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Liquid chromatographic–thermospray mass spectrometric analysis of crude plant extracts containing phenolic and terpene glycosides

J.L. Wolfender, M. Maillard and K. Hostettmann*

Institut de Pharmacognosie et Phytochimie, École de Pharmacie, Université de Lausanne, CH-1015 Lausanne (Switzerland)

ABSTRACT

In crude plant extracts, constituents of biological or pharmaceutical interest often exist in the form of glycosides. Mass spectral investigations of these metabolites require soft ionization techniques such as desorption chemical ionization (D/CI) or fast atom bombardment if information on molecular mass or sugar sequence is desired. Thermospray (TSP) provides mass spectra similar to those obtained with positive-ion D/CI-MS using NH_3 and thus is potentially applicable to on-line analyses for these compounds and can be applied to plant extract analysis. Extracts of Gentianaceae species (containing secoiridoids and xanthone mono- and diglycosides), Polygalaceae (containing flavonol di- and triglycosides), Pedaliaceae (containing iridoids, phenylpropanoid glycosides) and Leguminosae (containing triterpene glycosides) have been screened by LC–TSP-MS. The plant extracts were analysed under standard LC–TSP-MS conditions on reversed-phase columns using methanol–water or acetonitrile–water gradients. Good optimization of the temperature of the source and the vaporizer was crucial for the observation of pseudo-molecular ions of glycosides.

INTRODUCTION

Natural products often exist in the form of glycosides and these conjugates may or may not occur together with their respective aglycones in the plants. Glycosides are thermally labile, polar and non-volatile compounds. Mass spectra investigation requires soft ionization techniques such as desorption chemical ionization (D/CI) or fast atom bombardment (FAB) [1,2], if information on molecular masses or sugar sequences is desired. These off-line techniques, however, require preliminary isolation and purification of the compounds. The development of LC–MS in the early 1980s now allows MS analysis to be coupled on-line with analytical HPLC separation. Hence it is possible to analyse many classes of non-volatile compounds without isolation from their biological matrices. The two pioneering techniques of moving belt (MB) and direct liquid introduction (DLI) have been widely replaced by thermospray (TSP) [3] and, more recently, by the atmospheric pressure ionization (API) technique [4], whereas continuous-flow (CF)-FAB and Frit-FAB [5] have not gained popularity owing to their technical complexity.

LC–TSP-MS using ammonium acetate as buffer provides mass spectra nearly identical with those obtained with D/CI-MS using NH_3 and thus is potentially applicable to the on-line measurement of glycosides containing up to three sugar units [2,6]. TSP allows the use of high flow-rates, and standard reversed-phase HPLC conditions (4 mm I.D. column, gradient capability flow-rate, 1–2 ml/min) are compatible with this interface. Parameters developed for routine HPLC–UV analysis of crude plant extracts are thus straightforwardly applicable to

* Corresponding author.

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LC–TSP-MS. Only the use of non-volatile buffers has to be avoided.

LC–TSP-MS has been widely used for bio-environmental investigations, but surprisingly few examples of glycoside analyses of crude plant extracts are known [7,8]. In the context of our studies on the active principles of higher plants [9] and in our search for more rapid and powerful methods for plant extract screening [10], conditions have been established for the TSP-MS analysis of different types of naturally occurring glycosides.

LC–TSP-MS analyses were performed on extracts containing secoiridoids, xanthone mono- and diglycosides (Gentianaceae), flavonol di- and triglycosides (Polygalaceae), iridoids and phenylpropanoid glycosides (Pedaliaceae) and saponins (Leguminosae). In all these examples, the correct tuning of TSP for the observation of molecular ion species was of great importance.

EXPERIMENTAL

Chemicals

HPLC-grade water was prepared by distillation on a Buchi (Flawil, Switzerland) Fontavapor 210 distillation instrument and passed through a 0.50-μm filter (Millipore, Bedford, MA, USA). HPLC-grade acetonitrile and methanol from Maechler (Reinach, Basle, Switzerland) was passed through a 0.45-μm filter. Ammonium acetate and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany) and diaminoethane from Fluka (Buchs, Switzerland).

