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# Liquid chromatography combined with thermospray and continuous-flow fast atom bombardment mass spectrometry of glycosides in crude plant extracts

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**Abstract**

In crude plant extracts, constituents of biological or pharmaceutical interest often exist in the form of glycosides. Off-line mass spectral investigations of these metabolites require soft ionisation techniques such as desorption chemical ionisation (DCI) or fast atom bombardment (FAB) if information on molecular mass or sugar sequence is desired. In LC–MS, glycosides can be ionised by using thermospray (TSP), continuous-flow fast atom bombardment (CF-FAB) or other interfaces. These techniques are thus potentially applicable to the on-line analysis of glycosides and can be applied to plant extract analysis.

Thermospray (TSP) used with ammonium acetate as buffer provides mass spectra similar to those obtained with DCI–MS using $NH_3$ and is potentially applicable to the on-line analysis of relatively small glycosides bearing no more than three sugar units. CF-FAB provides cleaner MS spectra than static FAB due to the lower concentration of the matrix used and can be applied to more polar compounds such as glycosides with a larger number of sugars. The use of a special setup involving post-column addition of the buffer or the matrix and splitting allows LC–UV, TSP LC–MS and CF-FAB LC–MS to be performed with the same standard HPLC conditions. Different crude plant extracts containing various types of glycosides with one to eight sugar units have been analysed by both TSP and CF-FAB. Cardenolides from *Nerium odorum* (Apocynaceae) and saponins from *Swarzia madagascariensis* (Leguminosae), *Aster scaber* and *Aster tataricus* (Asteraceae) have been studied by LC–MS. The combination of these two interfaces for the HPLC screening of crude plant extracts is discussed.

## 1. Introduction

Plant constituents often exist in the form of glycosides. 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 spectral investigation requires soft ionisation techniques such as desorption chemical ionisation (DCI) or fast atom bombardment (FAB) [1,2] if information on molecular masses or sugar sequences is desired. These off-line techniques, however, require a preliminary isolation and purification of the compounds. The development of LC–MS in the early 1980s allows nowadays MS analysis to be coupled on-line with analytical HPLC separation. Thus, it is possible to analyse many

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classes of non-volatile compounds without isolation from their biological matrices.

In this respect, cardenolides and saponins have been studied by two complementary interfaces, thermospray (TSP) [3] and continuous flow-FAB (CF-FAB) [4]. TSP has already proved to be a valuable technique for the analysis of small glycosides of xanthones, flavones, monoterpenes and triterpenes [5,6]. In the case of CF-FAB, only very few applications to naturally occurring glycosides have been described. However, the analysis of some saponins has been performed with frit-FAB, a related technique [7,8].

Thermospray LC–MS using ammonium acetate as buffer provides mass spectra nearly identical to those obtained with DCI-MS using $NH_3$ and is thus potentially applicable to the on-line analysis of glycosides containing up to three sugar units [2,9]. Due to the use of an additional mechanical pumping in the source chamber of the MS instrument, TSP can be run with high LC flow-rates. Thus standard reversed-phase HPLC conditions (4 mm I.D. column, gradient capability, 1–2 ml/min flow-rate) are compatible with this interface. Parameters developed for routine HPLC–UV analyses of crude plant extracts are directly applicable to TSP LC–MS. Only the use of non-volatile buffer has to be avoided [10].

Continuous flow-FAB produces the same type of soft ionisation as static FAB but cleaner spectra are obtained because less matrix is used (5–20%) [4]. This interface is applicable for all compounds usually analysed with static FAB, including glycosides with large number of sugars. CF-FAB requires very low flow-rates (5–10 μl/min) because, contrary to TSP, no additional pumping of the MS source chamber is used.

In order to keep LC-MS conditions as close as possible to those employed in standard reversed-phase HPLC (1 ml/min, gradient with aqueous solvent systems) and with the aim of using the same columns without changing the chromatographic conditions, the LC–UV and TSP LC–MS or LC–UV and CF-FAB LC–MS configurations shown in Fig. 1 have been employed.

An HPLC pump equipped with a gradient controller provides the eluent for the HPLC separation of the plant extracts. A reversed-phase column (I.D. about 4 mm) is most often used. At the column outlet, the eluent passes through a photodiode array detector (DAD) equipped with a high-pressure cell. At the exit of the UV detector, two configurations, according to the LC–MS mode chosen (TSP or CF-FAB), are possible:

For TSP LC–MS operation, post-column addition of the buffer needed for "TSP buffer"

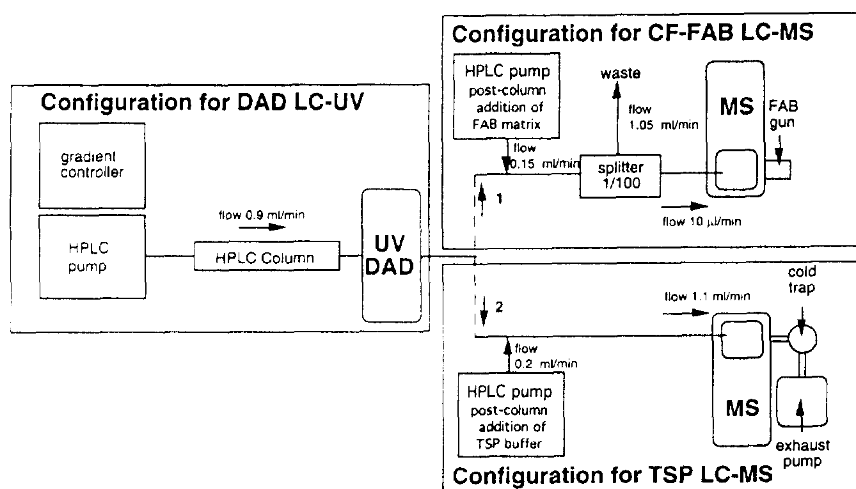



Fig. 1. Schematic representation of the experimental setup used for LC–UV and TSP or CF-FAB LC–MS analysis. The mass spectrometer used is a triple quadrupole instrument.

ionisation is provided by an additional HPLC pump (usually ammonium acetate 0.5 $M$, 0.2 ml/min). The total eluent (1.2 ml/min) containing the buffer passes through the TSP interface and the exhaust eluent is pumped away by a mechanical pump and trapped in a cold trap.

