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Determination of saponins in crude plant extracts by liquid chromatography–thermospray mass spectrometry

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ABSTRACT

The determination of saponins in crude plant extracts by liquid chromatography–thermospray mass spectrometry is described. The method is shown to be suitable for the separation, identification and determination of saponins carrying up to three sugars. The mass spectra recorded on-line provide information on the molecular mass, the nature of the sugars and their sequence. Selected applications for saponins from the molluscicidal plant *Tetrapleura tetraptera* are described.

INTRODUCTION

The inability of high-performance liquid chromatography (HPLC) to detect substances that lack a good chromophore poses several problems to phytochemists for the identification of these compounds in crude plant extracts. This is particularly true with saponins, which generally occur together with phenolic glycosides such as flavonoids and xanthones and/or pigments in plant extracts of high polarity.

Saponins are glycosides that commonly occur in higher plants. They are biosynthesized by more than 500 species belonging to almost 80 different families [1]. This class of natural compounds is also found in marine organisms [2]. They are classified into two groups according to the structure of their aglycone moiety (sapogenin): the triterpene group, in which the aglycone is usually an oleanane, ursane or dammarane skeleton, and the steroid group. The latter also includes the steroid alkaloids. The most common sugars encountered in saponins are hexoses (glucose, galactose, mannose), 6-deoxyhexoses (rhamnose), pentoses (arabinose, xylose), uronic acids (glucuronic acid, galacturonic acid) or amino sugars (glucosamine, galactosamine). Sugars may be linked to the sapogenin at one or two glycosylation sites (through an ether or/and an ester linkage), giving the corresponding monodesmosidic or bidesmosidic saponins, respectively.

Analyses of saponins are often performed by HPLC using an ultraviolet (UV) detector at short wavelength. Separations are achieved on reversed-phase columns with detection at 206 nm as there is only poor absorption by saponins at higher wavelengths [3]. Consequently, there are limitations concerning the solvents and gradients that can be used. When a gradient solvent system is used, refractive index measurement is not practicable. Thus, an alternative is to derivatize the saponins, in order to attach a chromophore that facilitates UV detection at higher wavelength (254 nm). Encouraging results have been obtained by derivatization of the saponins with 4-bromophenacyl bromide in the presence of a crown ether. This method has previously been employed for the analysis of fatty acids and prostaglandins. Details of the procedure have been published [4].

However, with all these methods, the identities of peaks can be confirmed only by their

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retention times and comparison with authentic samples. Application of mass spectrometry (MS) as a tool for identification of the peaks is therefore a useful alternative.

The combination of HPLC and MS has been attempted since the early 1980s and systems with several types of interface for direct and indirect introduction of the column effluent have been described [5].

Different systems capable of coupling micro HPLC directly with fast atom bombardment (FAB) have been developed [6–8]. These techniques, which, depending on the interface, are termed Frit FAB and continuous flow (CF)-FAB types, have been successfully applied to the separation, identification and determination of saponins [9–12]. Using these methods, the HPLC elution profiles of saponins were well traced by total ion current (TIC) and MS, and the positive- and negative-ion FAB mass spectra allowed the identification of each peak. However, operation with this kind of interface is troublesome, and flow-rates of the LC effluent have to be around 5 μl/min in the ion source of the mass spectrometer. This requires the splitting of the effluent after the column separation. Further, there are problems such as poor peak sharpness, peak tailing and lack of sensitivity.

For the problem under investigation, one of the main disadvantages of CF-FAB and Frit-FAB was the very low flow-rates. We tried to operate with an interface capable of introducing aqueous phase into the mass spectrometer at a flow-rate that is compatible with that usually used in phytochemical analysis. The thermospray (TSP) interface was chosen for this purpose, owing to its simplicity, its ability to handle a variety of difficult samples and its operating flow-rate of about 1–2 ml/min.

Thermospray interface (TSP)

Since its invention by Blackley and Vestal [13], the TSP interface has proved to be ideal for on-line LC–MS. Its mechanically simple inmentation means that it is now widely used for the routine application of LC–TSP-MS to the direct identification of organic compounds in complex mixtures. This interface is now available for nearly all mass spectrometers on the market [14].