HPLC conditions

Separations were performed on different RP-18, RP-8 or DIOL columns. Gradients of acetonitrile–water or methanol–water (1 ml/min) were used. In some instances, to avoid the tailing of phenolic compounds, 0.05–0.1% of trifluoroacetic acid was added to the solvents, giving a pH of 3.

LC–TSP-MS analyses

A Finnigan MAT (San Jose, CA, USA) TSQ-700 triple quadrupole instrument equipped with a TSP 2 interface was used for the data acquisition and processing. Depending on the type of compounds to be analysed and the eluent composition, the temperature of the TSP source block was set to 220–300°C and the vaporizer to 90–110°C. The electron multiplier voltage was 1800 V, dynode voltage 15 kV and the filament and discharge were off in all instances. Usually full-scan spectra from m/z 150 to 800 in the positive-ion (PI) mode were recorded (scan time 12 s). Concerning the LC part, the eluent delivery was provided by an HPLC 600-MS pump (Waters, Bedford, MA, USA) equipped with a gradient controller. The UV trace was recorded on-line with a Waters 490-MS programmable multi-wavelength detector. Postcolumn addition of buffer (0.5 *M* ammonium acetate) was effected with a Waters 590-MS programmable HPLC pump (0.2 ml/min).

Samples

Extracts were prepared from the dried plant material by maceration at room temperature with methanol. Solutions to be analysed were usually prepared by dissolving 30 mg of extract in 1 ml of a methanol–water mixture. The injected volumes varied from 10 to 20 μl.

RESULTS

The plant extracts were separated under standard HPLC conditions (RP-8 or RP-18, methanol–water or acetonitrile–water gradients at 1–1.5 ml/min, 0.05 or 0.1% TFA for suppression of tailing). To avoid any alteration of the chromatographic conditions, buffer [0.5 *M* aqueous ammonium acetate or diaminoethane (0.2 ml/min)] was added postcolumn. The HPLC separation was followed by the use of an on-line multi-wavelength detector. Details of HPLC conditions for each extract are given in the figure captions.

TSP tuning

As glycosides are thermolabile compounds, the ability to observe their molecular ions is a function of temperatures set for the TSP interface. With polyphenolic diglycosides, the observation of pseudo-molecular ions is, for example, greatly dependent on the vaporizer tempera-

ture. In order to reveal these pseudo-molecular ions, the parameters [11] of the TSP interface were tuned in the positive-ion mode with a solution of rutin (M_r 610) (0.16 mM), a common flavonoid diglycoside, in acetonitrile–water (50:50). Measurement of ion intensities at various temperatures of the vaporizer (50–110°C) (source block temperature 250°C) showed that the best ion intensities for the observation of the pseudo-molecular ion $[M + H]^+$ were obtained with a setting of 90°C (Fig. 1). The intensity plot of the ion at m/z 465 is not indicated in Fig. 1 but is similar to that of $[M + H]^+$, showing also a maximum intensity at 90°C. Nevertheless, the intensity of these two ions remained low in comparison with the corresponding fragment ion of the aglycone $[A + H]^+$, which was the base peak of the spectrum (Fig. 1). For vaporizer temperatures below 75°C or higher than 100°C detection of the molecular ion was almost impossible and only the aglycone ion $[A + H]^+$ which was still of high intensity at these temperatures was observed. This point is of crucial importance when extracts are screened for unknown compounds, as the relative pseudo-molecular ion intensities of glycosides are weak if the vaporizer is badly tuned and peaks due to glycosides could be wrongly attributed to aglycones.