For CF-FAB operation, the additional HPLC pump allows the post-column addition of the glycerol matrix needed for FAB ionisation (usually glycerol 50%, 0.2 ml/min). The viscous matrix is efficiently mixed in the eluent with a visco mixer. The mobile phase is then split (splitter: 1/100), and only 10 $\mu$l/min of the total eluent enters the CF-FAB interface through a fused-silica capillary.

With such a setup, standard HPLC conditions for crude extract analysis (1 ml/min, 4 mm I.D. column) can be maintained without alteration in both TSP or CF-FAB LC–MS modes. Furthermore DAD LC–UV detection is not affected by the buffer or the matrix used.

In the context of our studies on the active principles of higher plants [11] and in our search for more rapid and powerful methods for plant extract screening [12], conditions have been established for both TSP LC–MS and CF-FAB LC–MS analysis of different types of naturally occurring glycosides.

In order to compare the potential and limitations of both TSP and CF-FAB ionisation techniques, LC–MS analyses of glycosides bearing one to eight sugar units have been investigated. Crude plant extracts containing cardenolides (Apocynaceae) and saponins (Leguminosae and Asteraceae) were studied.

## 2. Experimental

### 2.1. Chemicals

HPLC grade water was prepared by distillation on a Buchi Fontavapor 210 distillation instrument (Flawil, Switzerland) and passed through a 0.50-$\mu$m filter Millipore (Bedford, MA, USA). HPLC grade MeCN and MeOH from Maechler AG (Reinach, Basel, Switzerland) were passed through a 0.45-$\mu$m filter. Ammonium acetate and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany) and glycerol from Fluka (Buchs, Switzerland).

### 2.2. HPLC conditions

Separations were performed on a Nova-Pak $C_{18}$ column, 4 $\mu$m (150 × 3.9 mm I.D.) from Waters (Bedford, MA, USA), equipped with a Nova-Pak Guard-Pak $C_{18}$ precolumn. MeCN–$H_2O$ gradients (0.9 ml/min) were used. To avoid the tailing of phenolic compounds, 0.05% of trifluoroacetic acid was added to the solvents, giving a pH of 3. The eluent delivery was provided by a HPLC 600-MS pump (Waters-Millipore, Bedford, MA, USA) equipped with a gradient controller.

### 2.3. LC–UV (DAD) analyses

The UV trace was recorded on-line with a HP-1050 photodiode array detector from Hewlett-Packard (Palo Alto, CA, USA) (Fig. 1).

### 2.4. TSP LC–MS analyses

A Finnigan MAT TSQ-700 (San Jose, CA, USA) triple quadrupole instrument equipped with a TSP 2 interface was used for the data acquisition and processing. As all the analyses were carried out with approximately the same eluent composition (20 to 50% MeCN), the TSP parameters were set to average values for glycosides. The temperature of the TSP source block was maintained at 280°C and the vaporiser was set to 100°C giving aerosol temperatures varying between 305–330°C (according to the eluent composition). The electron multiplier voltage was 1800 V, dynode 15 kV and the filament and discharge were off in all cases. Usually full-scan spectra from $m/z$ 150–1500 in the positive ion (PI) mode were recorded (scan time 3 s). Post-column addition of buffer (ammonium acetate 0.5 $M$) was achieved by a Waters 590-MS programmable HPLC pump (0.2 ml/min) (Fig. 1).

### 2.5. CF-FAB LC–MS analyses

The same mass spectrometer as described for TSP was used, equipped with the continuous-flow FAB interface series-70 BioProbe from Finnigan MAT. Post-column addition of the matrix (glycerol 50% aqueous solution) was achieved by a Waters 590-MS programmable HPLC pump (0.15 ml/min) producing a matrix concentration of 7% in the eluent. In order to obtain an LC flow-rate compatible with CF-FAB operation, the total eluent flow-rate (1.05 ml/min) was reduced to 10.5 $\mu$l/min with a splitter [microflow processor splitter: 1/100 (Acurate)] from LC-Packings (Zürich, Switzerland). For stable operation, the copper tip of the CF-FAB interface was maintained at 50°C and the source block was kept at 100°C. The FAB gun was set to 4 kV and 1.2 mA; xenon gas was used for bombardment.

### 2.6. Samples

Extracts were prepared from the dried plant material by maceration at room temperature with MeOH. In the case of *Aster scaber* (Asteraceae), the saponin-rich fraction from the MeOH extract [13] was chromatographed into two fractions (A and B) on a polystyrene resin (Diaion HP-20). These fractions were treated with an ion-exchange resin, Amberlite IRC-84, and the acidic products were converted to methyl esters with $CH_2N_2$. Solutions to be analysed were usually prepared by dissolving 30–100 mg of extract in 1 ml of a MeOH–$H_2O$ mixture. The injected volumes varied from 10 to 20 $\mu$l.

## 3. Results

The crude MeOH extracts of plants and the saponin fractions of *Aster scaber* were separated by HPLC on reversed-phase columns with various MeCN–$H_2O$ gradients at 0.9 ml/min. Suppression of tailing was achieved with 0.05% TFA. The LC conditions for each extract were first established by LC–UV. For the LC–MS analyses, as the buffer or the matrix needed for ionisation were added post-column (Fig. 1), no alteration of the chromatographic separation was observed.

### 3.1. TSP tuning

Since glycosides are thermolabile compounds, the ability to observe their molecular ions is a function of the temperatures set for the TSP interface [5,14].

For the study of terpene glycosides, the ionisation of different pure mono-, di- and triglycosylated triterpenes was investigated by repeated loop injection while varying different TSP parameters. In the positive ion mode with ammonium acetate as buffer (0.1 *M*) at a total flow-rate of 1.1 ml/min (MeCN–$H_2O$, 50:50), the vaporiser and the source block temperature were set at average values of 100 and 280°C respectively. These settings represented a compromise, allowing the observation of the molecular ions and a satisfactory total-ion current intensity for the different types of glycosides studied. The influence of the repeller voltage was negligible. The filament "off" mode was preferred as no significant changes were observed in the filament "on" mode.

