids showed a pseudomolecular ion at *m/z* 385. In order to obtain better sensitivity and resolution in the same experiment, acquisition was further performed using single ion monitoring (SIM). Typical HPLC-MS chromatograms of the alkaloids from *U. tomentosa* are shown in Fig. 2, in which the total ion current (TIC) profile for the *m/z* range 300–550, the HPLC-SIM/MS for *m/z* 369, and HPLC-SIM/MS for *m/z* 385 are depicted [Fig. 2(a)–(c), respectively]. Figure 2(d) and (e) shows the complete ES/MS of a pentacyclic alkaloid and of a tetracyclic alkaloid, respectively.

Whilst the sensitivity and the resolution of the method described enabled quantitative analysis of the alkaloids to be carried out, identification of the alkaloids could not be obtained from MS data alone since each compound produced the same spectrum with the same fragmentation pattern. For this reason the designation of each alkaloid was realised by means of HPLC retention times and ^{1}H-NMR data in comparison with literature data.

Quantitative analysis of alkaloids in wild bark and cultivated bark and leaves of *U. tomentosa*

The HPLC-ES/MS method described was selective for the alkaloids and the IS since no interfering compounds co-eluted at the elution time of the alkaloids. Quantitative information was obtained using external and internal standards. IS was introduced into both calibration standards and samples before the SPE extraction in order to improve the precision and accuracy for the quantitative analysis. The calibration graphs obtained by plotting the area ratio between external and internal standard vs. the known concentration of each compound were linear ($r^2 = 0.998$) in the range 0.5–5 µg/mL.

The method was employed to study the content of alkaloids in crude extracts of wild bark, cultivated bark and cultivated leaves (three aliquots of each) of *U. tomentosa*. The results shown in Table 1 indicate that pteropodine (**1**), isopteropodine (**2**) and isomytraphylline (**6**) were the most abundant alkaloids in the bark, whereas in the leaves the major compounds were mytraphylline (**5**) and isomytraphylline (**6**). Whilst the total content of alkaloids in each sample differed, the variations were not extreme. In the absence of reference compounds, the results for the tetracyclic compounds allowed a percentage value to be calculated only in terms of the total amount of the oxindole alkaloids.

ES/MS and ES/MS/MS analysis of quinovic acid glycosides

Since two main classes of metabolites are present in *U. tomentosa*, namely oxindole alkaloids and quinovic acid glycosides, a full characterisation of an extract necessitates the analysis of both types of constituents. Whilst quinovic acid glycosides typically show a very low absorbance in the UV range 206–210 nm, this wavelength range is not selective for such compounds and is, therefore, not suitable for the direct analysis of crude extracts by HPLC-UV. Moreover, sample preparation techniques are laborious and, unfortunately, there is no rapid method that can isolate all of the compounds representative of this fraction; for this reason, present studies were aimed towards the study of crude extracts. There was clearly a need for a rapid analytical method to act as a screening test of an extract before detailed HPLC-PAD or HPLC-MS analyses, and this guided us to develop a method to obtain a finger-print of the extract directly using flow injection analysis (FIA) together with ES/MS, a soft ionisation technique which produces mainly molecular ions and very few fragments and hence is highly suitable for the characterisation of complex mixtures such as plant extracts.

The ES/MS analytical conditions were optimised using standard quinovic acid glycosides previously isolated in our laboratory (Fig. 1). The best analytical selectivity for this class of metabolite was obtained by acquiring spectra in the negative ion mode. Plant extracts from bark and leaves were dissolved in methanol and centrifuged before being analysed by FIA at a flow rate of 10 µL/min. Figure 3 shows the ES/MS spectrum of an extract of wild bark of *U. tomentosa*. The most abundant

Table 1. Analysis of the alkaloidal content of bark and leaves of *Uncaria tomentosa*