The mass spectra of rutin were also recorded at different repeller potential values (0–200 V) but only a small influence on the ion intensities was observed and only extreme values below 20 V or higher than 160 V induced an important decrease in ion intensities. An increase in buffer concentration (higher than 0.1 M) in the eluent and the use of the filament- or discharge-on mode were found to have no significant influence on the ionization of glycosides. The source block temperature was set between 200 and 300°C, depending on the eluent composition.

These optimized tuning parameters are theoretically applicable only for this specific flavonoid. However, these parameters provide a good starting point for studying other related compounds in crude plant extracts. For this purpose,

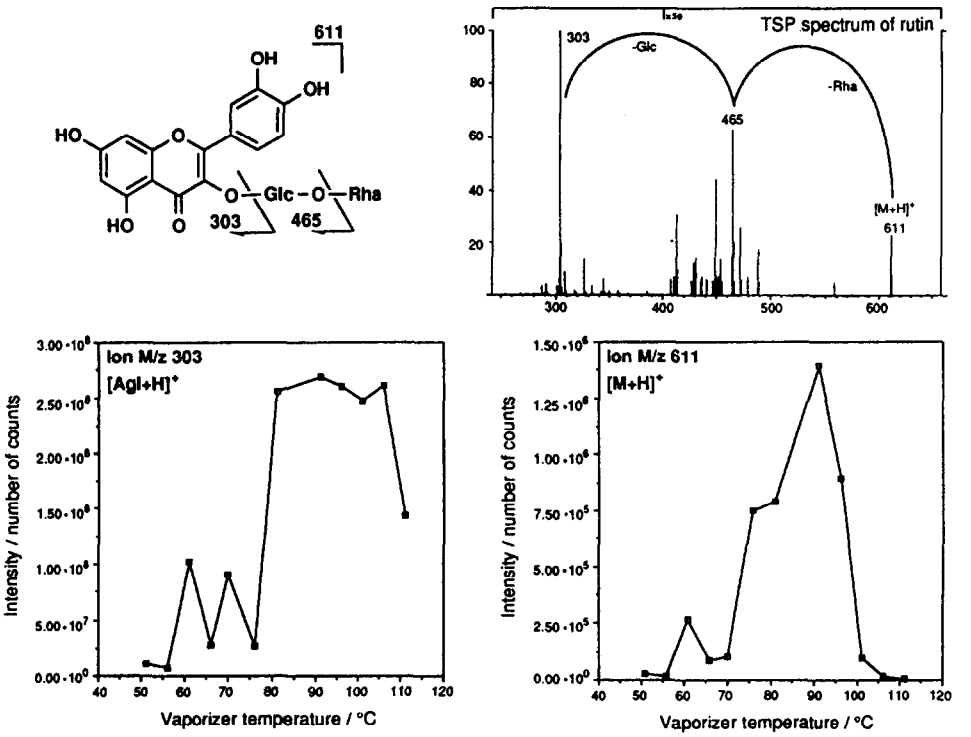

Fig. 1. Influence of vaporizer temperature on the aglycone $[Agl + H]^+$ and pseudo-molecular ions $[M + H]^+$ of rutin.

the optimization of the parameters is usually improved by performing several injections of the extract, varying slightly the starting parameters (obtained with pure products) and looking at the aspect of the total ion current trace.

Extract analysis

The mass spectra obtained for naturally occurring glycosides after injection of crude plant extracts were similar to those obtained by loop injection of the pure products if the peak resolution of the HPLC separation was good enough. Most of the extracts required a solvent gradient to ensure a good separation of the great variety of their metabolites. When subjected to reversed-phase HPLC, glycosides eluted faster than their corresponding aglycones in an extract. Some examples of TSP-MS analyses for naturally occurring glycosides in plant extracts are described below.

Iridoid and secoiridoid glycosides

Iridoids and secoiridoids represent a large and still expanding group of cyclopentane[*c*]pyran monoterpenoids. They are found as natural constituents in a large number of plant families, usually, but not invariably, as glucosides. They also often exist as coumaroyl, caffeoyl, sinapoyl, feruloyl or diphenyl esters. Ionization of these compounds by conventional methods is difficult owing to their high lability [12]. To illustrate the use of TSP with iridoids and secoirdoids, two examples of plant extract analyses have been selected.