### 3.2. CF-FAB tuning

In the case of CF-FAB, the different settings were tuned in order to obtain stable operation. The glycerol matrix (glycerol 50% aqueous solution 0.15 ml/min) was efficiently mixed post-column with a visco mixer in order to obtain a homogeneous eluent. The total eluent 1.05 ml/min was split accurately (1/100) with a microflow processor allowing a uniform microflow (10.5 $\mu$l/min) even in the gradient mode. The temperatures of the source and the FAB tip were optimised by monitoring the intensity of the glycerol ion at $m/z$ 185 in order to obtain stable conditions (variation of intensity of $m/z$ 185 $< 10\%$) at eluent compositions corresponding to the beginning and the end of the LC gradient used. Under these conditions, a source temperature of 100°C and a tip temperature of 50°C were found to be satisfactory for eluent compositions

varying from 20:80 to 50:50 $MeCN-H_2O$. Several loop injections of various triterpene glycosides have showed that the negative ion mode produced intense deprotonated molecular ions $[M-H]^-$ and distinctive fragment ions. The positive ion mode produced less clear results; various salt adduct ions were observed and fragment ions resulting from the sugar losses were not always clearly visible. The negative ion mode was thus chosen for this study.

### 3.3. *Cardenolides from Nerium odorum (Apocynaceae)*

Cardiac glycosides have been isolated from both vegetable and animal sources. Some of them are important drugs. Their characteristic structural features are a $C_{23}$ or $C_{24}$ steroid aglycone with a sugar moiety containing from one to five sugars attached at position C-3.

In order to illustrate the potentialities of both TSP and CF-FAB LC–MS for the analysis of cardenolides, the crude root bark MeOH extract of *Nerium odorum* (Apocynaceae) has been investigated. This Indian plant is known to possess acetophenones, pregnenolone glucosides, pregnanes, cardiac glycosides and steroid glycosides [15,16].

For a rapid survey of the chemical composition of the MeOH root extract of *N. odorum*, 1 mg was separated by HPLC on a $C_{18}$ column ($MeCN-H_2O$ gradient, 20:80→50:50 in 40 min, 0.9 ml/min). The UV chromatogram recorded at 210 nm showed a satisfactory separation of the main metabolites. Under the same LC conditions, this extract was injected twice: once for TSP LC–MS detection and once for CF-FAB LC–MS detection (Fig. 2).

Under the given TSP conditions, the total-ion current trace recorded in TSP LC–MS was very similar to the UV trace at 210 nm, showing the efficient ionisation of the major part of the metabolites detected at 210 nm in the extract. *N. odorum* is known to contain cardenolides bearing only one to three sugar units (Fig. 3). For all these cardiac glycosides, the TSP LC–MS analysis of the crude extract gives on-line molecular mass and sugar sequence information. In order to illustrate the results obtained, the TSP spectra of four representative cardiac glycosides [15] [odoroside A (1), neritaloside (2), bioside G (3) and odoroside G (4)] have been selected (Fig. 4A). Odoroside A (1) and neritaloside (2) are cardiac monoglycosides. The TSP spectra of 1 and 2, recorded on-line, exhibited the intense protonated molecular ions $[M+H]^+$ at $m/z$ 519 and 593 respectively and the main fragment ions corresponding to the aglycone moiety $[A+H]^+$ ($m/z$ 375 and 433). In the case of 1, the mass difference between the proton adduct ion of the aglycone $[A+H]^+$ and the $[M+H]^+$ ion was characteristic for the loss of a diginosyl unit (144 u), while for 2, this loss was due to a digitalosyl unit (160 u). The aglycone of 1 is digitoxigenin ($M_r$ 374), which corresponded to the main ion of the TSP spectra at $m/z$ 375. In the case of 2, the aglycone is digitoxigenin-16-acetate ($M_r$ 432). This aglycone was more labile than digitoxigenin and gave, for example, an intense ion at $m/z$ 373 corresponding to the successive loss of the acetyl group and water. Bioside G (3) and odoroside G (4) contain two and three sugar units respectively and have the same aglycone moiety as 1 ($[A+H]^+$, $m/z$ 375). Contrary to 1 and 2, these two polar cardiac glycosides exhibited weak ammonium adduct $[M+NH_4^+]$ molecular ions and no or very weak protonated molecules. For 3, a first loss of 162 u gave two ions at $m/z$ 552 $[M+NH_4^+-162]$ and $m/z$ 535 $[M+H-162]^+$ which corresponded to the loss of a terminal glucose unit. The loss of 160 u between $m/z$ 535 and $m/z$ 375 was characteristic for a digitalosyl unit. Odoroside G (4) produced identical ions to those described for 3 except that the $[M+NH_4^+]$ ion was shifted by 162 u to $m/z$ 876, indicating a supplementary terminal glucose unit. The display of the different molecular ions permitted their precise localisation in the chromatogram (Fig. 2A).

Contrary to TSP LC–MS, the total-ion current trace observed in the CF-FAB analysis of the same extract was rather different from the UV trace at 210 nm. The peak sharpness was diminished and the ion intensity differed largely among the peaks compared (Fig. 2). Concerning the spectra recorded on-line, only deprotonated

