*Journal of Chromatography*, 647 (1993) 147–154
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2798

# Use of liquid chromatography–thermospray mass spectrometry in phytochemical analysis of crude plant extracts

Marc P. Maillard, Jean-Luc Wolfender and Kurt Hostettmann*

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

ABSTRACT

Qualitative analyses of selected compounds by high-performance liquid chromatography of crude plant extracts are not always straightforward owing to the lack of a suitable chromophore for UV detection or because insufficient material is present. In both instances, however, coupling liquid chromatography with thermospray mass spectrometry has proved to be ideal for the direct detection of these compounds. Different examples of analyses of bioactive constituents (including ginkgolides, sesquiterpene lactones, acetogenins and saponins) in crude plant extracts are described. Total ion current traces and mass chromatograms allow the easy detection of these compounds in the extracts. Depending on the ionization pattern, $[M + H]^+$ or $[M + NH_4]^+$ ions are used for selected ion monitoring studies.

## INTRODUCTION

Analyses of natural products of plant origin are generally problematic. These substances usually occur in complex mixtures, containing up to several thousand different constituents. Sometimes only one compound is responsible for the pharmacological and/or toxic properties of the plant. Hence the phytochemist needs very efficient methods to analyse such extracts and identify their active principles.

Two points have to be noted. First, the method for separating the different constituents of the extract has to be as selective as possible. Chromatographic techniques are the main tools for analyses of drug extracts, and different methods have been developed (thin-layer chromatography, gas chromatography and liquid chromatography), with different ranges of application, depending on the substances being analysed [1]. Second, optimum detection of the separated peaks is required. Normally, an ultraviolet (UV) detector is used.

However, different problems can arise. In some instances, where the extract is very complex, it may be difficult to identify a specific peak. Insufficient resolution of the separation and peak overlapping can perturb the analysis. In other instances, the substances of interest do not possess suitable chromophores for easy detection by conventional methods, or the amount of compounds to be detected is too small for the sensitivity of the detector. All these problems require the development of new types of detector, more universal and more versatile.

The aims of qualitative analyses in phytochemistry are numerous. Some of them are as follows. Chromatographic analyses are used to "pilot" the preparative isolation of natural products (optimization of the experimental conditions, checking of the different fractions throughout the separation) and to control the final purity of the isolated compounds. For chemotaxonomic purposes, the botanical relationships between different species can be indicated by chromato-

* Corresponding author.

0021-9673/93/$06.00 

graphic comparisons of the chemical composition. Finally, comparison of chromatographic "fingerprints" between authentic samples and unknowns permits the identification of drugs and/or the detection of adulterants.

However, in many applications it may be necessary not only to detect but also to identify compounds in extracts. With conventional detection, the identities of peaks can be confirmed only from their retention times and by comparison with authentic samples. Hence, the development of new techniques has been necessary.

Interesting results have been obtained by coupled techniques, such as gas chromatography–mass spectrometry (GC–MS) [2], gas chromatography–Fourier transform infrared spectrometry (GC–FT-IR) [3], liquid chromatography–UV spectrometry with diode-array detection (LC–UV) [4] and liquid chromatography–mass spectrometry (LC–MS) [5].

The main advantage of these techniques is their ability to provide qualitative information on the detected peaks. The use of a diode array UV detector allows, for example, the whole UV spectrum of a specific product in a crude extract to be obtained on-line. This technique has been particularly useful for the identification of polyphenolic compounds [6] and bitter principles [7].

LC–MS techniques are more recent and are still generally regarded as pure research tools, without any major routine uses [8]. The principal consequence is that this technique does not yet appear to have gained the popularity that it merits and is still relatively little used in analytical work.

However, some less sophisticated interfaces have recently been developed, and are now widely available in laboratories, *e.g.*, the thermospray (TSP) interface [9].

This interface is capable of introducing an aqueous phase into the MS system at a flow-rate compatible with that usually used in phytochemical analysis (about 1–2 ml/min). From this point of view, the TSP interface is particularly well suited for this kind of analysis, and in fact TSP has already proved valuable, especially in the detection of alkaloids [10, 11]. More recently, LC–TSP-MS of flavonoids has also been reported [12]. However, so far, routine applications of LC–TSP-MS in phytochemistry are still rare.

