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# Analysis of taxol and related diterpenoids from cell cultures by liquid chromatography–electrospray mass spectrometry

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ABSTRACT

Authentic taxanes (taxol, 10-deacetyltaxol, cephalomannine, 10-deacetylcephalomannine, baccatin III) and extracts from cell cultures derived from various yew tree species have been analyzed by microbore high-performance liquid chromatography (HPLC)–electrospray mass spectrometry (ESMS). All gave excellent positive-ion ES spectra with dominant protonated molecules at low nozzle-to-skimmer bias value (45 V). By increasing the voltage value to 85 V, fragmentation increased and structurally informative spectra were obtained. The fragments found were both of the C-13 side-chain and of the taxane ring, so their analysis gave important information about the taxane structure and any chemical modifications at different positions of the molecule. When tandem MS was used (argon gas, 25 eV collision energy), fragments similar to those obtained from collision-induced dissociation in the source were detected. The cell culture extracts were analyzed by microbore HPLC–ESMS and excellent spectra were obtained on 5–10 ng of separated compounds; even greater selectivity and sensitivity were obtained through use of selected-ion monitoring (SIM). With SIM, 100 pg of all taxanes could readily be detected. In the HPLC–ESMS mode, only 10% of the eluent was mass-analyzed, so 90% would be available for recovery through fraction collecting.

## INTRODUCTION

Taxol, a natural component extracted from the bark of the yew tree, *Taxus brevifolia*, has been shown to be an efficient antitumor agent especially in the treatment of ovarian, breast and skin cancer [1,2] (Fig. 1). In 1992, in the United States, more than 200 000 patients suffered from these ailments and could have benefited from taxol administration. Unfortunately, naturally occurring taxol is only available in low amonts ($\approx$100 mg/kg of dry yew tree bark) and there are only a finite number of these slow-growing evergreens harvestable. Excessive cutting of these trees could endanger the species, so alternative sources of taxol are clearly required.

Partial synthesis of taxol and related diterpe-

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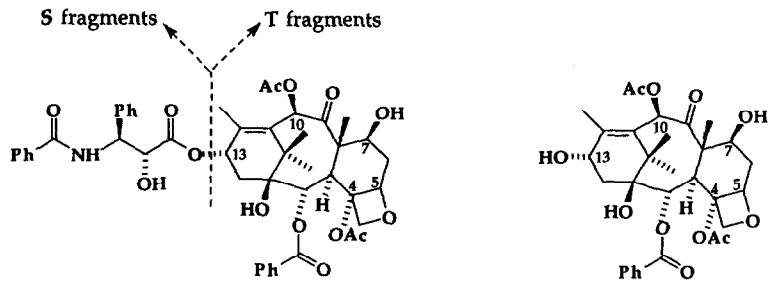


Fig. 1. Structures of taxanes, taxol and Baccatin III, illustrating the side-chain (S) and ring structure (T) fragments produced in ESMS.

noids (taxanes) from baccatin III or 10-deacetylbaccatin III (Fig. 1) has already been described [3,4]. Total synthesis has not yet been achieved. Recently, yew tree cell cultures have been shown to provide reasonable amounts of taxol and to offer a possible long-term solution to the availability of taxol (ESCAgenetics, unpublished results).

Among taxanes, many analogues have been shown to have potent antitumor activity [3–5], and for study of these different compounds efficient and sensitive methods for separation and characterization are required. Different methods of identification and quantitation of taxanes, such as high-performance liquid chromatography (HPLC) [6,7] and enzyme-linked immunosorbent assays (ELISA) [8], are currently used to screen plant extracts. Mass spectrometry (MS) has been shown to be a powerful technique for taxane identification. Electron impact (EI), chemical ionization (CI), desorption chemical ionisation (DCI), fast atom bombardment (FAB), particle beam and electrospray ionization (ES) techniques have all been used for the MS analysis of taxol and taxol-related diterpenoids [9–11]. Auriola *et al.* [12] have reported characterization and quantification of taxanes by HPLC–thermospray MS and Hoke *et al.* [13] recently described a tandem mass spectrometric (MS–MS) method using negative-ion DCI-MS–MS for the rapid screening of taxol, cephalomannine and baccatin III in the bark of different species of yew trees. Detection limits of approximately 300–500 pg were achieved.