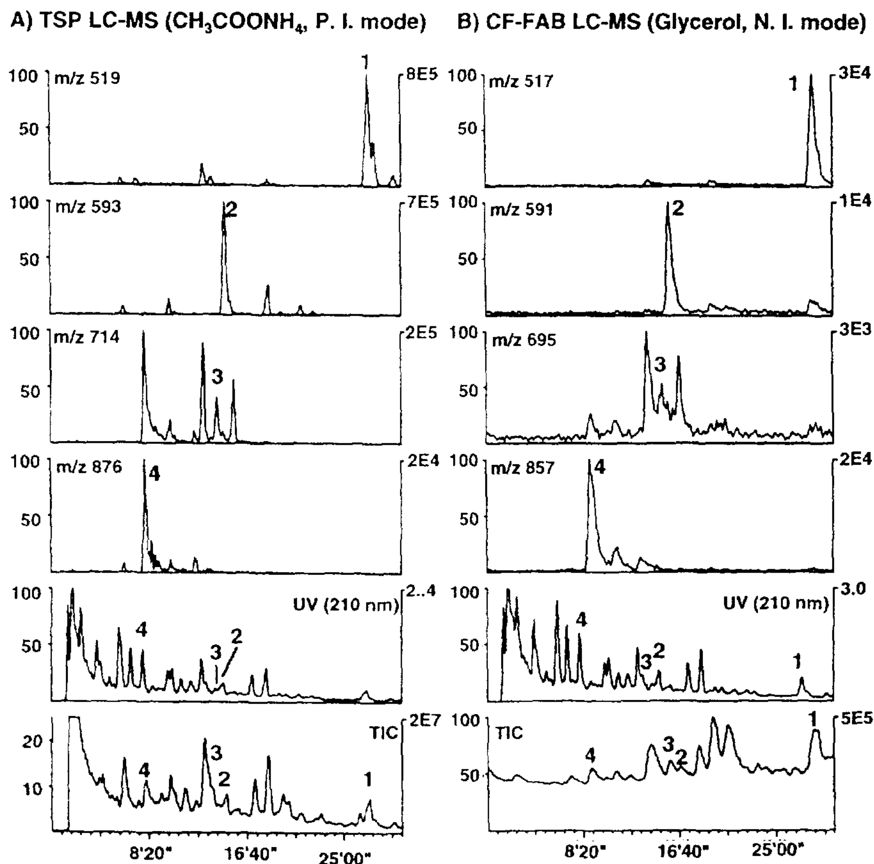
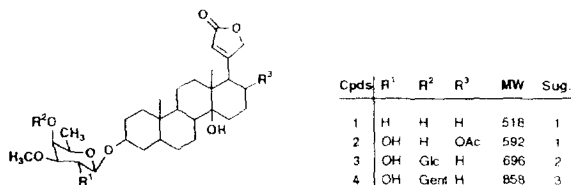



Fig. 2. Combined TSP (A) and CF-FAB (B) LC–MS of the methanolic extract of *Nerium odorum* (Apocynaceae). HPLC: $C_{18}$ Nova-Pak (4 $\mu$m, 150 × 3.9 mm I.D.); gradient, $CH_3CN$–$H_2O$ (0.05% TFA) 20:80→45:55 in 40 min (0.9 ml/min). For conditions of TSP and CF-FAB LC–MS see Experimental.

molecular ions $[M-H]^-$ were observed and no significant fragment ions were recorded (Fig. 4B). Furthermore, the MS responses of these $[M-H]^-$ ions were weaker than those of the corresponding molecular ions in TSP (Fig. 2). The non-labelled ions observable in the CF-FAB spectra of cardenolides 1–4 were due to the glycerol matrix or attributable to other co-migrating metabolites, as was the case for the ion at *m/z* 841 in the CF-FAB spectrum of 3.



| Cpds | R¹ | R² | R³ | MW | Sug. |
|---|---|---|---|---|---|
| 1 | H | H | H | 518 | 1 |
| 2 | OH | H | OAc | 592 | 1 |
| 3 | OH | Glc | H | 696 | 2 |
| 4 | OH | Gent | H | 858 | 3 |

Fig. 3. Structure of selected cardenolides isolated from *Nerium odorum* (Apocynaceae).

These results suggest that for the analysis of cardenolides bearing up to three sugar units, TSP LC–MS is probably the method of choice. It enables molecular mass information to be obtained, together with significant sugar sequence and aglycone fragment ions with almost no loss of peak resolution. CF-FAB in this case gives less structural information and is less sensitive. Nevertheless, this latter technique represents a good complement to TSP because it permits a precise confirmation of the on-line molecular mass attribution of the different cardenolides.

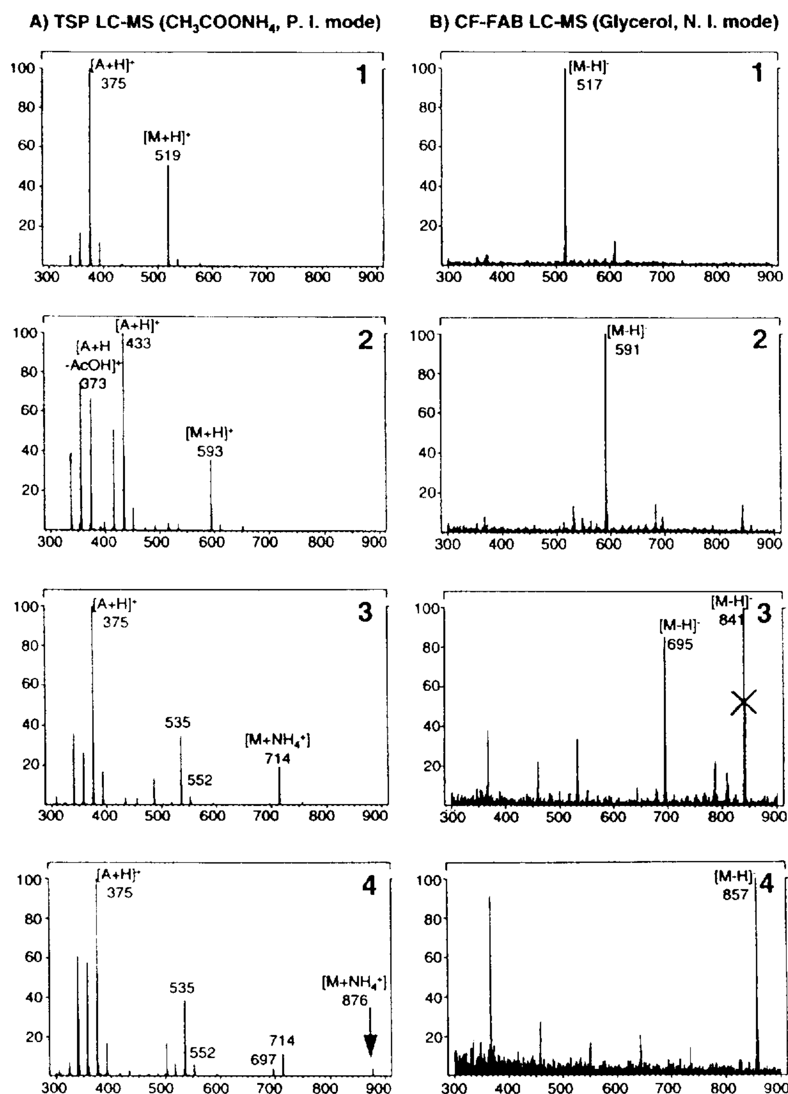


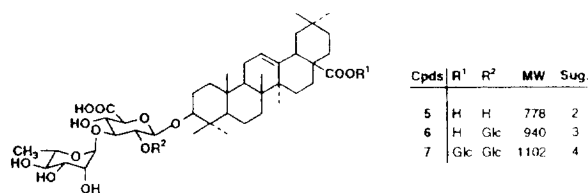

Fig. 4. TSP (A) and CF-FAB (B) MS spectra of cardenolides 1, 2, 3 and 4 from the root methanolic extract of *Nerium odorum* (Apocynaceae). Same experimental conditions as in Fig. 2.