A methanolic extract of *Sesamum angolense* Welw. (Pedaliaceae) [13] was separated on an RP-8 column with an acetonitrile–water (0.05% TFA) gradient system (Fig. 2). The temperature of the TSP vaporizer was set to 110°C and the source block to 220°C. The total ion trace recorded was in good accordance with the UV trace (220 nm). The spectra of iridoids **1** (phlomiol) and **2** (sesamoside) showed intense $[M + NH_4^+]$ pseudo-molecular ions and weak $[M + H]^+$ ions. Intense fragments at *m/z* 180 ($[Glc + NH_4^+ - H_2O]$) and *m/z* 198 ($[Glc + NH_4^+]$) were recorded in both spectra, characteristic of the presence of a hexose residue, glucose in this instance. Ions at *m/z* 259 (**1**) and 240 (**2**) corresponded to dehydrated aglycone

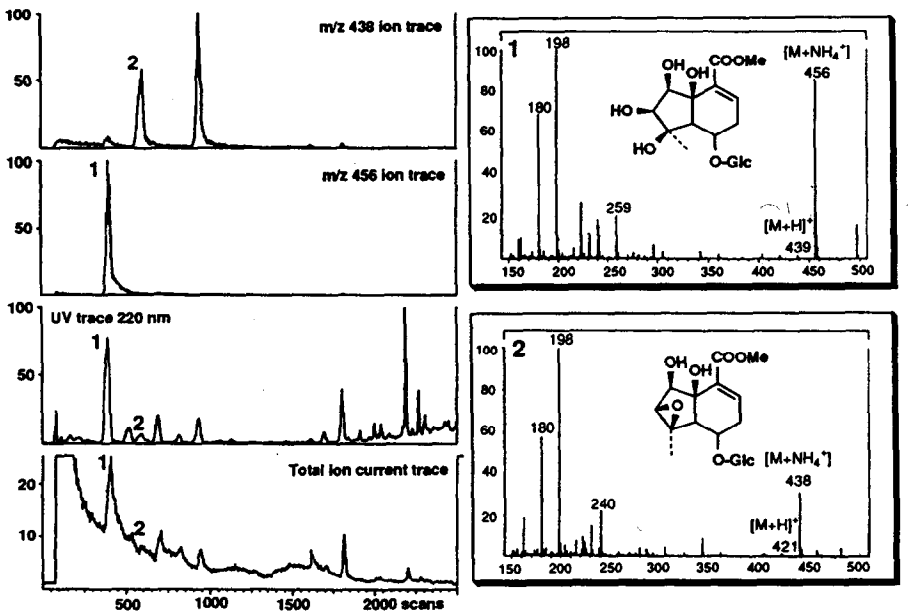

Fig. 2. LC–TSP-MS of the root methanolic extract of *Sesamum angolense* (Pedaliaceae), and TSP mass spectra of phlomiol (**1**) and sesamoside (**2**). HPLC: column, RP-8 Nucleosil (5 μm, 125 × 4 mm I.D.); gradient, CH_3CN–H_2O (0.05% TFA) 2:98→7:93 in 30 min and 7:93→37:63 in 30 min (1 ml/min). TSP: vaporizer, 110°C; source, 220°C; ammonium acetate buffer (0.5 *M*, 0.2 ml/min); PI mode.

moieties. Spectra recorded on-line with LC–TSP-MS were comparable to those obtained by D/CI of the corresponding pure products using NH_3 in the positive-ion mode [13]. However, the intensities of fragments corresponding to the aglycone ion adducts were greater with D/CI.