In the TSP interface, the analyte is ionized either by the use of additives (volatile salt solutions) or by the use of electron beams or electrical discharge. The ions pass through an orifice in a cone and are mass analysed. For saponins, which are very polar compounds, their analysis needs a postcolumn addition of a 0.5 *M* solution of ammonium acetate, giving a final concentration of *ca.* 80 m*M* after dilution with the column effluent, in order to provide the volatile buffer for ion evaporation ionization. Hence, the mass spectra recorded by this method look like chemical ionization (CI) mass spectra.

EXPERIMENTAL

Plant material

Fruits of *Tetrapleura teraptera* Taub. (Leguminosae) were collected near Ile-Ife, Nigeria. A voucher specimen is retained at the University of Ife. The dried powdered pulp of the fruits was extracted with methanol. The crude methanolic extract (30 mg/ml) was analysed on a Waters μ-Bondapak C_{18} column (10 mm, 300 × 3.9 mm I.D.), with a 30–80% gradient of aqueous acetonitrile within 30 min. The flow-rate was maintained at 1 ml/min.

Saponins **G** and **H** were previously isolated in our laboratory from the leaves of *Swartzia simplex* Spreng. collected in Panama (for isolation and purification procedures see the original reference [15]). Samples were injected directly into the loop (flow-injection mode).

Chemicals

HPLC-grade water was prepared by distillation on a Büchi (Flawil, Switzerland) Fontvapor 210 distillation instrument and passed through a 0.50-μm filter (Millipore, Bedford, MA, USA). HPLC-grade acetonitrile from Mächler (Reinach, Basle, Switzerland) was passed through a 0.45-μm Millipore filter. Ammonium acetate was obtained from Merck (Darmstadt, Germany).

LC–TSP-MS analysis

The LC–TSP-MS system used included a Waters 600 MS multi-solvent delivery LC system

and a Finnigan-MAT (San Jose, CA, USA) TSQ-700 triple quadrupole mass spectrometer equipped with a Finnigan-MAT TSP 2 interface. The electron multiplier voltage was 1800 V and the dynode voltage was kept at 15 kV, filament and discharge off. The repeller potential was optimized between 80 and 100 V, and the source temperature was kept at 270°C. The best results were recorded with temperatures of 100°C for the vaporizer and 310°C for the aerosol. Full-scan spectra from m/z 400 to 1000 (scan time 1.2 s) were obtained. Postcolumn addition of 0.2 ml/min of a 0.5 *M* solution of ammonium acetate was used to provide the volatile buffer for ion evaporation ionization. The TSP mass spectra were recorded by injecting 10 μl of a 1 mg/ml solution of crude extracts or 2 μg of pure compounds.

RESULTS AND DISCUSSION

LC–TSP-MS has been applied to the analysis of the molluscicidal saponins from the methanolic extract of fruits of *Tetrapleura tetraptera* (Leguminosae–Mimosoideae). This West African tree, locally known as Aridan, is mainly used by traditional healers in the management of convulsions, inflammation and rheumatic pains [16]. Previous reports have dealt with the strong molluscicidal activity of this plant [17,18] and *T. tetraptera* is now considered to be one of the most promising plants in the local control of the parasitic disease schistosomiasis in Africa [19]. We recently reported the isolation and identification of the five principal molluscicidal constituents of Aridan, which proved to be saponins. These compounds (**A–E**) are among the most powerful natural molluscicides and have very similar potencies to those of synthetic compounds [20,21].

Reversed-phase HPLC analysis with an acetonitrile–water gradient of the crude methanolic extract of the fruits of *T. tetraptera* showed a series of compounds whose UV spectra recorded with a diode-array detector indicated an absorption maximum at 200 nm. The same extract was analysed by LC–TSP-MS using a μBondapak C_{18} column for separation. Mass spectral detection was operated in the scan mode (m/z 450–1000). The LC–MS total ion current corresponded well with the UV trace at 206 nm. Six major peaks were visible, corresponding to the five known compounds (**A–E**) and to a sixth saponin (**F**), not yet identified. Plots of selected ion traces (*e.g.*, the $[M+H]^+$ ion) allow the location of each compound in the chromatogram (Fig. 1).

