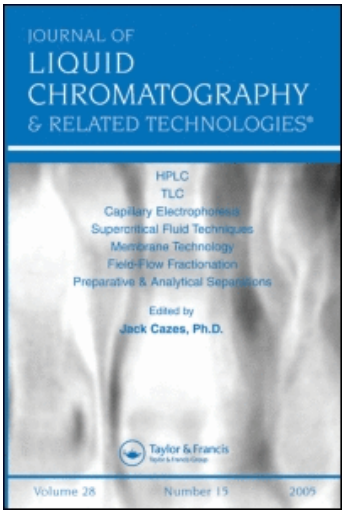

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ANALYSIS OF TAXINES IN *TAXUS* PLANT MATERIAL AND CELL CULTURES BY HPLC PHOTODIODE ARRAY AND HPLC-ELECTROSPRAY MASS SPECTROMETRY

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ABSTRACT

A semi-purified *Taxus baccata* needles extract was analysed by RP-HPLC. More than 18 taxines and cinnamates were detected by photodiode array detection and LC-MS, 10 of them being positively identified. Furthermore, 10-deacetyl baccatin III (paclitaxel's main precursor) and other taxanes were also found in the extract. Taxines were also detected in numerous extracts of plant and cell culture extracts of various origins. The identification of the taxines in the extracts was made by correlation of retention

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and spectral data. LC-electrospray MS verified the identification of the known taxines in *T. baccata* seeds, needles, and pollen.

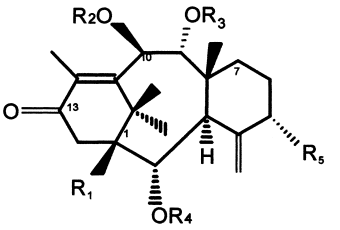
INTRODUCTION

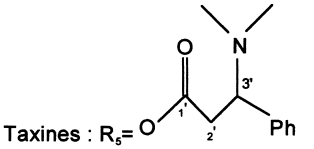
Taxine is known as the main toxic constituent of *Taxus* species and is associated with deaths of both humans and domestic animals. Lucas first reported about an alkaloid substance, which he called taxine and to which the poisonous properties were attributed(1). Taxine was later shown to be a mixture of at least 11 alkaloids,(2-4) with taxine A and taxine B as the main constituents. Taxines are very strong cardiotoxic agents; they cause convulsions, fall of blood pressure, and stopping of the heart in diastole. Phytochemical work on *Taxus* during the 1940's and 1950's was focused on taxines and their cinnamate analogues. The discovery of the potent cytostatic agent paclitaxel in *Taxus* in the late 1960's(5) overshadowed any interest in taxines. The last years effort was put again on the isolation and structural characterisation of taxines from *T. baccata* by the groups of Appendino and Potier(6-10) and from *T. x media* 'Hicksii(11).

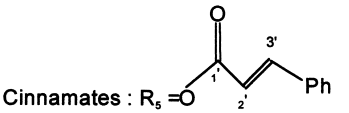
Taxines are relatively abundant in plants, especially in *T. baccata and T. cuspidata*; in contrast the taxine fraction is almost absent in *T. brevifolia*(12). Therefore, they can serve as an alternative starting material for semi-synthetic production of paclitaxel or paclitaxel derivatives. There are significant structural resemblances between taxine B and taxanes (see Figure 1). Both groups share the main taxane ring; moreover, the C-5 side chain of the taxines has a close spatial position to the C-13 side chain of the taxanes (the latter is essential for antitumor activity). A biosynthetic hypothesis involved the intermediacy of a C-5 to C-13 ester transfer; it was also synthetically demonstrated that taxine B was converted into a baccatin V derivative(12). However, taxines do not show intrinsic antitumor activity, whereas their cardiotoxicity is higher compared to paclitaxel. Pacliataxel's cardiotoxicity is an undesirable effect for its therapeutic use. Hence, it was assumed that the structural features necessary for the cardiotoxicity are different from those necessary for the antitumor activity.

Understanding the factors responsible for the toxicity of taxines could lead to the design of paclitaxel analogues with diminished cardiotoxicity(13). Sophisticated analytical methods are, therefore, necessary for their analysis and determination in plant or biological samples.