In this paper, the ability of this method to detect and identify biologically active compounds in crude plant extracts is reported; special emphasis is placed on the detection of substances that lack a strong chromophore and are thus unsuitable for conventional UV detection.

## EXPERIMENTAL

### *Plant material*

Commercially available leaves of *Ginkgo biloba* L. (Ginkgoaceae) (Dixa, St. Gallen, Switzerland) were extracted with methanol–water (1:1). The methanolic extract was then partitioned between ethyl acetate and water. The organic phase was analysed on a Nucleosil $C_{18}$ (5 $\mu$m) column (125 × 4.0 mm I.D.) (Macherey–Nagel, Düren, Germany) with methanol–water (40:60) as eluent at a flow-rate of 1 ml/min.

*Artemisia annua* L. (Asteraceae) was grown in Switzerland (Mediplant, Conthey, Switzerland). Its aerial parts (2.0 g) were extracted by maceration at room temperature in 200 ml of dichloromethane for 24 h. The extract was analysed on a Nucleosil $C_{18}$ (7 $\mu$m) column (250 × 4.0 mm I.D.) (Macherey–Nagel), with a gradient of aqueous methanol from 50 to 75% in 30 min. Trifluoroacetic acid (0.1%) was added to the solvents. The flow-rate was maintained at 1 ml/min.

*Annona purpurea* L. (Annonaceae) was collected at Cierro Jefe, Panama, and a voucher specimen is retained at the University of Panama, Panama City. Extraction was achieved at room temperature with dichloromethane. The crude dichloromethane extract was analysed on a Nucleosil $C_{18}$ (7 $\mu$m) column (250 × 4.0 mm I.D.) (Macherey–Nagel), with isocratic elution with acetonitrile–water (85:15) or with a gradient of acetonitrile–water from 60:40 to 85:15 in 30 min. The flow-rates were 1 ml/min.

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 ana-

lysed on a μBondapak $C_{18}$ (10 μm) column (300 × 3.9 mm I.D.) (Waters, Bedford, MA, USA) with a gradient of aqueous acetonitrile from 30–80% in 30 min. The flow-rate was maintained at 1 ml/min.

The retention times of the identified products in these extracts were all confirmed by injection of authentic samples under the same conditions.

*Chemicals*

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

*LC–TSP-MS analysis*

The LC–TSP-MS system 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 kept at 15 kV. Unless specified otherwise, the filament and discharge were off. The repeller potential was optimized between 80 and 100 V. The block source temperature and temperature for the vaporizer were optimized to maximize the intensities of the quasi-molecular peaks. Full-scan spectra from *m/z* 400 to 1000 (scan time 1.2 s per scan) were obtained. A postcolumn addition of 0.2 ml/min of a 0.5 *M* solution of ammonium acetate (giving a final concentration of about 80 m*M* after dilution with the column effluent) was used to provide the volatile buffer for ion evaporation ionization. The thermospray 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

*Ginkgo biloba*

Preparations containing *Ginkgo biloba* L. (Ginkgoaceae) leaf extracts have become a major market, with estimated annual sales of US$ 500 million world-wide [13]. The antagonistic activity of *G. biloba* on platelet aggregation induced by platelet aggregation factor (PAF) is associated with the non-flavonoid fraction, composed of ginkgolides, which are diterpenes [14]. Owing to the difficulty of identifying these compounds among other substances in crude leaf extracts (low concentrations, weak chromophores), analysis and standardization of *Ginkgo biloba* preparations have previously been carried out by HPLC of their phenolic constituents, which do not contribute to the inhibition of PAF [15]. Hence methods for the determination of ginkgolides in such preparations are urgently needed.

The TSP mass spectrum of ginkgolides revealed only a strong quasi-molecular ion $[M + NH_4]^+$ peak, without other adduct species, as is the case with desorption/chemical ionization (D/CI, $NH_3$ positive-ion mode). No subsequent fragmentation was shown (Fig. 1). However, as ginkgolides are easily ionized by TSP, on-line LC–TSP-MS was demonstrated to be a very efficient method to detect these diterpenes in a *Ginkgo biloba* leaf extract (Fig. 1). Ginkgolides were not visible in the UV trace, but the total ion current (TIC) trace showed the presence of these compounds. Further, the specific display of ion traces for masses corresponding to the respective quasi-molecular ion $[M + NH_4]^+$ peaks allowed the identification of all ginkgolides in the extract.