The TSP mass spectra acquired for saponins **A**, **B**, **D** and **E** in the extract displayed strong $[M+H]^+$ quasi-molecular peaks, together with adduct species such as $[M+CH_3CN+NH_4]^+$, confirming the molecular mass. In addition, an important $[A+H]^+$ peak for the aglycone was visible in these spectra (see also Table I for other fragments of importance).

Optimization of the experimental parameters (temperatures of the TSP vaporizer and ion source block) permitted "soft" ionization under sufficiently mild conditions to provide information on fragmentation of the osidic chain of the saponin. Thus, it was possible to observe peaks corresponding to the loss of one (mono- and diglycosides) and then two sugars (diglycosides) in all spectra. Additional losses of 18 and 42 u fragments accounted for the elimination of water (H_2O) and the acetamide moiety ($COCH_2$) from the inner sugar (Figs. 2–5 and Table I).

The mass spectrum obtained for saponin **C** showed a different pattern. Small quasi-molecular peaks at m/z 855 ($[M+CH_3CN+NH_4]^+$), 814 ($[M+NH_4]^+$) and 797 ($[M+H]^+$) confirmed the molecular mass. The base peak in this spectrum appeared at m/z 784 $\{[(M+NH_4)-30]^+\}$, resulting from the rapid elimination of a CHOH fragment. This phenomenon was also observed in the desorption chemical ionization (D/CI) mass spectrum of this saponin [21] and in those of other saponins containing a 14α-CH_2OH moiety [22]. A small peak resulting from the loss of one sugar was present at m/z 622 ($[784-\text{hexose}]^+$). In addition, important aglycone peaks at m/z 473 ($[A+H]^+$) and 490 ($[A+NH_4]^+$) were visible in this spectrum (Fig. 6).

Examination of the spectrum of the unknown compound **F** (Fig. 7) gave some information about its structure. In the first instance, the molecular mass was established as 837 {quasi-