Research work on taxines was hampered by their instability and loss of activity during storage, due to the loss of the dimethylamino function from the C-3' atom (See Figure 1) and formation of the corresponding cinnamates(14-16). Isolation of taxines is accomplished by alkaline/base extraction, but it is difficult to obtain them in a purified form, due to rapid acetate isomerisation and/or decomposition to cinnamates. Moreover, they often interfere in extraction, isolation, and analysis of paclitaxel. In a recent report, Jenniskens et al.(15) identified

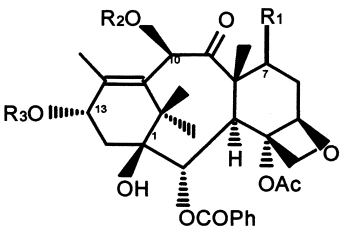




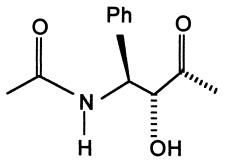


(1) R_1= OH, R_2 = R_3= R_4= H
(2) R_1= H, R_2= Ac, R_3= R_4= H
(3) R_1= OH, R_3= H, R_2= R_4= Ac
(4) R_1= OH, R_2= Ac, R_3= R_4= H
(5) R_1= OH, R_3= Ac, R_2= R_4= H
(6) R_1= H, R_3= Ac, R_2= R_4= H
(7) R_1= OH, R_2= H, R_3= R_4= Ac
(8) R_1= OH, R_2= R_3 = R_4= Ac
(9) R_1=H, R_2=R_3=R_4=Ac

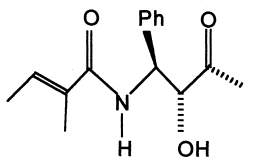
(10) R_1= OH, R_2= R_3 = R_4= H
R_1= R_2= R_3 = R_4= H
R_1= OH, R_2= R_3 = R_4= Ac
R_1= H, R_2= R_3 = R_4= Ac



Paclitaxel: R_1= OH (β), R_2= Ac, R_3= Ph



Cephalomannine: R_1= OH (β), R_2= Ac, R_3=



Baccatin III: R_1= OH (β), R_2= Ac, R_3= H

10-deacetyl baccatin III: R_1= OH (β), R_2= H, R_3= H

Figure 1. Molecular structures of taxines, cinnamates, and taxanes.

and isolated six taxine alkaloids from the needles of *T. baccata*. Acidic extraction with 0.5% (v/v) H_2SO_4 was followed by an extraction with Et_2O and basic extraction with 25 % aq. NH_3. Taxines were recovered with $CHCl_3$, with a yield of 0.5-1% for the total crude taxine fraction. This report comprises the first analytical methodology for the determination of taxines. HPLC analysis employed a C_{18} column, eluted by a linear gradient of acetonitrile over methanol and 0.05 M sodium dihydrogen phosphate buffer.

In a recently presented paper, Potier's group, in their search for high producing plants, studied the analysis of four taxines and 10-DAB III in *Taxus* needles.(17) The authors developed a normal phase separation system (silica eluted with chlorofom:methanol:triethylamine 99.4/0.5/0.1) for the evaluation of the total taxine content in *Taxus* needles. To separate the taxines, they used reversed phase (C_{18} eluted with acetonitrile:water:tetrahydrofuran 23/77/0.5). The method, although suffering from peak asymmetry and tailing, was useful for preparative objectives since it avoids the use of buffers. Taxines have also been determined by GC-MS(18) in a search of *Taxus* poisoning of cattle and horses. Since taxines are molecules that are too large to be analysed by GC, the authors actually measured an ion at 134 m/z^+, which was attributed to a specific fragment at the 2' atom of the taxine side chain (Figure 1).

The present paper reports on the analysis of taxines by HPLC-photodiode array detection (PDA) and HPLC-electrospray mass spectrometry. A semi-purified taxine extract, obtained with acid/base extraction of *T. baccata* needles,(15) was analysed in reversed phase HPLC; nine taxines, one cinnamte and six taxanes were found to be present in the sample. Identification of the peaks was made with on-line LC-MS and off-line NMR following fraction collection at the end of the HPLC. Retention and spectral data of the identified peaks were used as a tool to screen for the taxines and cinnamates in plant and cell culture extracts. Several members of both groups of compounds were found in samples of various origin. LC-MS which verified the presence of some of the known taxines and cinnamates in extracts of *Taxus* needles, pollen, and seeds.

EXPERIMENTAL

Materials and Methods

A semi-purified taxine extract, obtained according to Jenniskens et al.,(15) was described to contain at least six taxines with taxine B (**4**) as the main component. The mixture was also expected to contain some cinnamates as products of taxine's degradation. A solution of 2.8 mg/mL of the semi-purified taxine mixture in ACN was used for the analysis. Solutions of lower concentrations (1.4-0.35 mg/mL) were obtained by diluting the first solution with ACN.