ESMS has recently been demonstrated to be an excellent technique for the analysis of low-molecular-mass polar compounds, *i.e.* steroids, drugs and metabolites [14] as well as peptides and proteins [15–17]. It is an appropriate method for the direct coupling of liquid chromatographs to a mass spectrometer. We describe a fast and sensitive method for separation and characterization of taxanes in cell culture extracts using microbore HPLC combined with triple quadrupole ESMS.

## EXPERIMENTAL

### *Sample preparation*

A sample of biomass from cell culture (~2 g) was homogenized in 10 ml of methanol three times. The extracts were combined and evaporated to dryness. The residue was then partitioned between 10 ml of water and 10 ml of dichloromethane. The organic fraction was applied to a Bond Elut $NH_2$ SPE cartridge (Alltech, Deerfield, IL, USA) and eluted with 4 ml of dichloromethane and 4 ml of 15% methanol in dichloromethane. The methanol–dichloromethane eluate was evaporated to dryness and redissolved in 50–100 μl methanol for HPLC and HPLC–MS.

### *Reversed-phase high-performance liquid chromatography*

Microbore reversed-phase HPLC (RP-HPLC) was performed on a Michrom microbore HPLC system (Michrom BioResources, Pleasanton, CA, USA) using a 150 mm × 1 mm I.D. Vydac $C_{18}$ column (5 μm particle size: 300 Å pore size).

The flow-rate was typically 40 μl/min. Splitting 10:1 of the flow was achieved directly in the UV cell allowing 4 μl/min to be analyzed by the mass spectrometer (Fig. 2). Eluent A was a mixture of 75% water, 20% methanol and 5% acetonitrile. Eluent B was a mixture of 80% acetonitrile and 20% methanol. Separation of the different taxanes was achieved by running a linear gradient from 5 to 100% B in 30 min.

*Electrospray ionization mass spectrometry*

ESMS was performed on a VG Bio-Q triple quadrupole mass spectrometer (Fisons VG BioTech, Altrincham, UK). The instrument was controlled, and data were analyzed using LabBase software (VG BioTech). The electrostatic-spray ion (electrospray) source was operated at 3.2 kV. The nozzle-to-skimmer bias value (*Δns*) was typically varied from 45 to 100 V. Full-scan mass spectra were recorded from mass-to-charge ratio (*m/z*) 230 to 1030 in 3.4 s. Selected-ion monitoring (SIM) was achieved by taking data for each selected ion for 0.6 to 1 s.

MS–MS experiments were carried out on the same instrument. The collision energy for collision-induced dissociation (CID) was 25 eV. The collision gas (argon) was used at a pressure sufficient to suppress the precursor ion beam to about 30% of its initial value.

Pure taxanes were dissolved in a 1:1 water–methanol solution at a concentration of approximately 10 pmol/μl. Typically, 4 μl/min was introduced into the ionization source using the standard probe provided by the manufacturer.

Fractions eluted from RP-HPLC were analyzed directly using a VG BioTech probe modified in this laboratory (Fig. 2). Typically, 2 μl of methanol containing 0.2% of trifluoroacetic acid were added per min as a sheath liquid prior to pneumatically assisted electrospray nebulization.

## RESULTS AND DISCUSSION

*Mass spectrometry of taxanes*

Samples of different authentic compounds were analyzed in order to determine the capabilities of electrospray ionization for taxane analysis. Table I gives the *m/z* values of the protonated molecules for five common taxanes. Fig. 3A shows the ES mass spectrum of 20 pmol of taxol using direct infusion particularly illustrating the protonated molecules $[M + H]^+$ at *m/z* 854.3.