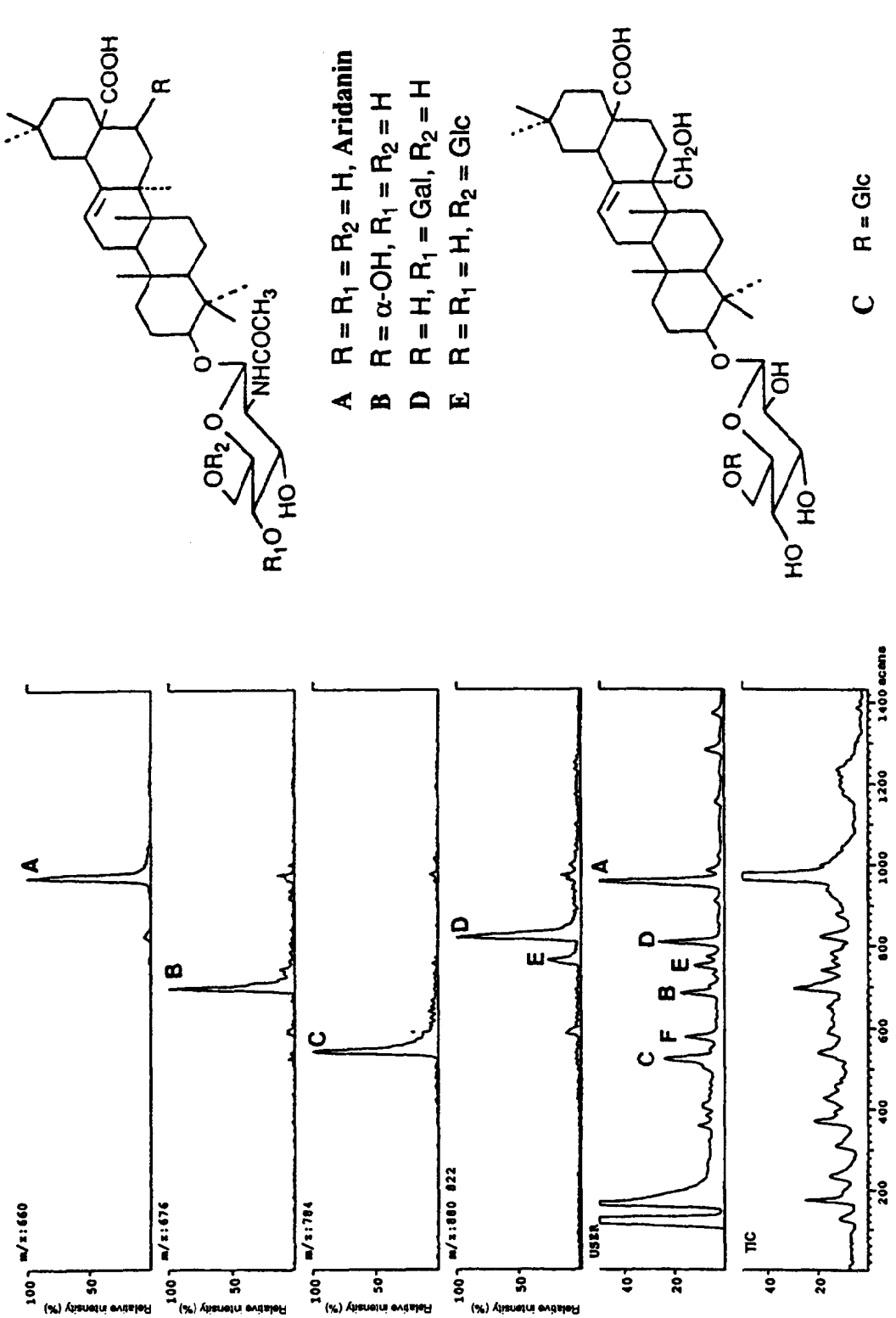

Fig. 1. LC–TSP-MS of the methanolic extract of the fruits of *T. tetraptera* and selected-ion current profiles of five known saponins. HPLC conditions: column, μBondapak C_{18}; eluent, CH_3CN–H_2O, 30:70→80:20 over 30 min flow-rate; 1 ml/min. TSP conditions: vaporizer temperature, 100°C; source temperature, 270°C; buffer, 0.5 *M* ammonium acetate; flow-rate, 0.2 ml/min; 1.5 s per scan; detection, UV at 206 nm.

TABLE I

MAJOR IONS OBSERVED IN THE TSP MASS SPECTRA (*m/z*) TOGETHER WITH THEIR RELATIVE INTENSITIES (%)

Ion[a]	Saponin					
	A	**B**	**D**	**E**	**F**	**C**
$[A + H]^+$	457 (46)	473 (95)	457 (78)	457 (100)	473 (66)	473 (49)
$[A + NH_4]^+$	474 (22)	490 (57)	474 (39)	474 (46)	490 (47)	490 (39)
$[(M + H) - hex - 42]^+$	–	–	618 (23)	618 (16)	–	–
$[(M + H) - hex - H_2O]^+$	–	–	642 (36)	642 (36)	–	–
$[(M + H) - hex]^+$	–	–	660 (76)	660 (17)	676 (39)	–
$[(M + CH_3CN + NH_4) - hex]^+$	–	–	718 (42)	718 (15)	734 (35)	–
$[(M + H) - 42]^+$	618 (22)	634 (19)	780 (23)	780 (15)	–	–
$[(M + H) - H_2O]^+$	642 (38)	658 (38)	–	–	–	–
$[M + H]^+$	660 (100)	676 (95)	822 (42)	822 (25)	838 (37)	797 (3)
$[(M + NH_4) - CHOH - hex]^+$	–	–	–	–	–	622 (8)-
$[(M + NH_4) - CHOH]^+$	–	–	–	–	–	784 (100)
$[M + NH_4]^+$	–	–	–	–	–	814 (17)
$[M + CH_3CN + NH_4]^+$	718 (85)	734 (80)	880 (100)	880 (42)	896 (55)	855 (4)