Repetitive quantitative analyses of the same *Ginkgo biloba* leaf extract showed that the reproducibility of detection (*i.e.*, the area under the curve) was good (standard deviation of about 10%), as long as all the parameters of ionization (source block temperature, vaporizer temperature, aerosol temperature, pressure in the source) were well stabilized. Different temperatures for the source and aerosol were tried, and the best results were obtained with the block source temperature kept at 225°C and the vaporizer temperature fixed at 62°C. Ionization was induced by the use of ammonium acetate and the filament was operated at 600 V with a 0.200 mA emission current.

Using these parameters for ionization and isocratic elution with methanol–water (40:60), it was possible to analyse different

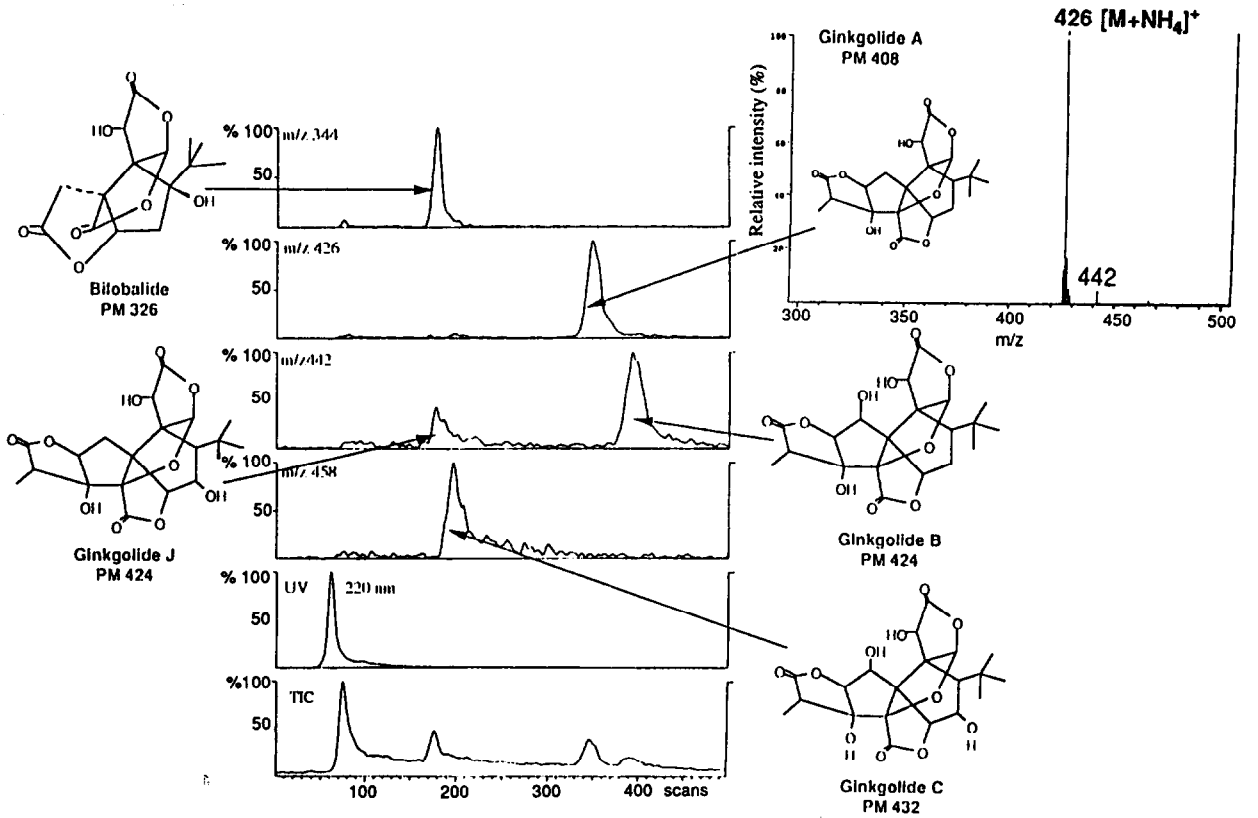


Fig. 1. LC–TSP-MS analysis of *Ginkgo biloba* extract and TSP mass spectrum of ginkgolide A. Column, Nucleosil $C_{18}$; eluent, MeOH–$H_2O$ (40:60), 1 ml/min; detection: UV 220 nm. TSP: vaporizer 62°C; source 225°C; filament on, 600 V and 200 mA; buffer, 0.5 *M* $CH_3COONH_4$ at 0.2 ml/min; 1.2 s per scan.

phytotherapeutic preparations containing *Ginkgo biloba* extracts. Low levels of detection, in the range of 1 ng (*ca.* 0.3 pmol/$\mu$l) were obtained, and even homeopathic preparations could be analysed by this technique. Thus, the sensitivity of the LC–TSP-MS analysis of ginkgolides is comparable to those obtained with GC-MS, as the latter technique permits the detection of gingkolides at concentrations as low as 0.1 pmol/$\mu$l of injected purified material [16].

*Artemisia annua*