These independent results are particularly useful in this case due to the number of adducts possible in TSP.

### 3.4. Saponins from *Swarzia madagascariensis* (*Leguminosae*)

Saponins are glycosides that commonly occur in higher plants. They are found in more than 500 species belonging to almost 80 different families. Their aglycones are triterpenes (usually with oleanane, ursane or dammarane skeletons) or steroid. Monodesmosidic saponins (glycosylated at position C-3 of the aglycone, with a free carboxylic group in position C-28) are known to exhibit important mollucisicidal activities [17].

Schistosomiasis (bilharzia) affects millions of people living in African, Asian and South-American countries. This disease is linked to certain species of aquatic snails because they serve as




| Cpds | R¹ | R² | MW | Sug. |
|---|---|---|---|---|
| 5 | H | H | 778 | 2 |
| 6 | H | Glc | 940 | 3 |
| 7 | Glc | Glc | 1102 | 4 |

Fig. 5. Structure of selected saponins isolated from *Swartzia madagascariensis* (Leguminosae).

intermediate hosts of the parasite. Molluscicidal plants are of special importance for the control of schistosomiasis as they seem to be less expensive than synthetic compounds. For this reason, a broad screening of snail-killing plants has been undertaken. Different plants containing saponins have thus been studied [17].

In order to have a rapid idea about the saponin content of a plant extract, complementary TSP and CF-FAB LC–MS analyses have been undertaken. For example, this approach has been used in the study of the molluscicidal water extract of the fruit of *Swartzia madagascariensis* (Leguminosae), a tree widespread in Africa [18]. As in the case of *Nerium odorum* the crude extract of *Swarzia madagascariensis* (0.6 mg) was separated on a $C_{18}$ column (MeCN–$H_2O$ gradient, 30:70→50:50 in 30 min, 0.9 ml/min).

The TSP LC–MS analysis of the extract exhibited the presence of triterpene glycosides derived from oleanolic acid ($M_r$ 456) (Fig. 5). Indeed, strong ionisation of this aglycone moiety was observed for different peaks in the extract (compounds 5, 6 and 7 in Fig. 6A). The ion characteristic of the sapogenin was $m/z$ 439 $[A + H - H_2O]^+$ (TSP spectra, Fig. 7A). Another ion at $m/z$ 502 was not identified but was present in

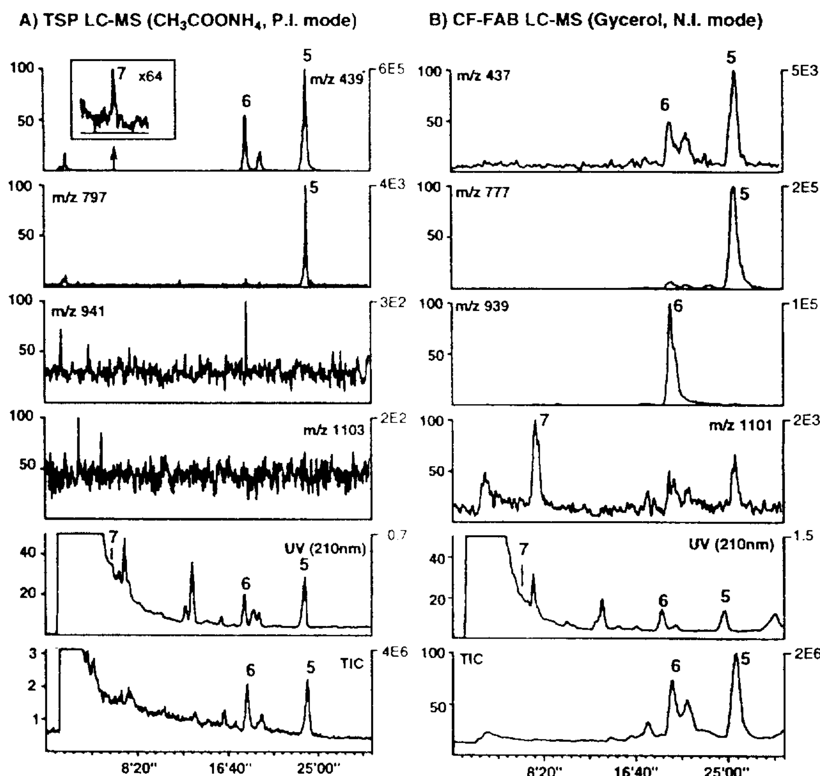



Fig. 6. Combined TSP (A) and CF-FAB (B) LC–MS of the methanolic extract of *Swartzia madagascariensis* (Leguminosae). HPLC: $C_{18}$ Nova-Pak (4 μm, 150 × 3.9 mm I.D.); gradient $CH_3CN$–$H_2O$ (0.05% TFA) 30:70→50:50 in 30 min (0.9 ml/min). For conditions of TSP and CF-FAB LC–MS see Experimental.