Alkaloids	Wild bark (mg/g plant)[a, b]	Cultivated bark (mg/g plant)[a, c]	Cultivated leaves (mg/g plant)[a, d]
Pentacyclics			
(1) Pteropodine	9.542 ± 0.11	7.281 ± 0.17	0.791 ± 0.09
(2) Isopteropodine	8.572 ± 0.21	5.291 ± 0.31	1.044 ± 0.08
(3) Speciophylline	1.443 ± 0.15	0.946 ± 0.08	0.351 ± 0.04
(4) Uncarine F	2.889 ± 0.18	0.707 ± 0.03	2.845 ± 0.12
(5) Mytraphylline	0.902 ± 0.02	0.902 ± 0.11	3.372 ± 0.11
(6) Isomytraphylline	3.550 ± 0.11	5.621 ± 0.05	5.813 ± 0.08
Total pentacyclics	*26.895 ± 0.78*	*17.723 ± 0.75*	*14.25 ± 0.52*
Tetracyclics			
(7) Ryncophylline	1.936 ± 0.11	2.188 ± 0.21	2.925 ± 0.09
(8) Isoryncophylline	0.425 ± 0.14	0.213 ± 0.02	0.228 ± 0.01
(9) Corynoxeine	0.364 ± 0.04	0.456 ± 0.05	trace
(10) Isocorynoxeine	trace	trace	trace
Total tetracyclics	*2.753 ± 0.28*	*2.854 ± 0.28*	*3.213 ± 0.10*
Percentage tetracyclics	*10.2%*	*16.1%*	*22.5%*

[a] Mean values ± standard deviation ($n = 3$); referenced to dried plant material.
[b] Yield of extract 10.4%.
[c] Yield of extract 10.6%.
[d] Yield of extract 36.8%.