[a] hex = Hexose.

molecular peaks at *m/z* 838 ($[M + H]^+$) and 896 ($[M + CH_3CN + NH_4]^+$)}. In a second step, elimination of a terminal hexosyl moiety was observed, generating peaks at *m/z* 676 {$[(M + H) - hexose]^+$} and 734 {$[(M + CH_3CN + NH_4) - hexose]^+$}, followed by the loss of an N-acetylglucosyl unit. Hence this compound should be a glycoside of saponin **B**. The base peak in this spectrum was recorded at *m/z* 784. It could represent a residual presence of saponin **C** in this spectrum. Isolation of compound **F** is in progress.

In the first example described in this paper, LC–TSP-MS of mono- and diglycosides is

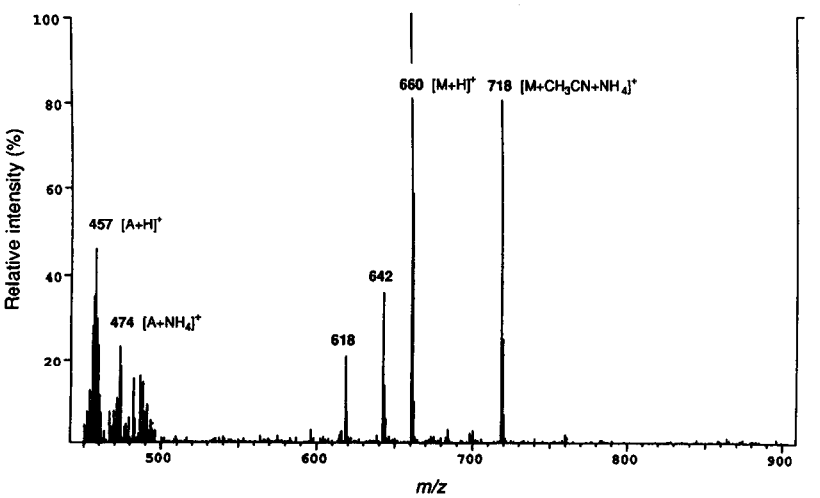

Fig. 2. LC–TSP-MS of aridanin (**A**) obtained after on-column analysis of the methanolic extract of *T. tetraptera*. For conditions, see Fig. 1.

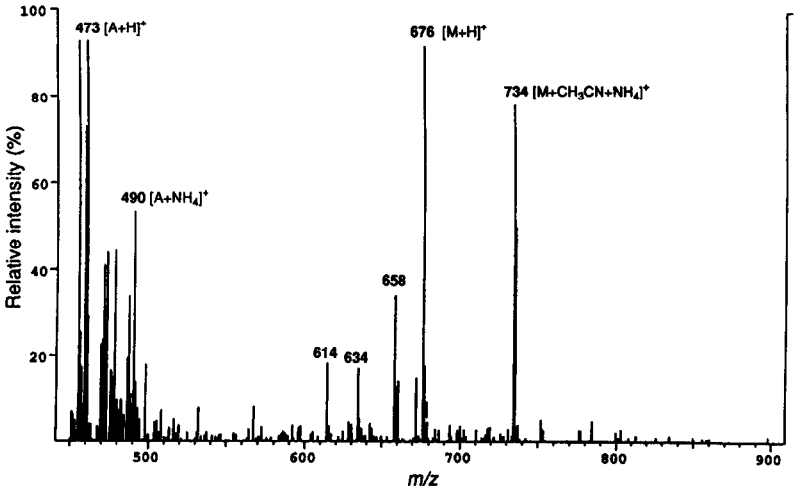

Fig. 3. LC–TSP-MS of saponin (**B**) obtained after on-column analysis of the methanolic extract of *T. tetraptera*. For conditions, see Fig. 1.

shown. However, after optimization of the experimental parameters, it was also possible to analyse triglycosides. The mass spectrum recorded on-line provided information on the molecular mass, the nature of the sugars and their sequence.