The antimalarial compound artemisinin is found in extracts of *Artemisia annua* L. (Asteraceae), a Chinese plant, locally known as Qinghao [17]. Artemisinin represents one of the most remarkable success stories of antimalarial compounds from plants. This metabolite is a sesquiterpene lactone, too complex to be synthesized on a large scale. Hence, the only way to obtain this active principle remains its isolation from dried plant material. In order to increase the efficiency of the method, cultures of different strains of *A. annua* have been performed for the optimization of their artemisinin content. In addition, suitable analytical methods for its determination have had to be developed.

HPLC–UV determination of this compound in a crude plant extract is not straightforward because it lacks a suitable chromophore for detection with conventional UV detectors. It also occurs with phenolic components which interfere in the analysis. LC–TSP-MS can provide a solution to this detection problem, because the TSP mass spectrum of artemisinin shows different quasi-molecular peaks *i.e.*, at *m/z* 283 [M+

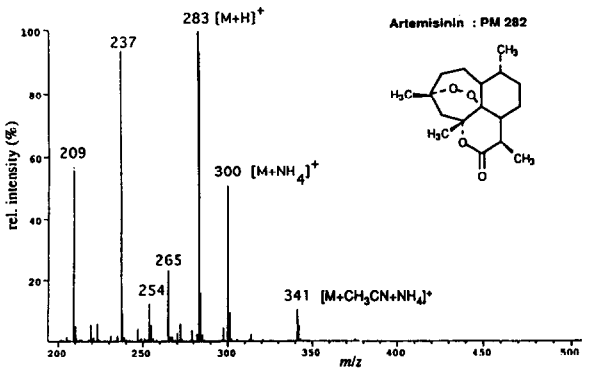


Fig. 2. TSP mass spectrum of artemisinin. Column, Nucleosil $C_{18}$, eluent $CH_3CN$–$H_2O$ gradient from 50:50 to 75:25 in 30 min; flow-rate, 1 ml/min; detection UV at 208 nm; TSP vaporizer, 90°C; source, 280°C; filament on, 600 V and 200 mA; buffer, 0.5 *M* at $CH_3COONH_4$ 0.2 ml/min; 1.2 s per scan.

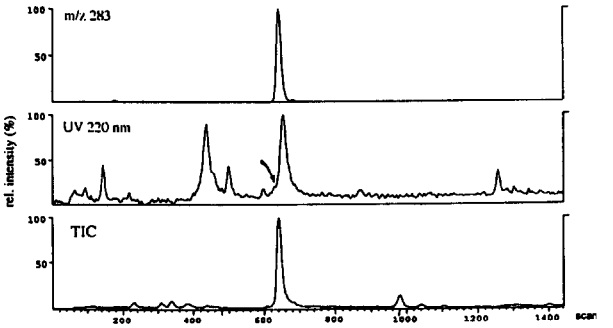


Fig. 3. LC–TSP-MS analysis of a dichloromethane extract of the aerial parts of *Artemisia annua*. Conditions as in Fig. 2. Artemisinin is indicated by an arrow in the UV trace.