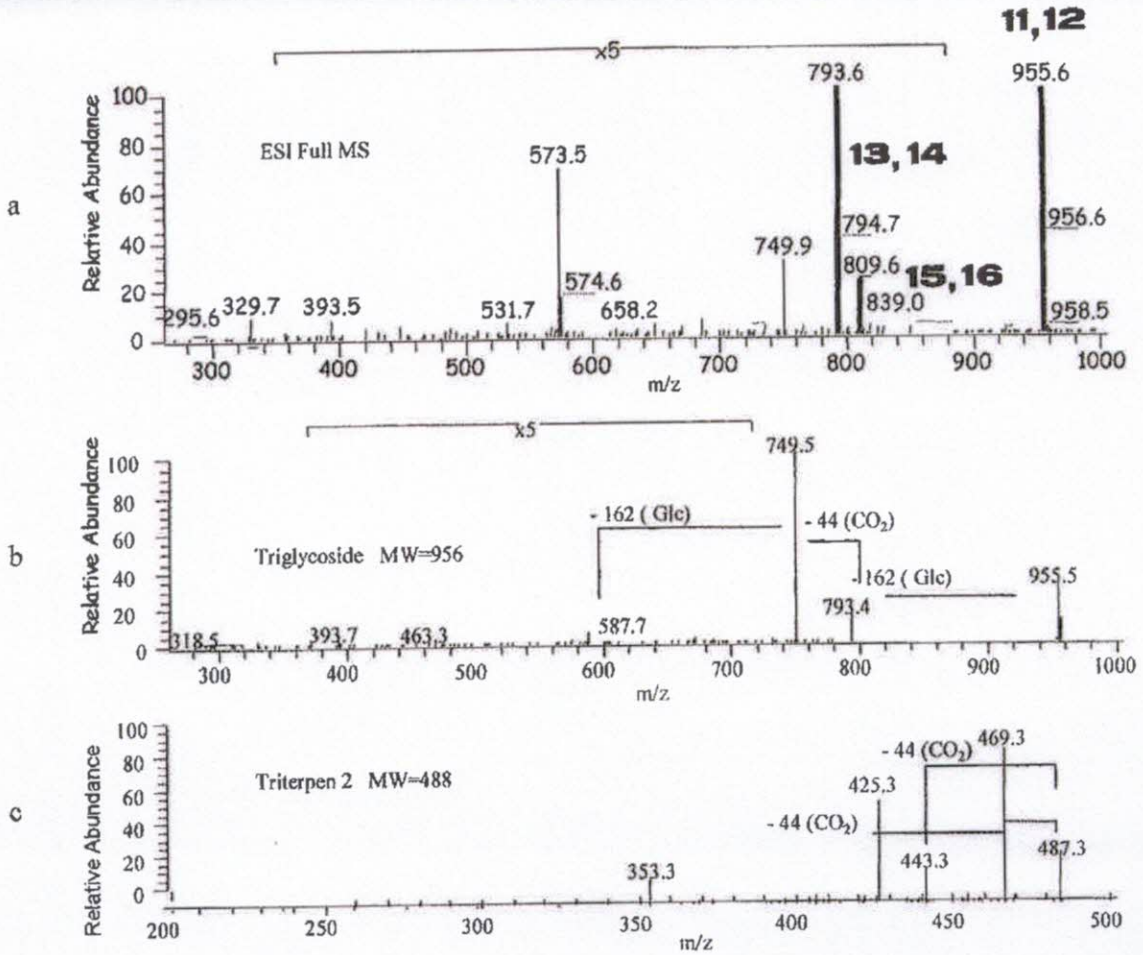


Figure 3. (a) ES/MS spectrum of an extract from the bark of a wild plant of *Uncaria tomentosa*; (b) ES/MS/MS analysis of the triglycosylated quinovic acid glycosides **11** and **12** with pseudomolecular ions at *m/z* 955; and (c) ES/MS/MS analysis of a triterpenoid compound with a pseudomolecular ion at *m/z* 487 (peak numbers correspond to the respective structures shown in Fig. 1).

ions are the pseudomolecular ions of quinovic acid glycosides and triterpenes. In particular, the ions at *m/z* 793.6, 809.6 and 955.6 are associated with three different glycoside derivatives of the same quinovic acid aglycone (see Fig. 1); the ion at *m/z* 793.6 is due to the addition of the pseudomolecular ions of isobaric compounds **13** and **14** (diglycosylated compounds), the ion at *m/z* 809.6 is due to the addition of the pseudomolecular ions of isobaric compounds **15** and **16** (also diglycosylated compounds), and the ion at *m/z* 955.6 is due to the addition of the pseudomolecular ions of isobaric compounds **11** and **12** (triglycosylated compounds).

Using an MS equipped with an ion trap analyser, it was possible to obtain information about the ion fragmentation pattern by measuring an MS/MS spectrum for each signal present in the full spectrum. Figure 3(b) shows the characteristic fragmentation pattern