Two saponins (**G** and **H**), previously isolated from a methanolic extract of the leaves of *Swartzia simplex* [15] (Leguminosae–Caesalpinaceae), were used to demonstrate the ability of TSP to perform such analyses. With post-column addition of ammonium acetate to provide the volatile buffer for ion evaporation ionization, the TSP mass spectra acquired for both saponins showed $[M + NH_4]^+$ quasi-molecular peaks. Subsequent losses of sugars were observed.

For saponin **G**, simultaneous cleavage of a

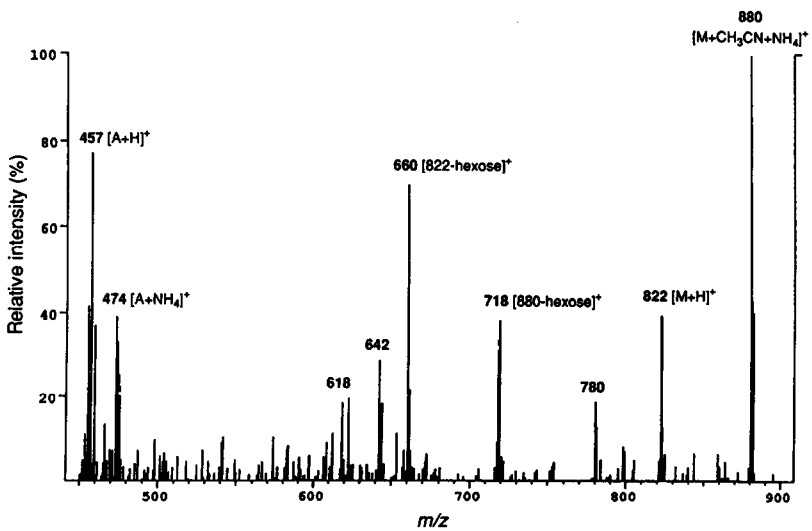

Fig. 4. LC–TSP-MS of saponin (**D**) obtained after on-column analysis of the methanolic extract of *T. tetraptera*. For conditions, see Fig. 1.

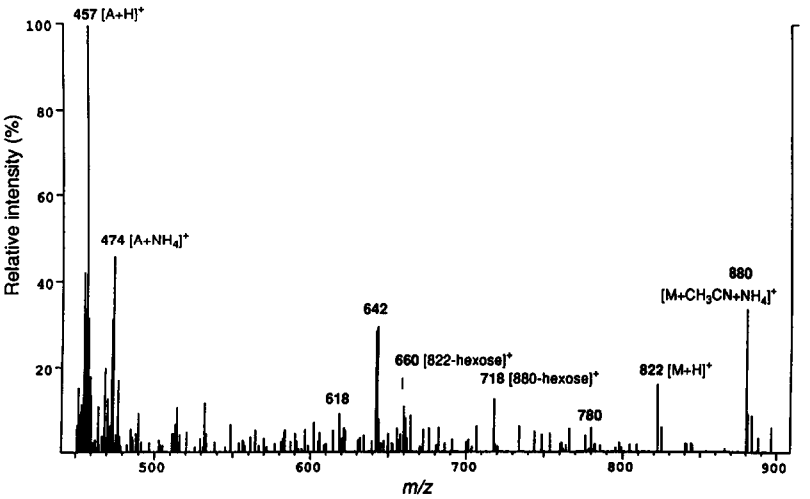

Fig. 5. LC–TSP-MS of saponin (**E**) obtained after on-column analysis of the methanolic extract of *T. tetraptera*. For conditions, see Fig. 1.

rhamnosyl $[(M + NH_4) - 146]^+$ moiety and a glucosyl $[(M + NH_4)-162]^+$ moiety indicated that both sugars were in terminal positions. Additional signals could be observed at *m/z* 650, 636 and 474, which corresponded to the elimination of both sugar moieties together with an additional inner glucuronic acid residue. The fragmentation patterns indicated clearly that rhamnose was attached to the glucuronic acid and that glucose belonged to a second chain (Fig. 8).