$H]^+$, 300 $[M + NH_4]^+$ and 341 $[M + CH_3CN + NH_4]^+$ (Fig. 2). The base peak in this spectrum is the $[M + H]^+$ ion, as is the case for the D/CI spectrum ($NH_3$ or other reagent gas, positive-ion mode) [18]. Peaks resulting from the subsequent elimination of water (*m/z* 265) and HCOOH (*m/z* 237) were also visble in the TSP spectrum.

Chromatographic analysis of the dichloromethane extract of the aerial parts of *A. annua* showed, even at lower wavelengths, a UV trace that was not suitable for artemisinin detection. Phenolic components are present, co-migrate with artemisinin and interfere with the analysis. However, observation of the TIC trace, together with selective display of the *m/z* 283 $[M + H]^+$ ion trace permits a very efficient detection of the sesquiterpene lactone (Fig. 3).

*Annona purpurea*

The third example shown here is the identification of acetogenins in the leaves of *Annona purpurea* L. (Annonaceae). Acetogenins are fatty acid derivatives that possess tetrahydrofuran rings and a methylated γ-lactone with various substituents along the hydrocarbon chain. They exhibit a broad range of potent biological activities (cytotoxicity, antitumour activity, antimalarial, antimicrobial and immunosuppressant potencies and antifeedant and pesticidal actions) [19]. This kind of metabolite seems to occur only in the Annonaceae family, and so far more than 80 different products have been isolated from different *Annona* species and four other genera [20].

The 208-nm trace of an HPLC-UV analysis of a dichloromethane extract of *A. purpurea* shows some small peaks which can be related, after observation of their whole UV spectra, to the acetogenin group.

As is the case with D/CI ($NH_3$, positive-ion mode) [21], acetogenins are easily ionized in TSP, displaying strong quasi-molecular peaks with $[M + H]^+$ ions as the base peak, together with adduct species $[M + NH_4]^+$ (relative intensity *ca.* 70%) and $[M + CH_3CN + NH_4]^+$ (*ca.* 50%). Further, LC–TSP-MS analysis of the dichloromethane extract is straightforward (Fig. 4).

In the mass range 600–750 (in which the molecular masses of the acetogenins can be found), it was possible to see a series of different compounds under the HPLC conditions used [isocratic, acetonitrile–water (85:15)].

Modification of the chromatographic conditions with a gradient of acetonitrile from 60% to 85% within 30 min led to an improvement in the resolution and allowed the separation of eight compounds (**1–8**) (Figs. 5 and 6) [22].

Compounds **3–6** are known substances, whereas **1**, with a new type of skeleton for an acetogenin, and **2**, a stereoisomer of cherimoline (**3**), are new natural products. Structure determinations of acetogenins **7** and **8** are in progress.

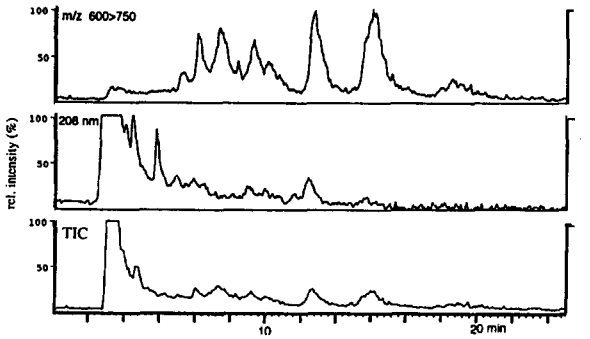


Fig. 4. LC–TSP-MS analysis of the crude dichloromethane extract of *Annona purpurea* leaves. Column, Nucleosil $C_{18}$, eluent, $CH_3CN$–$H_2O$ (85:15), at 1 ml/min; detection, UV at 208 nm; TSP: vaporizer, 90°C; source, 250°C; filament off; buffer, 0.5 *M* $CH_3COONH_4$, at 0.2 ml/min; 1.2 s per scan.