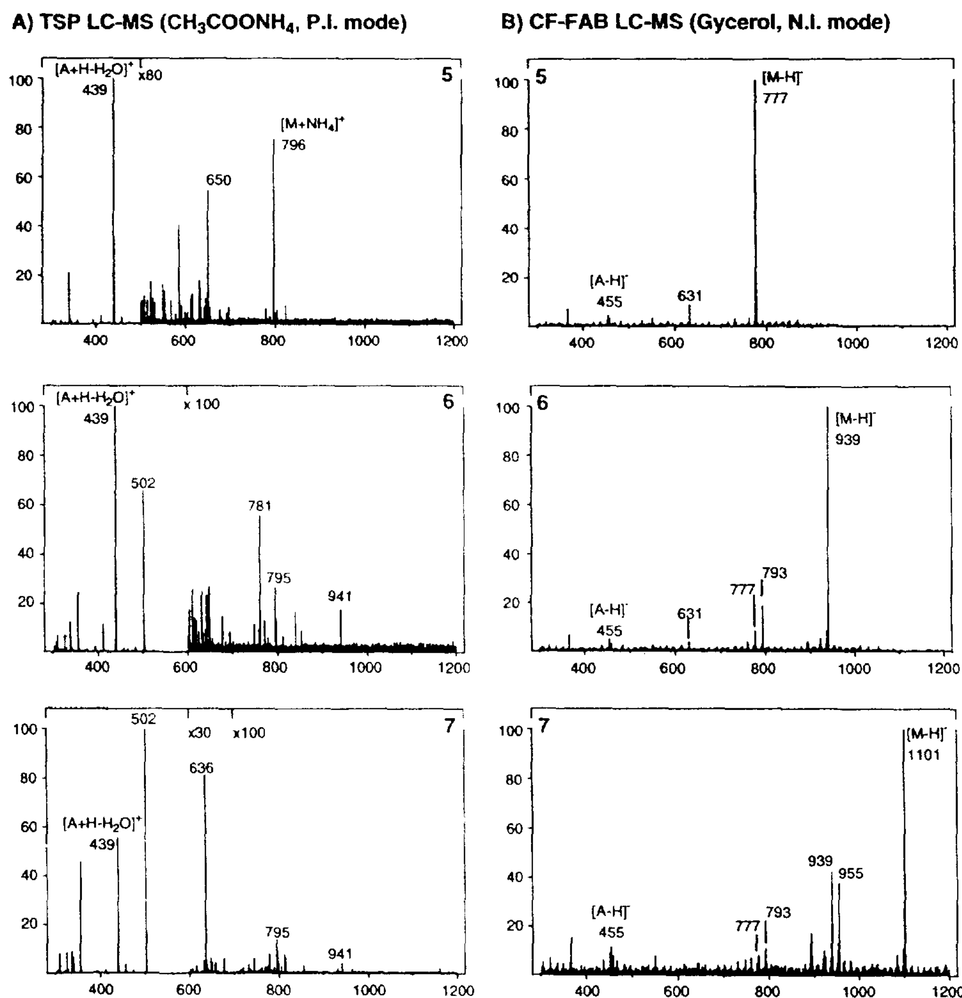



Fig. 7. TSP (A) and CF-FAB (B) MS spectra of saponins 5, 6 and 7 from the root methanolic extract of *Swartzia madagascariensis* (Leguminosae). Same experimental conditions as in Fig. 6.

the extract and the reference sample. In this case and contrary to the cardenolides from *Nerium odorum*, only very weak or no molecular ions of the corresponding saponins were recorded. For 5, a distinctive ion at $m/z$ 796 and a fragment ion at $m/z$ 650 were characteristic for a saponin bearing a diglycosidic moiety consisting of a terminal deoxyhexose unit (146 u) and a glucuronic acid (176 u) moiety. As rhamnose is the most frequent deoxyhexose occurring in saponins, it can be assumed from these on-line MS data that 5 was a saponin of oleanolic acid, substituted by a glucuronic unit and a rhamnose in the terminal position. The TSP spectra of saponins 6 and 7 were less clear than those of 5. In both cases, characteristic signals for the oleanolic acid moiety were present, and fragment ions at $m/z$ 795 were indicative of the presence of at least glucuronic acid with a hexose unit. No clear molecular ions for tri- or higher glycosylation (TSP spectra, Fig. 7A) were visible. An ion at $m/z$ 941 was discernable in the spectra of 6 and 7 but was so weak that it could not be correlated to any "real" peak (see trace $m/z$ 941 in Fig. 6A). In the case of 7, the ion at $m/z$ 781 arose from a coeluting compound in the

extract and was not recorded on the TSP spectrum of the corresponding pure standard. For these metabolites, the TSP analysis alone could not give enough structural information.

In the CF-FAB analysis of the same extract (Fig. 6B) and unlike the TSP results, all the saponins found in the extract exhibited intense deprotonated molecular ions $[M-H]^-$ and very weak ions characteristic for the aglycone moiety $[A-H]^-$ (*m/z* 455) and $[A-H-H_2O]^-$ (*m/z* 437). Furthermore, different characteristic cleavages were distinctive. For 5, ions at *m/z* 777 $[M-H]^-$, *m/z* 631 $[M-H-146]^-$ and *m/z* 455 $[A-H]^-$ confirmed the results obtained with TSP. For 6 an intense $[M-H]^-$ ion at *m/z* 939 was observed in the CF-FAB spectrum showing that it was a triglycosylated saponin (Fig. 7B). The different fragments ions recorded in the CF-FAB spectrum of 6 (*m/z* 777 $[M-H-162]^-$ and *m/z* 793 $[M-H-146]^-$) confirmed that 6 is probably similar to 5 with one more hexose unit in position C-28 or branched on the diglycoside moiety. The CF-FAB spectra of 7 exhibited an intense molecular ion at *m/z* 1101 $[M-H]^-$. This indicated that 7 has one hexose unit (164 u) more than 6. Saponin 7 was thus a tetraglycosylated triterpene. This was also confirmed by its high polarity (see retention time of 7 in Fig. 6). The molecular ion $[M+H]^+$ of 7 was not detected during the TSP analysis, showing the limit of TSP in this field.

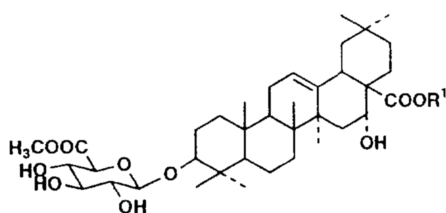
### 3.5. *Saponins from Aster scaber and Aster tataricus (Asteraceae)*

Plants from the Asteraceae family are known to possess a large number of saponins; in particular, triterpenes having long saccharide chains have been described in many species of the genera *Solidago*, *Bellis* and *Aster* [19]. In order to show the potentialities of CF-FAB or TSP for the analysis of these glycosides, two plants from this family, *Aster scaber* and *Aster tataricus*, have been selected.