In the mass spectrum of saponin **H**, the signals at *m/z* 796 $\{[(M + NH_4)-132]^+\}$ and 765 $\{[(M + NH_4)-146]^+\}$ corresponded to the simultaneous elimination from the quasi-molecular ion of a xylosyl moiety and of a rhamnosyl moiety. Addi-

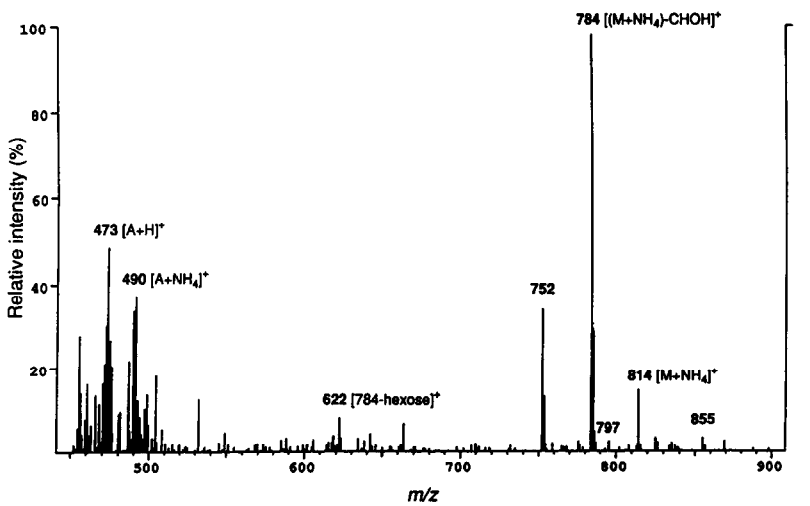

Fig. 6. LC–TSP-MS of saponin (**C**) obtained after on-column analysis of the methanolic extract of *T. tetraptera*. For conditions, see Fig. 1.

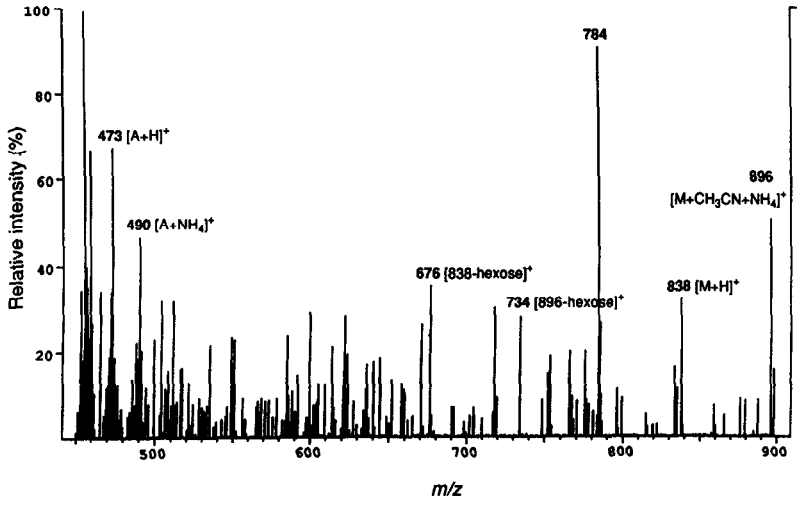

Fig. 7. LC–TSP-MS of the unknown saponin (**F**) obtained after on-column analysis of the methanolic extract of *T. tetraptera*. For conditions, see Fig. 1

tional signals for the glucuronic acid–oleanolic acid moiety and the oleanolic acid moiety were observed at *m/z* 650 $\{[(M+NH_4)-278]^+\}$ and 474 $\{[(M+NH_4)-454]^+\}$, respectively. From this fragmentation pattern, it could be deduced that rhamnose and xylose were both terminal sugars and that they were linked to a glucuronic acid (Fig. 9).