Paclitaxel and 10-deacetyl baccatin III (10-DAB III) standards were obtained from Sigma (St. Louis, MO, USA).

Methanol, ethanol, acetonitril, chloroform, and dichloromethane were all distilled in house prior to use. Tetrahydrofuran was from Janssen (Beerse, Belgium). Ammonium acetate p.a. was obtained from Merck (Darmstadt, Germany). Water was of Milli-Q quality. All solvents were filtered through a 0.45 μm RC 55 membrane filter (Schleicher & Schuell, Dassel, Germany).

Chromatographic Conditions

HPLC analysis was carried out with a Waters gradient HPLC system consisting of a 600 E pump, a WISP 712 autosampler, a 991 photodiode array detector, and a 5200 printer/plotter. Injection volume was 20 μL throughout the study. Detection with the photodiode array system was in the range 190-400 nm; quantitation was accomplished in three wavelengths: 227, 249, and 280 nm.

Two Phenomenex columns (Torrance, CA, US) were used for the HPLC separation: Ultracarb C_{18}, 5 μm, 60 Å, 150 × 4.6 mm and Luna C_{18}, 60 Å, 5 μm, 150 × 4.6 mm. The columns were eluted with the use of gradient programs (see Table 1).

A number of experiments was performed with a chromatographic system used earlier for the determination of taxanes(19): Column: Novapak phenyl, 4 μm, 60 Å, 150 x 3.9 mm from Waters (Milford, MA, USA), eluted with gradient program 2 (see Table 1).

During the whole study the columns were protected by a precolumn 20 x 4.6 mm, filled, in-house, with Lichrosorb RP-18, 10 μm material, obtained from Merck.

Sample Pre-treatment

Solid Phase Extraction

SPE was performed as reported previously.(20) LC-18 SPE cartridges (Supelco, Bellefonte, PA, USA) were conditioned by consecutive washing with 2 mL of MeOH and 2 mL of water. Following sample application, the cartridges were washed with 2 mL of water and then eluted with 2 mL of 20% MeOH in water, 2 mL of 50% MeOH in water, 2 mL of MeOH, and 2 mL of $CHCl_3$.The last two fractions were collected and subsequently evaporated to dryness in a speed vac. The residues were reconstituted in 100 μL of ACN and 10 μL of the resulting solutions were analysed by HPLC.

The recovery of the SPE for the taxines was evaluated by extraction of three different known amounts (56, 140, and 280 μg) of the reference samples used for quantitative analysis.

Table 1. Gradient Separation Programs for the Analysis of Taxines

Program 1 Column: Novapak; Flow Rate : 0.8 mL/min; Solvents: A: 0.05 M CH_3COONH_4 : ACN 7: 3; B: H_2O : ACN 1 : 9.			Program 2 Column: Ultracarb; Flow Rate : 0.6 mL/min; Solvents: A: 0.05 M CH_3COONH_4 : ACN 7: 3; B: H2O : ACN 1 : 9.			Program 3 Column: Luna; Flow Rate : 0.8 mL/min; Solvents: A: 0.05 M CH_3COONH_4; B: MeOH.		
t (min)	A %	B %	t (min)	A %	B %	t (min)	A %	B %
0	100	0	0	80	20	0	65	35
30	66	34	30	0	100	38	25	75
32	100	0	32	0	100	38.1	0	100
			34	80	20	44.5	0	100
						45	65	35

Needles

An amount of 2.8 g of *T. baccata* needles was treated in a blender and was homogenised in a Ystral homogeniser with 50 mL MeOH. The homogenate was sonicated for 10 min, soaked by agitation for 2 hours, and subsequently filtered. The residue was extracted twice with 50 mL of MeOH following the same procedure. The three methanolic fractions were combined and evaporated to dryness in a rotary evaporator. The residue was reconstituted in 3 × 1 mL of MeOH. An aliquot (0.2 mL) of the solution was brought up to 10 % of MeOH in water, and applied on SPE cartridge.

Seeds

Five grams of *T. baccata* seeds were treated in the blender and an amount of 4.8 g was further processed for extraction, following the scheme described above for the extraction of needles.

Pollen

Pollen (2 g) were macerated with 3 × 10 mL of a $CHCl_3$-MeOH mixture (1:1) and the resulting solution was filtered through a Whatman paper filter. The filtrate was evaporated to dryness under vacuum. After washing with n-C_7H_{16} to

remove the lipophilic contaminants the residue was resuspended in $CHCl_3$. The organic layer was extracted twice with water and subsequently evaporated to dryness. The residue was reconstituted to solution with 2 mL of MeOH and processed further by SPE as already described below.