In the case of *Aster scaber*, two saponin fractions of the root MeOH extract were studied [13,20]. All the acidic saponins known to be present in the plant were converted into their methyl esters by treatment of the crude fractions with $CH_2N_2$ before the LC–MS analysis, in order to aid separation (see experimental). The two fractions A and B, which were known to possess triterpenes bearing from 2 to 6 sugar units [13,20], were separated (1 mg each) on a $C_{18}$ column (MeCN–$H_2O$ gradient, 20:80→50:50 in 60 min, 0.9 ml/min).

The TSP LC–MS analysis of the saponin fractions A and B failed to give any molecular mass information. Nevertheless, intense ions corresponding to the protonated aglycone moieties and their corresponding dehydrated fragments were recorded. These results showed that fraction A consisted mainly of saponins having echinocystic acid as sapogenin ($M_r$ 472), while fraction B was composed of oleanolic acid ($M_r$ 456) glycosides.

On the contrary, the CF-FAB analyses of fractions A and B gave molecular mass and sugar sequence information on-line for all the saponins previously isolated [13,20]. In order to illustrate the results obtained, the CF-FAB analysis of the fraction A containing saponins bearing 4 to 6 sugar units is presented here (Fig. 8). Four saponins, the scaberosides A1, A2, A3 and A4 (8,9,10, and 11 respectively), have been selected (Fig. 8). The CF-FAB spectrum of scaberoside A2 (9) (Fig. 9) exhibited a clearly discernible deprotonated molecular ion $[M-H]^-$ at *m/z* 1071. In addition, a main fragment ion was found at *m/z* 661 and a very intense aglycone ion $[A-H]^-$ was recorded at *m/z* 471. These observations suggested that 9 has an oligosaccharide unit of 410 u (1071 − 661) esterified at position C-28 and a glucuronic methyl ester unit at C-3 (190 u = 661 − 471). Indeed the ester bond at C-28 is more easily cleaved than a glycoside ether bond and gave a main fragment ion. The fragment ion recorded at *m/z* 939 was due to the loss of the terminal pentose unit of the oligosaccharide chain linked at C-28. This oligosaccharide moiety was in fact composed of two xylose units and a rhamnose unit (410 u = 2 × 132 + 146). Compound 9 was thus a tetraglycosylated bidesmosidic saponin. Saponin 8 was an isomer of 9 and presented the same




| Cpds | $R^1$ | MW | Sug. |
|---|---|---|---|
| 8 | -Ara$^2$-Rha$^3$-Api | 1072 | 4 |
| 9 | -Xyl$^2$-Rha$^4$-Xyl | 1072 | 4 |
| 10 | -Ara$^3$-Rha$^3$-Api (with Xyl at 4) | 1204 | 5 |
| 11 | -Xyl$^2$-Rha$^3$-Xyl$^3$-Xyl (with Xyl at 4) | 1336 | 6 |

Fig. 8. Structure of selected saponins isolated from *Aster scaber* (Asteraceae).

CF-FAB spectrum. The difference between 9 and 8 was due to the presence of an apiose and an arabinose unit in 8 instead of the two xylose units in 9. This difference was not detectable by MS only since these sugars are isomeric. Compound 10 exhibited the same fragments as 9 except that its intense deprotonated molecular ion at $m/z$ 1203 was shifted by 132 u in comparison to 9, indicating a supplementary pentose unit on the oligosaccharide chain at C-28. The saponin 11 had also a protonated molecular ion shifted by 132 u in comparison to 10, indicating in this case that the glycoside had 6 sugar units (5 pentoses and a deoxyhexose) at C-28 and a glucuronic methyl ester unit at C-3. On the $m/z$ 1335 ion trace (Fig. 10) a peak eluting just before 11 was found to be an isomer of the latter having also 5 pentoses and a deoxyhexose at C-28 and a glucuronic methyl ester unit at C-3. The nature of the sugars and/or they interglycosidic linkages were probably different from those of 11. However, this compound, which is a minor constituent of the extract (see UV trace 210 nm), has not been isolated. All these on-line MS results were in good agreement with the data obtained for the isolated scaberosides A1–A4 [20].

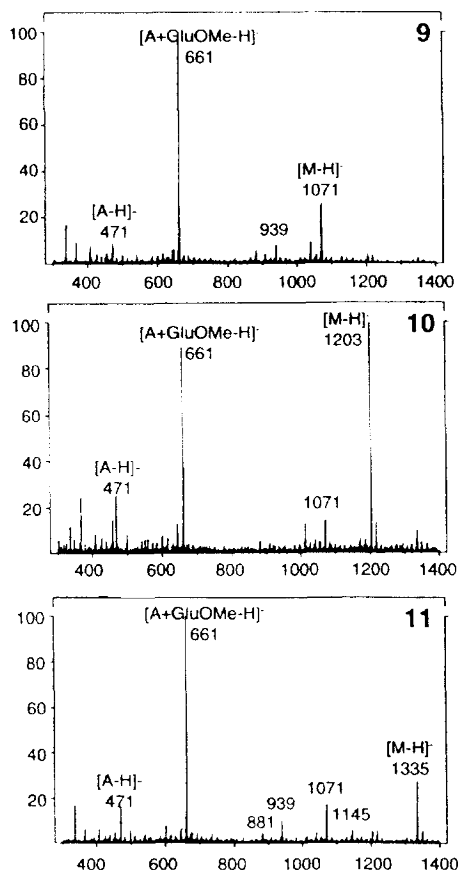



Fig. 9. CF-FAB MS spectra of saponins 9, 10 and 11 from the root methanolic extract of *Aster scaber* (Asteraceae). Same experimental conditions as in Fig. 10.