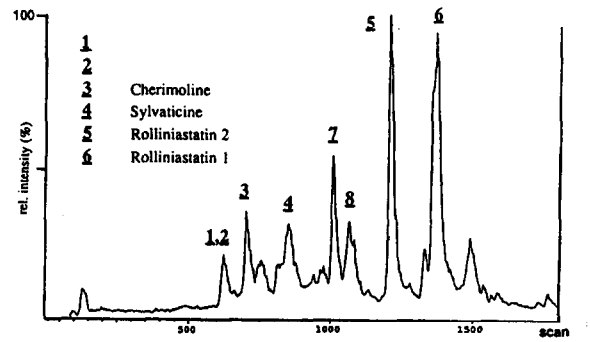


Fig. 5. LC–TSP-MS analysis of acetogenins from *Annona purpurea*. Column, Nucleosil $C_{18}$, eluent $CH_3CN$–$H_2O$ gradient from 60:40 to 85:15, flow-rate, 1 ml/min; detection UV at 208 nm, TSP vaporizer, 90°C; source, 250°C; filament off; buffer, 0.5 *M* $CH_3COONH_4$ at 0.2 ml/min.

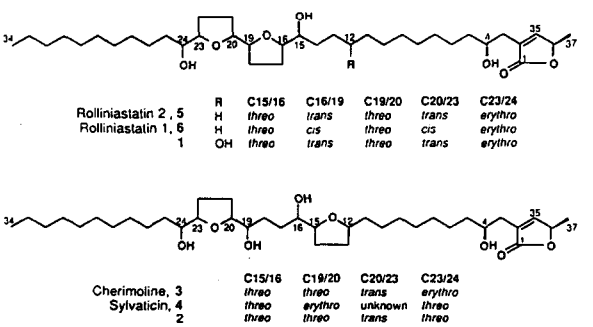


Fig. 6. Structures of the acetogenins isolated from the leaves of *Annona purpurea*.

*Tetrapleura tetraptera*

The above-mentioned applications of TSP in the analysis of crude drug extracts of plant origin all involve the detection of known compounds in complex mixtures. However, LC–TSP-MS can also be used in the detection and on-line identification of unknown products in vegetable extracts. In order to illustrate this potential, results of the LC–TSP-MS analysis of the methanolic extract of the fruits of the African tree *Tetrapleura tetraptera* Taub (Leguminosae) are described (see also the paper by Maillard and Hostettmann [23], elsewhere in this volume).

*T. tetraptera* is considered to be one of the most promising plants for the local control of the parasitic disease schistosomiasis in Africa [24]. Its fruits contain a large amount of molluscicidal saponins which kill the intermediate snails at concentrations similar to those of synthetic compounds [25].

Analysis of the methanolic extract of these fruits by LC–TSP-MS was also performed with postcolumn addition of ammonium acetate.

The TSP total ion current trace (mass range 450–900 u) coincided well with the UV trace at 208 nm (Fig. 7). Each peak on these chromatograms corresponded to a saponin. Retention times were confirmed by injection of pure material. The TSP spectra acquired on-line for each saponin (*e.g.*, saponins **A** and **D** in Fig. 8) displayed strong $[M+H]^+$ quasi-molecular peaks, together with adduct species such as $[M+CH_3CN+NH_4]^+$, confirming their molecular masses.

Fragmentation of the sugar moiety was observed and peaks corresponding to the loss of one (mono- and diglycosides) and then two sugars (diglycosides) were present. For example, in the spectrum of saponin **D** (Fig. 8), the peaks at m/z 718 $[(M+CH_3CN+NH_4)-162]^+$ and *m/z* 660 $[(M+H)-162]^+$ accounted for the elimination of the terminal galactosyl moiety. In addition, an important peak $[A+H]^+$ {or $[(M+H)-162-203]^+$} for the aglycone was visible in these spectra.