A dimeric secoiridoid, lisianthioside (**3**), has been found in the root methanolic extract of *Lisianthius seemanii* Robyns *et* Elias (Gentianaceae) [14]. The LC–TSP-MS analysis of the crude extract was carried out on a RP-18 column with an acetonitrile–water (0.05% TFA) gradient system. The total ion trace recorded for the whole chromatogram showed a very intense response for this metabolite, which was the main compound of the extract. The spectrum (Fig. 3A) recorded with ammonium acetate as buffer exhibited an intense pseudo-molecular ion at m/z 734 and an important fragment at m/z 359. This information was insufficient for the attribution of the pseudo-molecular ion at m/z 734 to $[M + NH_4^+]$ or $[M + NH_4^+ - H_2O]$, as no $[M + H]^+$ ion was present. Therefore, a second TSP-MS analysis of the extract was carried out with another buffer to confirm the molecular mass of the secoiridoid. Diaminoethane (0.5 *M*, 0.2 ml/min), which has a higher proton affinity than ammonium acetate, was used. This buffer is known to produce only $[M + 61]^+$ ($[M + (H_2N(CH_2)_2NH_3)^+]$) pseudo-molecular ion adducts [15]. The spectrum (Fig. 3B) recorded on-line under the same conditions as with the diamine buffer exhibited an important pseudo-molecular ion $[M + 61]^+$ at m/z 777, confirming the molecular mass to be 716 u. The intense fragment ion at m/z 359 (A) corresponded to a protonated entity with half the mass of the parent molecule. Compound **3** was therefore certainly a dimer (2 × 358 u). The ion of m/z 359 was found to be the pseudo-molecular ion ($[M + H]^+$) of sweroside, another secoiridoid common in the Gentianaceae, also present in the extract. On the basis of these results, **3** was formulated as a dimer of sweroside; the full structural determination of this novel type of secoiridoid was confirmed after isolation of the pure substance [14].

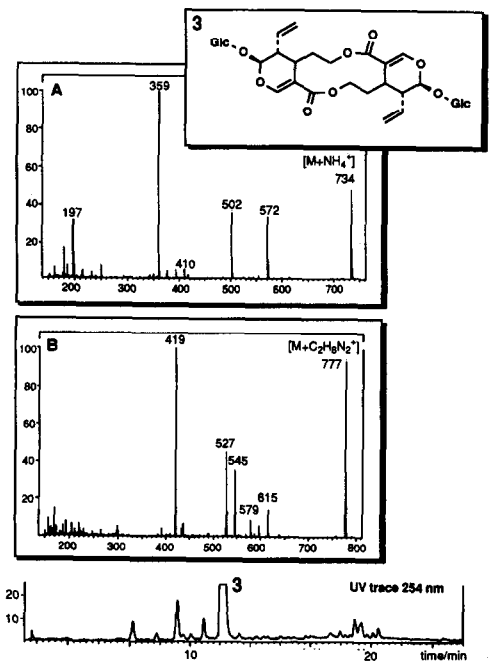

Fig. 3. TSP mass spectra of lisianthioside (**3**; molecular mass 716) recorded from the root methanolic extract of *Lisianthius seemanii* (Gentianaceae). HPLC: column, RP-18 Novapak (4 μm, 150 × 3.9 mm I.D.); gradient, CH_3CN–H_2O (0.05% TFA) 5:95→70:30 in 50 min (1 ml/min). TSP: vaporizer, 100°C; source, 250°C. (A) Ammonium acetate buffer (0.5 *M*, 0.2 ml/min); (B) diaminoethane buffer (0.5 *M*, 0.2 ml/min); PI mode.

This example shows that it is often helpful to have different types of pseudo-molecular ion adducts to ensure the on-line molecular mass determination of unknown metabolites.

To our knowledge, diaminoethane or other diamines [15] are the only alternative buffers to ammonium acetate which have been used in TSP analyses for glycosides. Further, it appears that diamines usually give adducts with compounds which form $[M + NH_4^+]$ ions with ammonium acetate.