of the ion at *m/z* 955 (associated with triglycosylated compounds **11** and **12**) with the loss of two sugar units and the characteristic intervening loss of CO_2; Fig. 3(c) shows the fragmentation pattern of a triterpene compound, corresponding to quinovic acid, having a pseudomolecular ion at *m/z* 487.3 and a fragmentation pattern associated with loss of water and/or CO_2.

Employing this strategy it was possible to perform a qualitative comparison between the extracts of *U. tomentosa* and thus to obtain information about the presence of quinovic acid glycosides by considering the relative intensities of the corresponding signals present in the spectra. Table 2 reports the qualitative results obtained

Table 2. Analysis of the quinovic acid glycoside content of bark and leaves of *Uncaria tomentosa*

Sample	Glycosides 11, 12[a] (molecular weight 956)	Glycosides 13, 14[a] (molecular weight 794)	Glycosides 15, 16[a] (molecular weight 810)	Triterpene[a] (molecular weight 486)	Triterpene[a] (molecular weight 488)	Triterpene[a] (molecular weight 502)
Commercial wild bark	+++	++	+	+	+	+
Cultivated bark	+++	++	+	+/–	+/–	–
Cultivated leaves	++	+	–	+/–	+	+

[a] +++ indicates presence at high intensity; ++ indicates presence at moderate intensity; + indicates presence at low intensity; +/– indicates presence in trace amounts; – indicates none detected.