All the structural information provided on-line by the LC–TSP-MS technique, *i.e.*, molecular

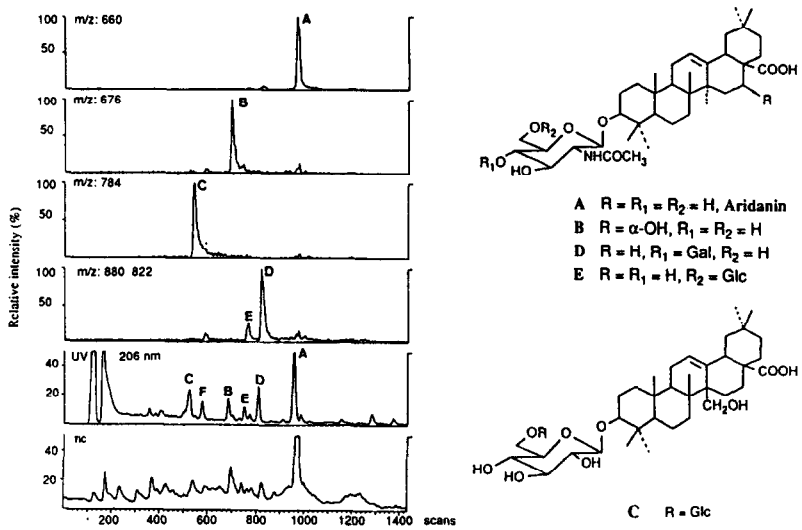


Fig. 7. LC–TSP-MS analysis of the methanolic extract from the fruits of *Tetrapleura tetraptera*. Column, μBondapak $C_{18}$; eluent, $CH_3CN$–$H_2O$ gradient from 30:70 to 80:20 in 30 min; flow-rate 1 ml/min; detection, UV at 206 nm; TSP vaporizer, 100°C; source, 270°C; filament off; buffer, 0.5 *M* $CH_3COONH_4$, at 0.2 ml/min, 1.5 s per scan.

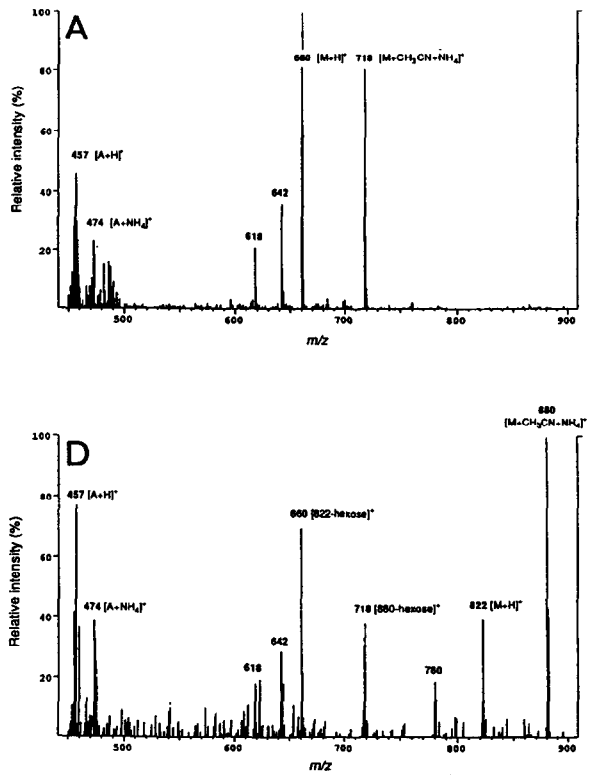


Fig. 8. TSP mass spectra of saponins **A** and **D** obtained after on-column analysis of the methanolic extract of *Tetrapleura tetraptera*. Conditions as in Fig.6.

mass, number, nature and sequence of the sugars and molecular mass of the aglycone, are of great help in structure elucidation processes.

## CONCLUSIONS

The coupling of LC and MS has not yet been widely used in phytochemical analysis. However, some encouraging results have already been obtained with the TSP interface. Qualitative information (including molecular mass and fragmentation pattern) on the detected peak are provided by this technique. In addition, when all the ionization parameters are optimized and well stabilized, semi-quantitative analyses can be performed, and low limits of detection, comparable to those obtained by GC–MS, for example, are obtained.

For all these reasons, LC–TSP-MS is very useful for different applications, such as the detection of known compounds in crude plant extracts, the determination of trace compounds in complex mixtures and the identification of unknown products in extracts.

The LC–TSP-MS offers an additional instrumental technique to the modern phytochemi-

cal laboratory. It is relatively simple to use, and therefore will find many applications in routine analysis.

ACKNOWLEDGEMENTS

Financial support was provided by the Swiss National Science Foundation, the Association of Swiss Pharmacy and the Mediplant Foundation.

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