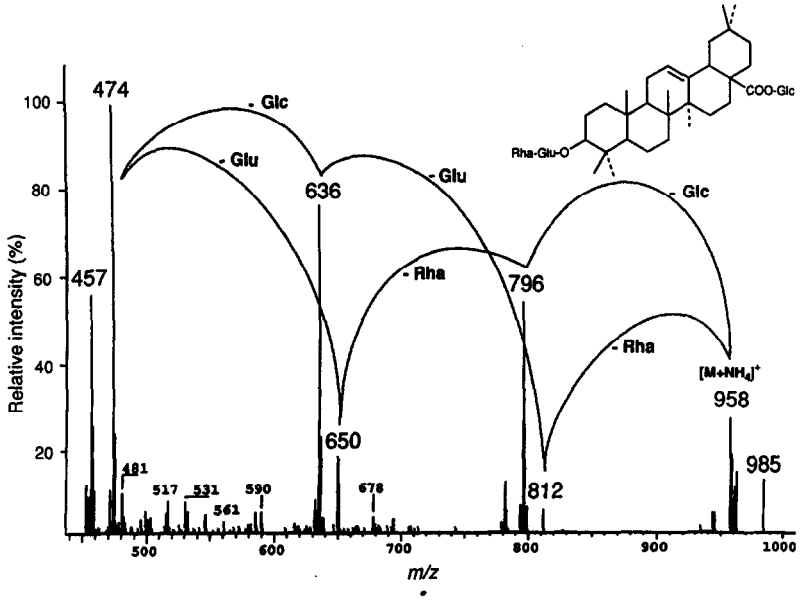

Fig. 8. TSP mass spectrum of saponin (**G**) obtained in the flow-injection mode. HPLC conditions: eluent, $CH_3CN–H_2O$ (50:50); flow-rate, 1 ml/min. TSP conditions: vaporizer temperature 100°C; source temperature, 270°C; buffer, 0.5 *M* ammonium acetate; flow-rate, 0.2 ml/min; detection, UV at 206 nm.

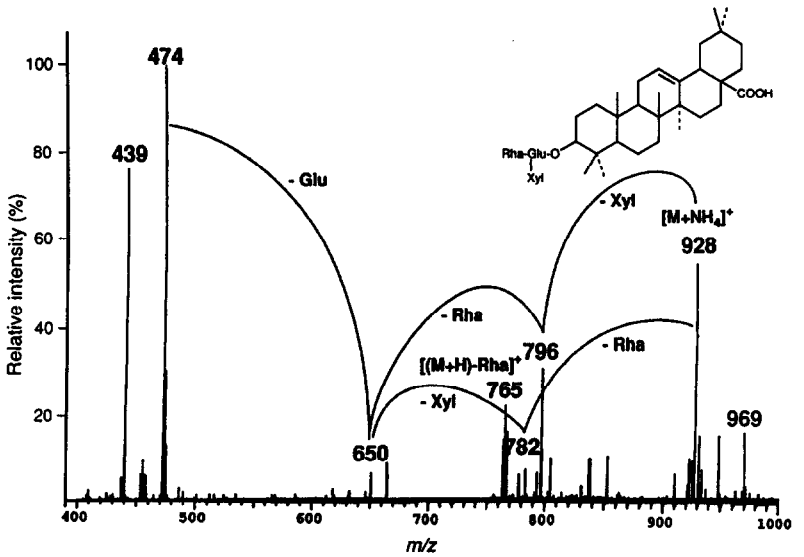

Fig. 9. TSP mass spectrum of saponin (**H**) obtained in the flow-injection mode. For conditions, see Fig. 8.

CONCLUSIONS

LC–TSP-MS is a suitable method for the detection of saponins in crude plant extracts, even in presence of other products such as phenolic compounds.

Addition of ammonium acetate as buffer provides an ionization similar to that obtained with D/CI (NH_3, positive-ion mode), and the same information can be recorded on-line. After optimization of the experimental parameters, different results such as molecular mass, the nature of the sugars and their sequence can be obtained.

Under the conditions described in this paper, it has not yet been possible to obtain molecular mass data for saponins carrying more than three sugars. However, experiments are in progress to increase the sensitivity of this method for polar glycosides of higher mass.

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