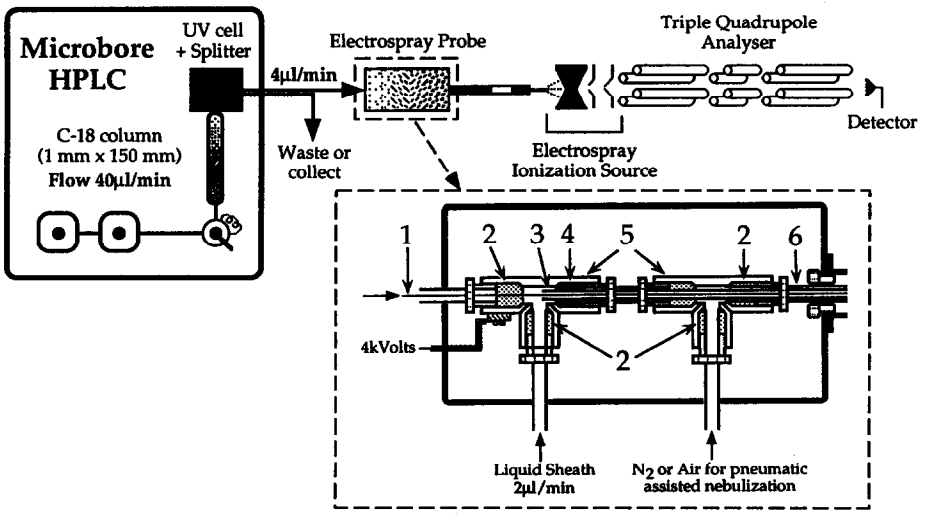


Fig. 2. Schematic of the microbore HPLC–ESMS system. The system was designed for using either pure electrospray or pneumatically assisted electrospray. 1 = Fused-silica tubing, 150 μm O.D., 50 μm I.D.; 2 = vespel ferrule; 3 = stainless-steel tubing, 175 μm I.D.; 4 = graphite ferrule; 5 = stainless-steel tee; 6 = PTFE tubing (insulator).

TABLE I

LIST OF TAXANES

Monoisotopic $m/z$ values are given.

| Compound | $[M + H]^+$ | Mass of taxane ring fragments | | | Mass of side-chain fragments | |
|---|---|---|---|---|---|---|
| | | $T^+$ | $T - H_2O^+$ | $T - AcOH^+$ | $SH_2^+$ | $S - O^+$ |
| Taxol | 854.3 | 569.2 | 551.2 | 509.2 | 286.1 | 268.1 |
| 10-Deacetyltaxol | 812.3 | 527.2 | 509.2 | – | 286.1 | 268.1 |
| Cephalomannine | 832.4 | 569.2 | 551.2 | 509.2 | 264.1 | 246.1 |
| 10-Deacetylcephalomannine | 790.3 | 527.2 | 509.2 | – | 264.1 | 246.1 |
| Baccatin III | 587.2 | 569.2 | 551.2 | 509.2 | – | – |

Using a low nozzle-to-skimmer bias value ($\Delta ns$ = 45 V), very few fragments were detected within the scanned mass range ($m/z$ 230–1030), and so little structural information was obtained under these conditions. Nevertheless, fragments can be produced in the atmosphere–vacuum interface by increasing the $\Delta ns$ as described previously [17–19]. Fig. 3B is the mass spectrum of taxol recorded with a $\Delta ns$ value of 85 V. The major peaks detected at $m/z$ 268, 286, 509, 551 and 569 correspond, respectively, to the C-13 side-chain ($m/z$ 268 and 286) and to fragments of the taxane ring ($m/z$ 569, 551, 509). Formation of the side-chain fragments (S fragments) and the taxane ring fragments (T fragments) in ESMS is similar to the fragmentation observed in the FAB mass spectra