In *Aster tataricus*, triterpene glycosides bearing up to eight sugar units (the astersaponins) have been isolated [21]. In order to check the ability of CF-FAB LC–MS to ionise these relatively large molecules, CF-FAB spectra were recorded for different astersaponins. To illustrate these results, the CF-FAB spectrum of an octaglycosylated triterpene, astersaponin D (12), is presented (Fig. 11). An intense deprotonated molecular ion $[M-H]^-$ at $m/z$ 1585 was obtained and two clearly discernible fragments were observed at $m/z$ 1453 ($M-H-132$) and $m/z$ 765, due to loss of the oligosaccharide chain

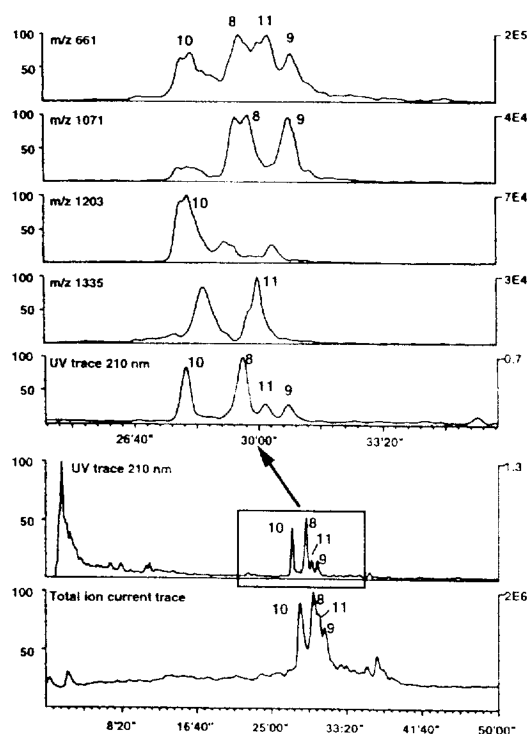



Fig. 10. CF-FAB LC–MS of the saponin fraction A of *Aster scaber* (Asteraceae). HPLC: $C_{18}$ Nova-Pak (4 $\mu$m, 150 × 3.9 mm I.D.): gradient, $CH_3CN-H_2O$ (0.05% TFA) 30:70→50:50 in 60 min (0.9 ml/min). For conditions of CF-FAB LC–MS see Experimental.

at C-28 (820 u = 4 × 132 + 2 × 146). In this case, the fragment ion corresponding to the aglycone moiety was not clearly observable.

These different LC–MS results obtained for saponins from *Swartzia madagascariensis* and *Aster* species show that CF-FAB provides molecular mass and sugar sequence information online for all the saponins, while TSP failed to give reliable molecular mass information as soon as the number of sugar units exceeded two or three. CF-FAB can be thus considered in this case as the method of choice for the LC screening of saponins. Nevertheless, TSP enables the assignment of the molecular mass of the aglycone moieties of the different saponins. This information is complementary to CF-FAB because in certain cases the aglycone ion is not always observed in the CF-FAB spectra. TSP can also be considered as an interesting tool for the detection of saponins in crude extracts. Indeed, due to its sensitivity to aglycone fragments and due to its ability to maintain good LC peak shapes, TSP LC–MS can be used, by displaying the ion traces of specific aglycone moieties, to detect saponins having a weak UV chromophore or present in very small amounts in the extract (Fig 6A).

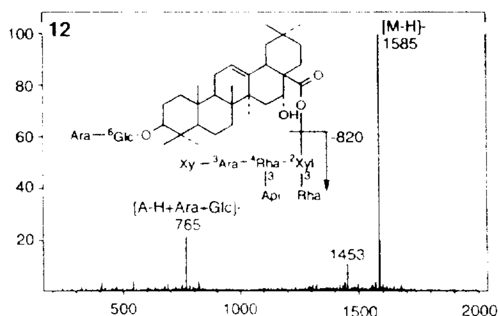



Fig. 11. CF-FAB MS spectrum of astersaponin 12 isolated from *Aster tataricus* (Asteraceae).

It should also be stated that TSP remains a powerful tool for the on-line identification of small saponins and thus the use of CF-FAB is not necessary in all cases. For example, LC–MS has proved to be a valuable method for the analysis of mono- and diglycosidic saponins related to aridanin, found in the fruit of *Tetrapleura tetraptera* (Leguminosae) [6].

## 4. Conclusions

It has been shown that TSP and CF-FAB are two valuable methods for the on-line analysis of glycosides in crude plant extracts. Nevertheless, depending on the nature of the glycosides and the type of information required, the use of one or the other or both techniques is needed.

TSP LC–MS is suitable for the investigation of small glycosides. Due to the thermal instability of glycosides, however, the TSP interface needs to be properly tuned in order to get all the structural information desired. While the technique permits an unambiguous molecular mass determination of mono- and diglycosides, the observation of triglycoside molecular ions is often difficult. The spectra obtained on-line by

TSP LC–MS using ammonium acetate as buffer in the positive ion mode are usually comparable to the DCI ($NH_3$) spectra of the corresponding pure products.

CF-FAB LC–MS permits the analysis of a broad range of polar compounds and it has been shown that molecular mass information can be obtained for triterpene glycosides and cardenolides bearing from one to eight sugar units. This technique, however, should be also applicable to larger glycosides. The main drawbacks of CF-FAB are the poor peak sharpness, peak tailing and lack of sensitivity due to the splitting device. The spectra obtained with CF-FAB are comparable to those of static FAB but are much cleaner due to the lower percentage of matrix used. In addition, more structural information can be obtained by the study of these glycosides in CF-FAB LC–MS–MS. LC–MS–MS studies are presently under investigation.

In this paper, examples of TSP and CF-FAB LC–MS of crude plant extracts containing known compounds have been described. The different LC–MS analyses performed have shown that it is possible to efficiently detect glycosides in the crude extracts and that the on-line MS spectra recorded on-line were comparable to those of the isolated products. However, these hyphenated techniques can also be used in order to screen unknown compounds in crude plant extracts and thus to target their isolation. Indeed, the MS information obtained on-line gives a rapid idea of the molecular mass, the number of sugars and also the type of aglycone of the different glycosides. The fragment ions corresponding to the intermediate loss of sugar units sometimes allow the elucidation of the sugar sequence. In combination with chemotaxonomical considerations, these on-line data often permit a partial structural determination of the metabolites of interest. LC–UV, TSP and CF-FAB LC–MS are now used routinely in our laboratories for the LC screening of crude plant extracts. They have already permitted, for example, the efficient targeted isolation of new types of secoiridoid glycosides in a *Gentiana* species (Gentianaceae) [22].

The use of these hyphenated techniques enables the early recognition of known products in extracts with a minute amount of plant material. This avoids their time-consuming isolation and, moreover, allows a targeted isolation of new interesting metabolites.

## Acknowledgement

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