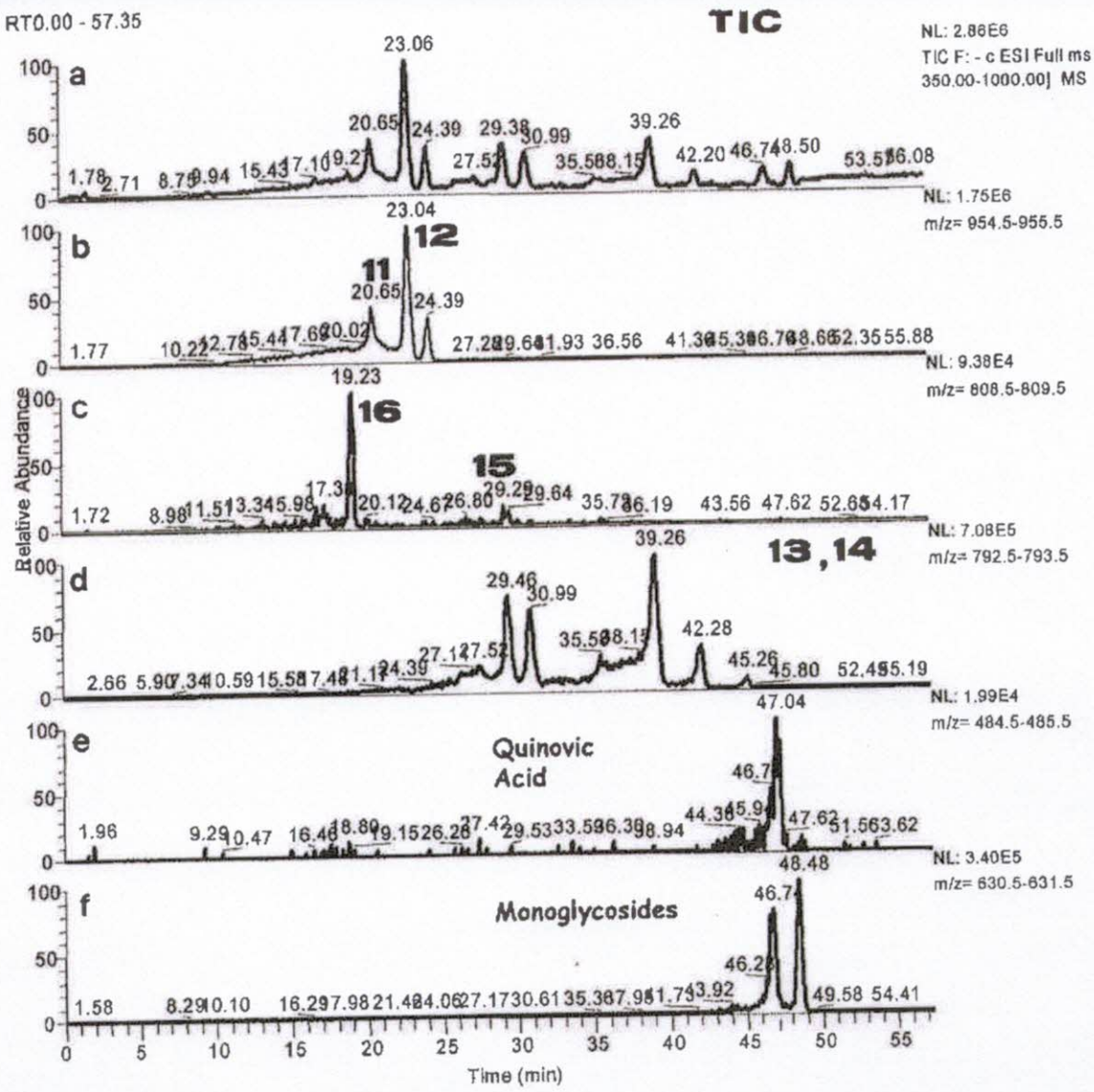


Figure 4. Typical HPLC-ES/MS chromatogram of the quinovic acid glycosides in a methanolic extract of *Uncaria tomentosa* showing: (a) the total ion current profile (*m/z* range 350–1000); and (b)–(f) the reconstructed ion chromatograms at *m/z* values of 955, 809, 793, 485 and 631, respectively (peak numbers correspond to the respective structures shown in Fig. 1: for chromatographic protocols see the Experimental section).

from a comparison of extracts from wild and cultivated bark and from cultivated leaves, and it can be seen that the bark from cultivated plants has a profile similar to that of wild bark, while the leaves contained proportionally less glycosides compared with the bark. However, the finding of quinovic acid glycosides, together with alkaloids, in the leaves is interesting with respect to the potential commercial use of the aerial parts of the plant.

HPLC-ES/MS of quinovic acid glycosides

Since the quantitative determination of quinovic acid glycosides using HPLC-PAD (wavelength range 206–400 nm) was not possible, we attempted to optimise the analytical conditions for the HPLC-MS analysis of these constituents in a crude extract. Operating in the negative ion mode, it was possible to obtain a selective ionisation of compounds structurally related to quinovic acid whilst completely avoiding interference from the co-occurring alkaloids.

Figure 4(a) shows the HPLC-MS analysis of a crude extract of wild bark of *U. tomentosa*. Interpretation of such a TIC chromatogram is typically achievable by studying the reconstructed ion chromatograms for each *m/z* value of interest [Fig. 4(b)–(f)], which provides information concerning the association of each peak in the TIC profile. The HPLC-MS method could then be used to compare the profiles of these compounds in different extracts. The results obtained confirmed that the profile of quinovic acid glycosides in extracts of bark from cultivated plants appeared to be very similar to that of bark from wild plants, while the profile obtained from leaves showed several differences and lower levels of constituents.

In the case of quinovic acid glycosides, some derivatives had the same molecular weight but a different structure, in particular with respect to the stereochemistry of one sugar unit. Nevertheless it was possible to distinguish these compounds with different substitutions by performing an HPLC-MS/MS product ion scan experiment. The MS was operated in the ES mode using the