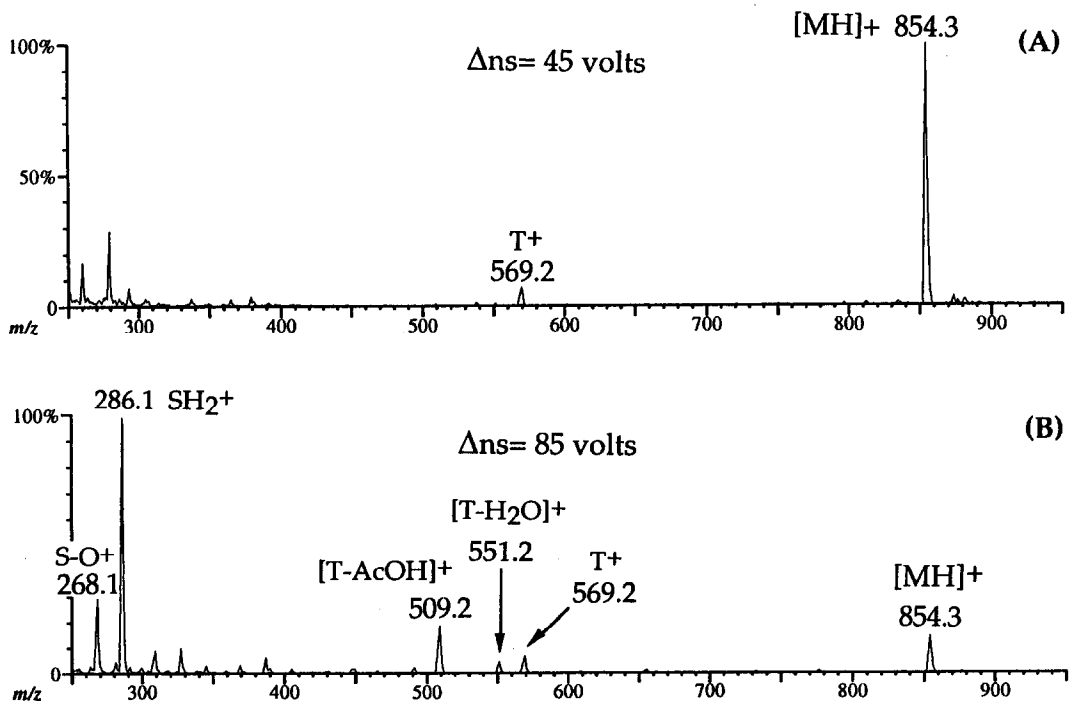


Fig. 3. ES mass spectra of taxol (17 ng, 20 pmol) recorded at $\Delta ns$ = 45 V (A) and 85 V (B). Direct infusion solvent: water–methanol (1:1) containing 1% acetic acid.

of taxol reported recently by McClure *et al.* [10] (*m/z* 286, $SH_2^+$; *m/z* 268, S − $O^+$; *m/z* 569, $T^+$; *m/z* 551, $[T - H_2O]^+$, *m/z* 509, $[T - AcOH]^+$). Analysis of these fragments gives important information about the structure of the taxane and any chemical modifications of different positions of the molecule. In addition to molecular mass information, Table I gives the *m/z* value of the S fragments ($[S + 2H]^+$) and the T fragments ($T^+$, $[T - H_2O]^+$, $[T - AcOH]^+$) of the different taxanes.

Among taxol-related diterpenoids, most of the structural variations occur on the C-13 side-chain and the taxane ring is generally conserved. Deacetylation at position 10 is the most frequent modification encountered on the taxane ring. The main consequence of 10-deacetylation is a higher yield of side-chain fragment ions by CID in the atmosphere–vacuum interface. The spectrum of 10-deacetylated taxol is dominated by the $SH_2^+$ fragment at *m/z* 286, and the fragments (*m/z* 527, $T^+$) *m/z* 509, $[T - H_2O]^+$) are of low abundance. The relative abundance of S fragments compared to the abundance of the $T^+$ fragment is higher in 10-deacetyltaxol than in taxol. This was a general observation when analyzing taxanes and their 10-deacetyl homologues. The same behavior of 10-deacetyltaxanes was observed in MS–MS (see below).