Phenolic and polyphenolic glycosides

Simple phenolic compounds such as phenylpropanoid glycosides are found, for example, in plants of the Pedaliaceae family. The TSP-MS analysis of the methanol extract of the aerial parts of *Rogeria adenophylla* J. Gay *ex.* Del. (Pedaliaceae) (Fig. 4) showed the presence of verbascoside (**4**), a phenylpropanoid derivative. Different pseudo-molecular ion adducts were recorded for this compound: an $[M + H]^+$ ion

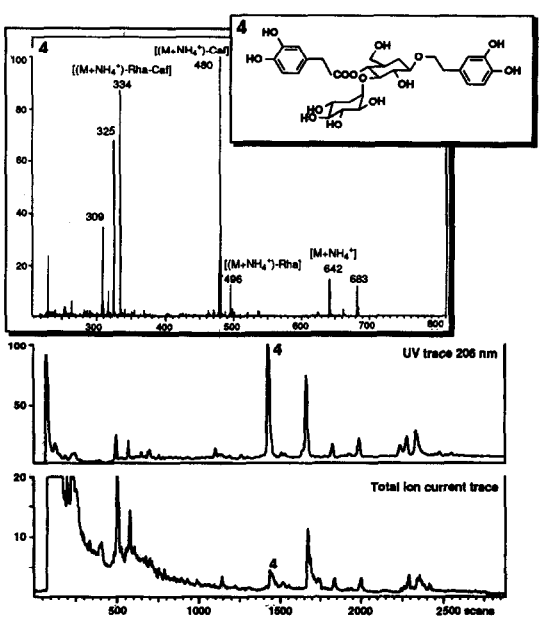

Fig. 4. LC–TSP-MS of the root methanolic extract of *Rogeria adenophylla* (Pedaliaceae) and spectrum of verbascoside (**4**). HPLC: column, RP-8 Nucleosil (5 μm, 125 × 4 mm I.D.); gradient, CH_3CN–H_2O (0.05% TFA) 0:100 (5 min), 0:100→10:90 in 5 min and 10:90→25:75 in 50 min (1 ml/min). TSP: vaporizer, 110°C; source, 280°C; ammonium acetate buffer (0.5 *M*, 0.2 ml/min); PI mode.

(*m/z* 625) and two intense ions at *m/z* 642 $[M + NH_4^+]$ and 683 $[M + CH_3CN + NH_4^+]$, confirming the molecular mass to be 624 u. The TSP mass spectrum also exhibited important significant fragment ions due to the loss of a rhamnosyl unit (*m/z* 496), a caffeoyl unit (*m/z* 480) or consecutive losses of both moieties (*m/z* 334). The spectrum obtained was similar to the D/CI (NH_3 positive-ion mode) of the corresponding pure product, with the exception of the sodium adduct ions [16].

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 [17]. They occur as O- or C-glycosides with various numbers of sugar units. Xanthones are found mainly in two families (Gentianaceae and Polygalaceae) [18], where they exist as aglycones and mono- or diglycosides.

The TSP mass spectra of these compounds were recorded from different plant extracts. To

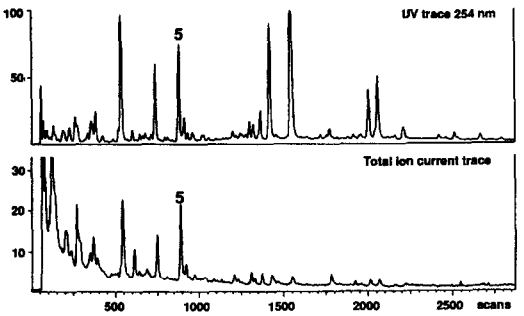

Fig. 5. UV trace (254 nm) and total ion current trace of the methanolic extract of *Gentiana dasyantha* (Gentianaceae). HPLC: column, RP-18 Novapak (4 μm, 150 × 3.9 mm I.D.); gradient, CH_3CN–H_2O (0.1% TFA) 10:90→50:50 in 60 min (1 ml/min). TSP: vaporizer, 100°C; source, 250°C. TSP mass spectrum of bellidifolin-8-O-glucoside (**5**) is shown in Fig. 6.

illustrate their ionization, the TSP spectra of mono-, di- and tri-O-glycosidic polyphenols is discussed here (Fig. 6).