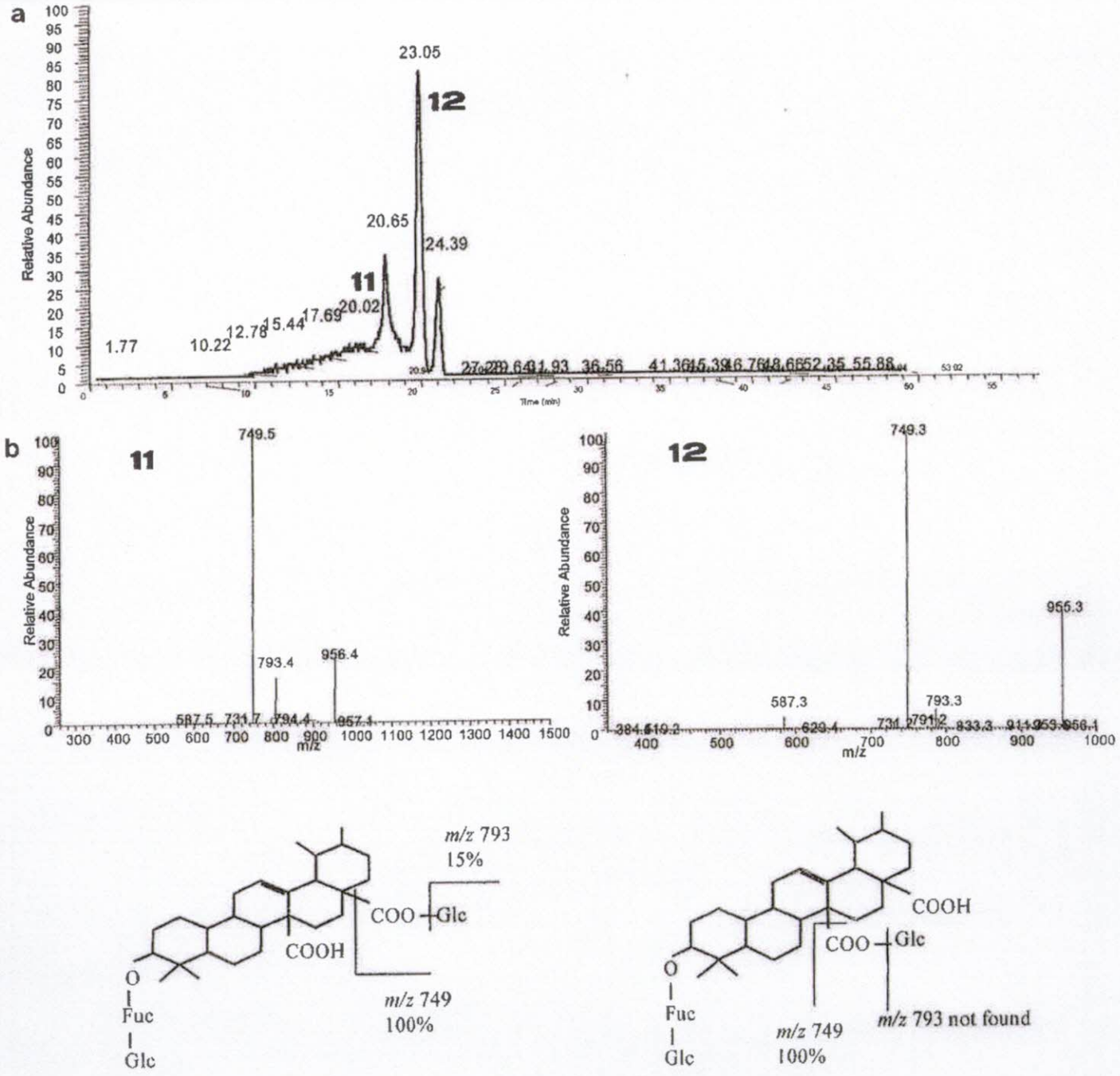


Figure 5. (a) Reconstructed ion chromatogram at an *m/z* value of 955 of an extract from the bark of a wild plant of *Uncaria tomentosa;* (b) ES/MS/MS analysis of the standard triglycosylated quinovic acid glycosides **11** and **12** (see Fig. 1) and their respective fragmentation patterns.

data-dependent scan function mode. One scan in the MS mode was made and the intensity of the base peak determined. When this intensity was lower than a preselected threshold, the instrument would acquire the next scan in the MS mode. However, if the base peak was above the preset threshold, then the instrument would acquire an MS/MS spectrum using the base peak in the previous MS scan as the precursor ion set mass. This process was repeated for all of the peaks in the chromatogram.

Two of the three different compounds appearing at *m/z* value of 955 [molecular weight 956: Figs 4(b) and 5(a)] were differentiated through their fragmentation patterns and were found to correspond to the triglycosylated compounds **11** and **12** which differed only in the carboxylic group supporting the glucose unit. The assignment was confirmed by analysis of standard pure compounds (Fig. 5). The similarity of the tandem MS of the third compound (at retention time 24.39 min) with those of **11** and **12** indicated that this was a triglycosylated compound, but the position of the three sugar moieties could not be determined.