*Tandem mass spectrometry*

The low-collision-energy MS–MS spectrum of the protonated molecules of taxol is shown in Fig. 4. The pattern observed at collision energy 25 eV, using argon as collision gas, is similar to that obtained by collision activation in the electrospray interface as illustrated in Fig. 3A and B. The major fragments observed are the $SH_2^+$ fragment (*m/z* 286) and T fragments at *m/z* 569, 551 and 509. Under these conditions, the ratio be-

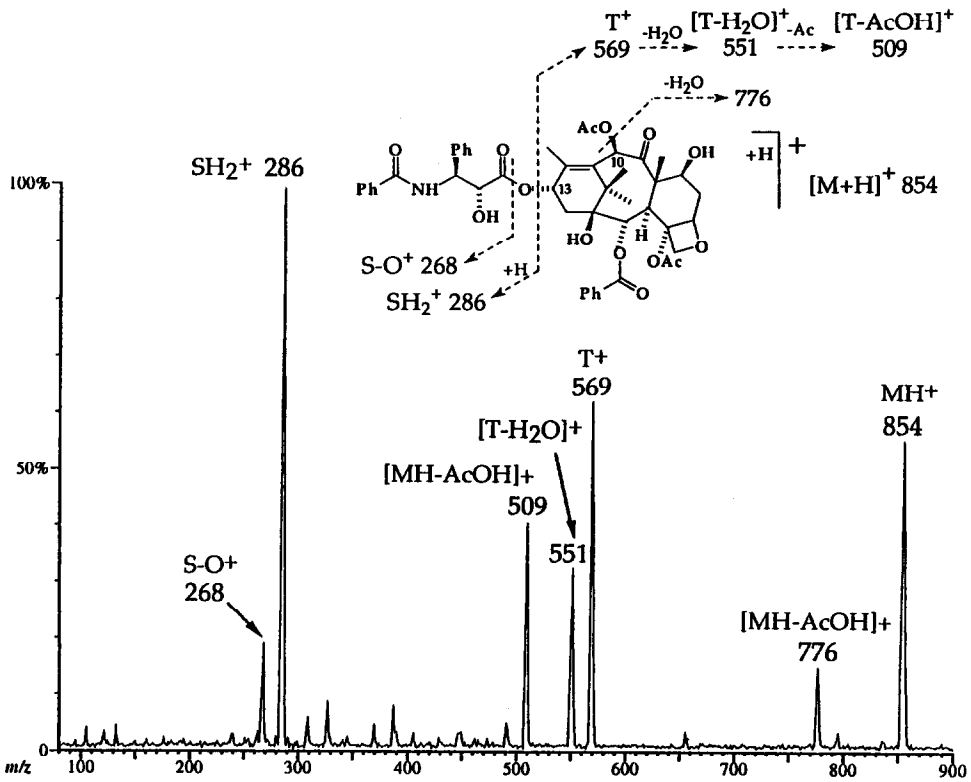


Fig. 4. CID mass spectrum of taxol $[M + H]^+$ 854.3. Collision energy: 25 eV. Collision gas: argon, $3 \cdot 10^{-4}$ mbar.