The TSP mass spectrum of 1,5-hydroxy-3-methoxy-8-O-glucosylxanthone (bellidifolin-8-O-glucoside) (**5**) was recorded in the crude methanolic extract of *Gentiana dasyantha* Gilg. [19] (Fig. 5) (Gentianaceae), whereas the spectrum of 3,5-dimethoxy-1-O-[xylosyl-(1→6)-glucosyl]xanthone (**6**) was recorded in the root methanolic extract of *Chironia krebsii* Griseb. [20], another Gentianaceous plant (chromatograms in Figs. 2 and 3 in ref. 21). The spectrum of the flavonoid triglycoside 3-O-(O-apiosyl-(1→2)-O-rhamnosyl-(1→6)-galactosyl]kaempferol (**7**) was obtained from the methanolic extract of *Monnina sylvatica* Schlecht. *et* Cham. (Polygalaceae) [22]. The xanthone monoglycoside **5** exhibited a single intense pseudomolecular ion ($[M + H]^+$) and a fragment corresponding to the protonated aglycone moiety $[A + H]^+$, which was the base peak of the spectrum (Fig. 6). The xanthone diglycoside **6** showed a less intense protonated pseudo-molecular ion at *m/z* 567. A very weak signal at *m/z* 435 ($[M + H - 132]^+$) was characteristic of the loss of the xylosyl terminal sugar unit. As with **5**, the base peak of the spectrum consisted of the aglycone fragment (Fig. 6). The triglycosidic flavonoid **7** presented only a very weak $[M + H]^+$ ion and two adducts ($[M + Na^+]$ and $[M + CH_3CN + NH_4^+]$), hardly discernible from the