Two different compounds appeared at *m/z* value of 809 [molecular weight 810; Figs 4(c) and 6(a)] and corresponded to the diglycosylated compounds **15** and **16**, which were characterised by a disaccharide unit (glc–glc) linked to an hydroxyl group in one compound, and to a carboxylic group in the other (Fig. 6). The fragmentation pattern observed in HPLC-MS/MS product ion scan permitted the discrimination of these compounds in that

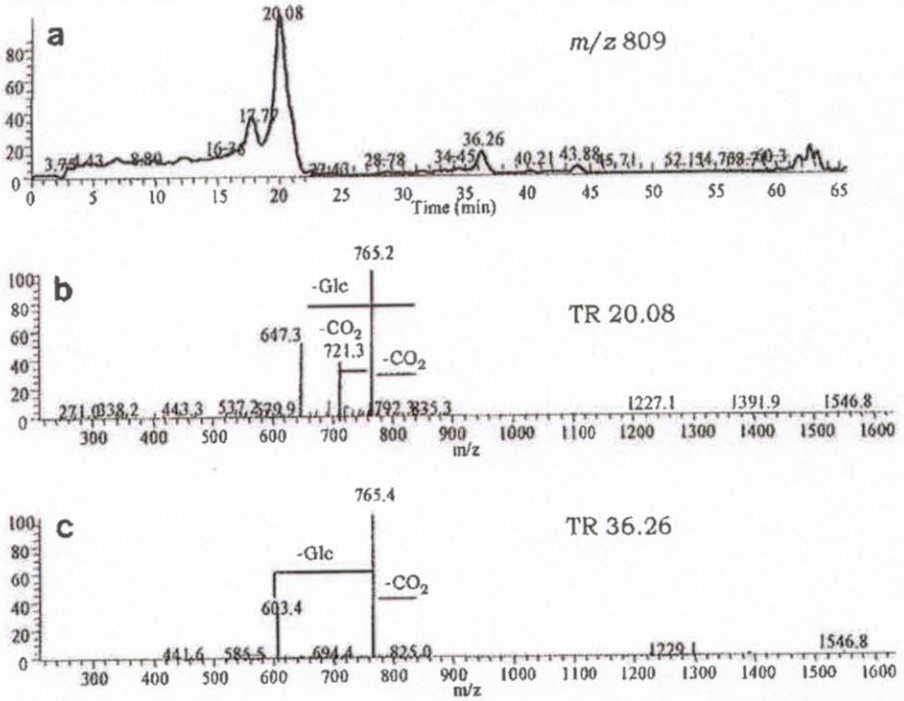


Figure 6. (a) Reconstructed ion chromatogram at an *m/z* value of 809 of an extract from the bark of a wild plant of *Uncaria tomentosa*; (b) and (c) ES/MS/MS analysis of the triglycosylated quinovic acid glycosides **16** and **15**, respectively (see Fig. 1), showing their fragmentation patterns.

the pattern for **16** differed from that for **15** by the absence of the second loss of CO_2, which was prevented by the substitution of a glycosidic chain on the carboxylic group.

CONCLUSION

It is concluded that HPLC-MS techniques are efficient in terms of sensitivity and specificity for the analyses of constituents in extracts of *U. tomentosa*, and provide two independent parameters, namely, retention time and MS information, for the identification of the compounds. Since both alkaloids and quinovic acid glycosides play important roles in the biological activities, as demonstrated for this plant both pharmacologically and clinically, a method for the determination of both classes of compounds is an appropriate method for the analysis of the extract and the products from this medicinal plant. A commercial product supposedly obtained from this plant must contain both classes of compounds. Moreover, because both bark and leaves of *U. tomentosa* grown in cultivation seem to contain the same metabolites as material from the wild plant, extracts from either root bark and aerial parts of cultivated plants could be used for similar applications. The proposed method of analysis could be of value for industrial quality control with respect to the quantification of marker compounds in raw materials and in final products.

REFERENCES

Aquino R, De Simone F, Pizza C, Conti C, Stein ML. 1989. Plant metabolites. Structure and *in vitro* activity of quinovic acid glycosides from *Uncaria tomentosa* and *Guettarda platypoda*. *J Nat Prod* **52**: 679–685.