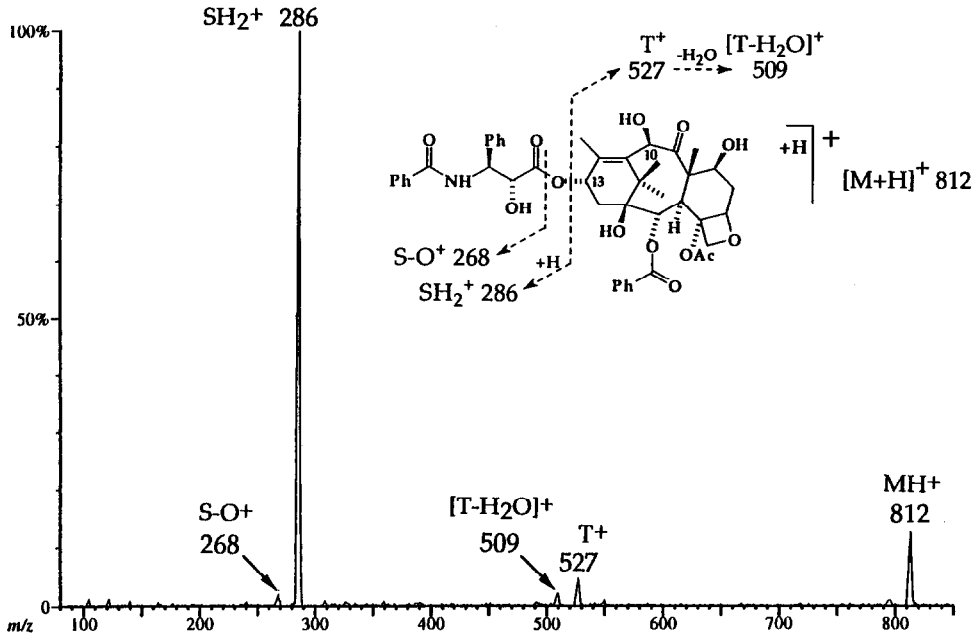


Fig. 5. CID tandem mass spectrum of 10-deacetyltaxol $[M + H]^+$ 812.3. Collision energy: 25 eV. Collision gas: argon, $3 \cdot 10^{-4}$ mbar.

tween the $SH_2^+$ fragment and the $T^+$ fragment is approximately 3:2. Fig. 5 shows the CID-MS–MS spectrum of 10-deacetyltaxol recorded under the same conditions used for taxol. The ratio between the $SH_2^+$ ion at *m/z* 286 and the $T^+$ fragment at *m/z* 286 is 15:1. The higher abundance of the $SH_2^+$ fragment (compared to taxol) may be explained by location of the charge principally on the C-13 side-chain. Intramolecular proton transfer from position 10 to the side-chain during the fragmentation process may also explain this finding. In a previous investigation using EI, the $SH_2^+$ fragments were considered to have been produced through a McLafferty rearrangement [10].

However, in all the examples we have studied, the formation of the S fragments and the taxane ring T fragments were shown to be informative and characteristic of the taxane structure.

*Microbore HPLC–ESMS of taxanes*

Direct analysis of taxane mixtures was achieved by using microbore HPLC separation combined with ESMS. The interface allowed 4 μl of the eluting solvent to be analyzed per min by the mass spectrometer necessitating a 10:1 flow split. The sensitivities achieved for taxane detection were in the order of 5–10 ng with a signal-to-noise ratio for the total ion current (TIC) signal greater than 10. However, in spite of the high sensitivity, the TIC signal corresponding to taxanes may be masked by the presence of other components present in the complex mixture. Better selectivity and higher sensitivity was achieved through use of SIM of the protonated molecules corresponding to the different taxanes. Fig. 6 shows SIM chromatograms of $[M + H]^+$ 586, 790, 832 and 856, respectively, from a mixture of 100 pg each of baccatin III, 10-deacetylcephalomannine, cephalomannine and taxol. The unsmoothed chromatogram yielded a signal-to-noise ratio greater than 5. This method is therefore excellent for the detection of low amounts of taxanes.

*Detection of taxanes from cell culture extracts by microbore HPLC–CID-ESMS*

When complex cell extracts are analyzed by HPLC–MS, taxane peaks may be masked by the presence of other components. As the taxane ring is generally conserved within different taxanes,