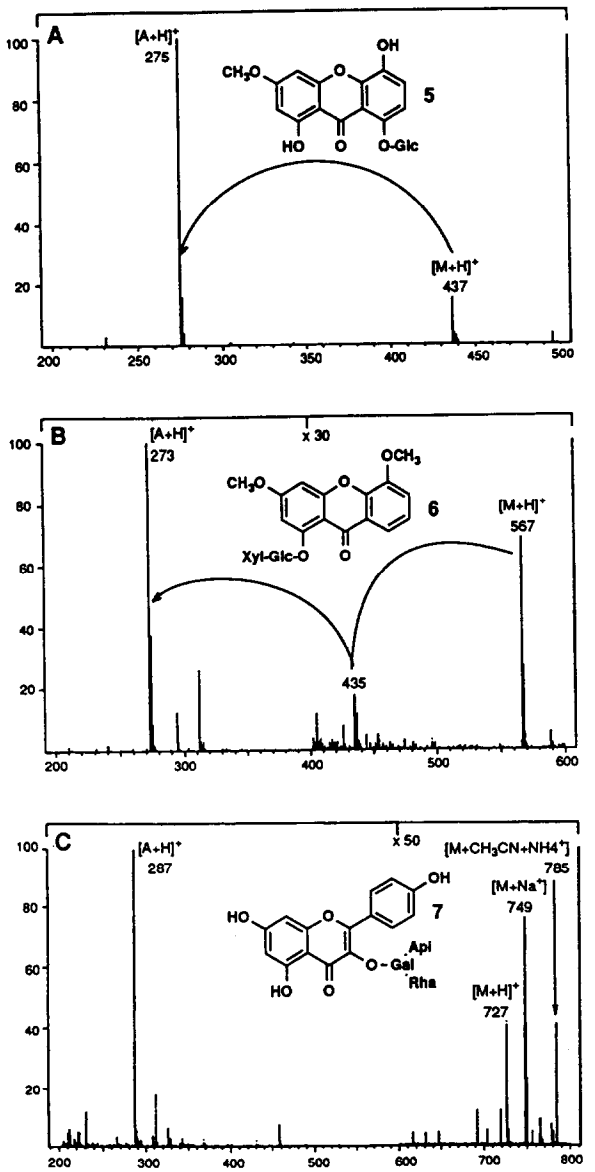

Fig. 6. Mass spectra of polyphenol glycosides. (A) TSP mass spectrum of bellidifolin-8-O-glucoside (**5**) recorded from the methanolic extract of *Gentiana dasyantha* (Gentianaceae). HPLC: column, RP-18 Novapak (4 μm, 150 × 3.9 mm I.D.); gradient, CH_3CN–H_2O (0.1% TFA) 10:90→50:50 in 60 min (1 ml/min). TSP: vaporizer, 100°C; Source, 250°C. (B) TSP mass spectrum of the xanthone **6** recorded from the methanolic root extract of *C. krebsii* (Gentianaceae). HPLC: column, RP-18 Novapak (4 μm, 150 × 3.9 mm I.D.); gradient, CH_3CN–H_2O (0.1% TFA) 5:95→70:30 in 50 min (1 ml/min). TSP: vaporizer, 100°C; source, 280°C. (C) TSP mass spectrum of **7** recorded from the methanolic extract of *Monnina sylvatica* (Polygalaceae). HPLC: column, LiChrosorb DIOL (7 μm, 250 × 4.6 mm I.D.); water (1 ml/min). TSP: vaporizer, 95°C; source, 270°C.

background noise and no fragments corresponding to the intermediate ions of the glycosidic unit were observed (Fig. 6). Ammonium adducts were not recorded for polyphenolic glycosides except for very polar triglycosides, but some salt adducts can occur.

Examples of mono- and diglycosidic flavonoids are not shown here but similar types of ions to those observed for xanthones are obtained [7].

Triterpene glycosides

Triterpene glycosides occur commonly in higher plants. The TSP mass spectra obtained usually gave molecular mass and sugar sequence information for glycosides up to three sugar units [23].

CONCLUSIONS

LC–TSP-MS is very suitable for on-line analyses for various naturally occurring glycosides encountered in crude plant extracts. Owing to the thermal instability of glycosides, the TSP interface needs to be properly tuned in order to obtain all the structural information desired. While the technique permits an unambiguous molecular mass determination of mono- and diglycosides, the observation of triglycoside parent ions is difficult, particularly with polyphenols. Other "softer" ionization techniques are then required (*e.g.*, CF-FAB or Frit-FAB) to record higher molecular mass ions [24]. The spectra obtained on-line by LC–TSP-MS using ammonium acetate as buffer in the positive-ion mode are usually comparable to the D/CI (NH_3) mass spectra of the corresponding pure products. However, salt adducts which do not appear in the D/CI mass spectra may occur in the TSP mass spectra.

Iridoids, secoiridoids and simple phenol glycosides are well ionized by TSP and usually form intense $[M + NH_4^+]$ ions, which may constitute the base peak of the spectra.

Polyphenol glycosides usually exhibit small but clearly discernible protonated pseudo-molecular ions for compounds with up to two sugar residues. The intensity of the molecular ion decreases with increase in the number of sugar

residues and the base peak of the spectra is the protonated aglycone ion.

The on-line detection of unknown compounds in crude plant extracts is possible with TSP, but often requires different techniques to confirm the results obtained. Hence the use of other buffers to confirm the molecular mass determination, the use of alternative on-line detection methods (such as photodiode-array detection to characterize the type of constituents by their UV spectra) and chemotaxonomic considerations are necessary for structure elucidation of unknown metabolites.

As TSP is compatible with high flow-rates and has a large field of ionization, it is a promising tool for the HPLC phytochemical screening of plants. As a "soft" ionization technique, it is not limited to stable, volatile compounds but is also an excellent tool for the mass spectrometric investigation of moderately volatile thermolabile compounds such as glycosides.

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