Aquino R, De Simone F, Vincieri FF, Pizza C. 1990. New polyhydroxylated triterpenes from *Uncaria tomentosa*. *J Nat Prod* **53**: 559–564.

Aquino R, De Feo V, De Simone F, Pizza C, Cirino G. 1991. Plant metabolites. New compounds and antiinflammatory activity of *Uncaria tomentosa*. *J Nat Prod* **54**: 433–459.

Aquino R, De Tommasi N, De Simone F, Pizza C. 1997. Triterpenes and quinovic acid glycosides from *Uncaria tomentosa*. *Phytochemistry* **45**: 1035–1040.

Cerri R, Aquino R, De Simone F, Pizza C. 1988. New quinovic acid glycosides from *Uncaria tomentosa*. *J Nat Prod* **51**: 257–261.

Ganzera M, Muhammad I, Khan RA, Khan IA. 2001. Improved method for the determination of oxindole alkaloids in *Uncaria tomentosa* by high performance liquid chromatography. *Planta Med* **67**: 447–450.

Keplinger K, Laus G, Wurm M, Dierich MP, Teppner H. 1999. *Uncaria tomentosa* (Willd) DC.–ethnomedicinal use and new pharmacological, toxicological and botanical results. *J Ethnopharmac* **64**: 23–34.

Lamaire I, Assinewe V, Cono P, Awang DVC, Arnason JT. 1999. Stimulation of interleukin-1 and -6 production in alveolar macrophages by the neotropical liana, *Uncaria tomentosa* (Uña de gato). *J Ethnopharmac* **64**: 109–115.

Laus G, Keplinger D. 1994. Separation of stereoisomeric oxindole alkaloids from *Uncaria tomentosa* by high performance liquid chromatography. *J Chromatogr A* **662**: 243–249.

Laus G, Brössner D, Senn G, Wurst K. 1996. Analysis of the kinetics of isomerisation of spiro oxindole alkaloids. *J Chem Soc Perkin Trans* **2**: 1931–1936.

Laus G, Brössner D, Keplinger K. 1997. Analysis of the kinetics of isomerisation of spiro oxindole alkaloids. *Phytochemistry* **45**: 855–860.

Lopez Avila V, Benedicto J., 1992. Supercritical fluid extraction of oxindole alkaloids from *Uncaria tomentosa*. *J High Resol Chromatogr* **20**: 231–236.

Rizzi R, Re F, Bianchi A, De Feo V, De Simone F, Bianchi L, Stivala LA. 1993. Mutagenic and anti-mutagenic activities of *Uncaria tomentosa* and its extracts. *J Ethnopharmac* **38**: 63–77.

Seki H, Takayama H, Aimi N Sakai S, Ponglux D. 1993. A nuclear magnetic resonance study on the eleven stereoisomers of heteroyohimbine-type oxindole alkaloids. *Chem Pharm Bull* **41**: 2077–2086.

Stuppner H, Sturm S. 1992. Capillary electrophoretic analysis of oxindole alkaloids from *Uncaria tomentosa*. *J Chromatogr* **609**: 375–380.

Stuppner H, Sturm S, Konwalinka G. 1992. HPLC analysis of the main oxindole alkaloids from *Uncaria tomentosa*. *Chromatographia* **34**: 597–600.

Toure H, Babadjamian A, Balansard G, Faure R, Houghton PJ. 1992. Complete ^{1}H- and ^{13}C-NMR chemical shift assignments for some pentacyclic oxindole alkaloids. *Spectrosc Lett* **25**: 293–300.

Verpoorte R, Niessen WMA. 1994. Liquid chromatography coupled with mass spectrometry in the analysis of alkaloids. *Phytochem Anal* **5**: 217–232.

Wagner H, Kreutzkamp B, Jurcic K. 1984. The alkaloids of *Uncaria tomentosa* and their phagocytosis-stimulating action. *Planta Med* **50**: 419–423.