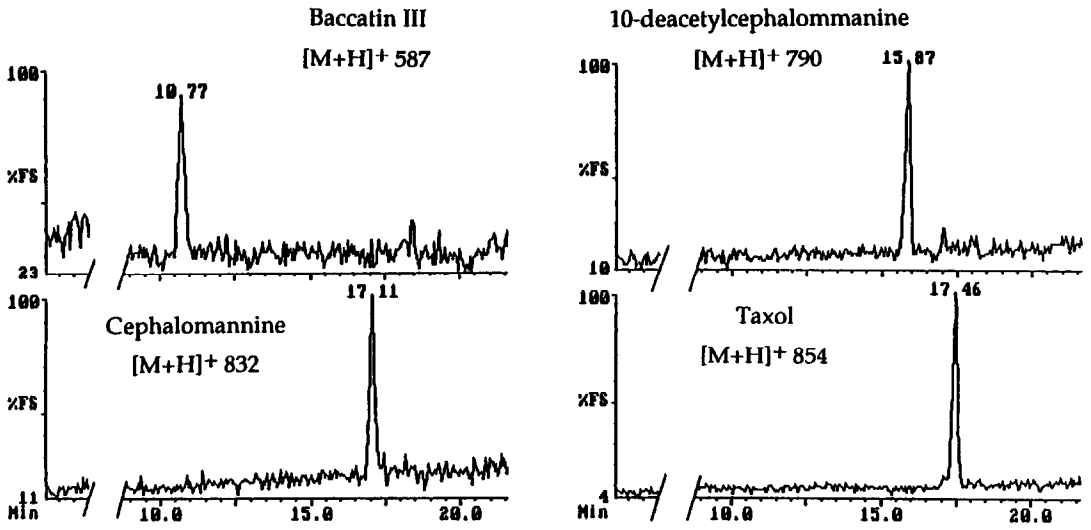


Fig. 6. SIM of the protonated molecules *m/z* 587, 790, 832 and 854 corresponding to baccatin III, 10-deacetylcephalomannine, cephalomannine and taxol from a mixture of 100 pg each.

we used the *m/z* values of the different T fragments obtained by CID in the ES interface as markers to locate taxanes in a mixture. Fig. 7 shows the selected-ion chromatograms (obtained from full scans 230–1030 a.m.u.) of the T fragment ions *m/z* 569, 551 and 509, produced by source CID at a *Δns* value of 85 V. Reporting only these ions greatly simplified the chromatogram. The simultaneous presence of the T fragments may strongly indicate the presence of a taxol-related compound. In our case, cephalomannine and taxol were located at retention times of 16.7 and 17.0 min, respectively. In addition to these taxanes, another compound present in the mixture with a retention time of 18.0 min showed the presence of the three T fragments. This compound was identified as a new taxane and its structure is currently under investigation (ESCAgenetics, unpublished results).

Using CID in the ES interface, adequate sensi-

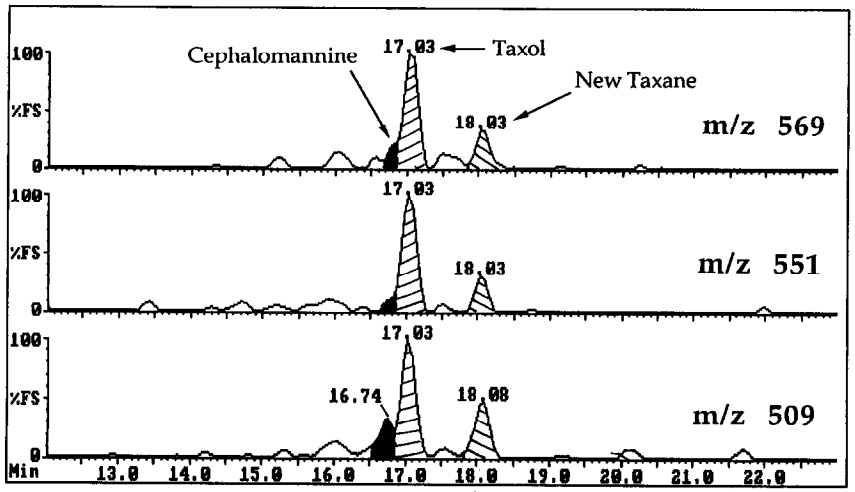


Fig. 7. Methanol extract of biomass from cell culture. Selected ion chromatogram constructed from full-scan data (*m/z* 230–1030) of the T fragments obtained by CID in the ES interface. *Δns* = 85 V.

tivity was achieved in the SIM mode since 200 pg could be detected with a signal-to-noise ratio of greater than 5. The separatory method is essentially non-destructive since 90% of the material eluting from the HPLC column is not mass-analyzed, and separate fractions could be collected and subjected to alternative analytical procedures.

## CONCLUSION

In this study, electrospray ionization was shown to be a sensitive and efficient method for the analysis of taxane-containing cell culture extracts. It certainly is the method of choice for interfacing with a microbore (or capillary) HPLC system. Detection at the level of 100–200 pg per component was achieved using SIM. Formation of specific side-chain fragments and taxane ring fragments by CID in the ES interface was used to indicate the presence of a series of taxanes in cell culture extracts. The HPLC–ESMS method has been shown to be effective for confirmation of taxane structure within a complex plant cell matrix and, in conjuction with other separation techniques and physicochemical analyses, the method has proved useful for discovery of new compounds within plant cell cultures.

## ACKNOWLEDGEMENTS

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