Cell Material

The sample pre-treatment of cell culture was according to methods previously described.(20) For the cell suspension medium, a volume of 10 mL was filtered through a filter paper and then applied onto SPE. As with the plant extracts, the cartridge was washed with 2 mL of water, 2 mL of 20% MeOH and 2 mL of 50% MeOH. The compounds of interest were eluted with 1.2 mL of MeOH. Calli and cell material were first lyophilised and amounts ranging from 50 to 150 mg were extracted twice with 20 mL of MeOH with the help of sonication and agitation. The methanolic fraction was evaporated to dryness; the residue was reconstituted in 3 × 1 mL of MeOH. An aliquot (0.5 mL) of the resulting solution was mixed with 4.5 mL of water and then applied onto a SPE cartridge. The above mentioned extraction scheme was followed. the compounds of interest were collected in the pure methanolic fraction and analysed on HPLC.

LC-MS and NMR Analysis

LC-MS analysis was performed on a Finnigan MAT (San Jose, USA) TSQ-70 equipped with a Finnigan MAT electrospray interface. Interfacing was performed with a custom-made electrospray interface with a heated capillary. Sampling capillary and ion source were kept at 250°C and 200°C, respectively. The repeller voltage was optimised for paclitaxel and 10-DAB III. In positive ionisation mode, the optimum voltage was 150 Volts. The optimisation of the mass spectrometer conditions was achieved with the analysis of paclitaxel and 10-DAB III in the constant infusion mode. Splitting of the flow at 19:1 was achieved before the probe allowing 50 μL/min to be analysed by the mass spectrometer. The rest of the flow was directed to a Waters 440 UV detector operating at 254 nm. For the chromatographic separation, two 2150 LKB (Bromma, Sweden) pumps were used to perform the gradient, under the control of an LKB (Bromma, Sweden) 2152 HPLC controller.

H^1-NMR spectra were recorded with a Bruker DPX-300 spectrometer. In order to obtain adequate NMR signals, repeated injections of the taxine mother solution (of 2.8 mg/mL) were performed on the Luna column eluted with the gradient program 3 (Table 1). The fractions were collected, evaporated to dryness, re-dissolved in $CDCl_3$, and further processed by NMR.

RESULTS AND DISCUSSION

Taxine Analysis-Validation

The initial purpose of this study was to analyse taxines with the already existing chromatographic systems for the analysis of taxanes.(19) However, analysis on the Novapak Phenyl column gave a poor fingerprint, with broad coeluting peaks. Yet, it was perceived that taxine peaks, in terms of both retention and spectral characteristics, resembled unknown peaks observed in previous analyses, during screening experiments in plant and cell culture extracts. However, since the chromatographic resolution was not satisfactory, further trials were made with the use of the Ultracarb column. This column resulted in better separation, but failed to separate taxine from isotaxine.

Peak assignment is very critical, especially due to the presence of many isomers which could not be distinguished by either PDA or MS detection. The chromatogram obtained depicted a satisfactory resolution of the taxines and cinnamates present in the purified extract within 40 min (Figure 2). Furthermore, the column showed outstanding reproducibility, robustness, and performance, even after months of use and analysis of several hundreds of cell culture extracts.

Utilisation of a newer column (Luna) resulted in complete separation of the two isomers **(4)** and **(5)**, as can be seen in Figure 3. A definite peak assignment was only achieved by fraction collection at the end of the HPLC column and NMR spectroscopy of the respective fractions. The HPLC fingerprint obtained under these conditions was similar to the one reported by Jenniskens et al.(15) This column was further used in the analysis of seeds, needles, and pollen extracts.

LC-PDA and LC-MS as Identification Tools

The spectrum index plots in Figures 2 and 3 depict the UV spectra of the analysed compounds (taxines, cinnamates and taxanes). A clearer prospect of the corresponding spectra is given in Figure 4. It can be seen that 227 nm represents the maximum for the UV spectrum of taxanes, whereas 280 nm is the λmax in the spectrum of cinnamates and, to a lesser extent, of taxines. Taxanes show low absorption at this wavelength, hence 280 nm can be used as a "selective" wavelength for the detection of taxines and cinnamates. It should be pointed out that taxines and cinnamates can cause problems in the HPLC analysis of paclitaxel, since they often coelute with paclitaxel.(21,22) Castor and Tyler(23) had also reported the detection of an impurity peak in the extracts of *Taxus x media* "Hicksii" needles. The uncharacterised impurity showed a UV maximum at 280

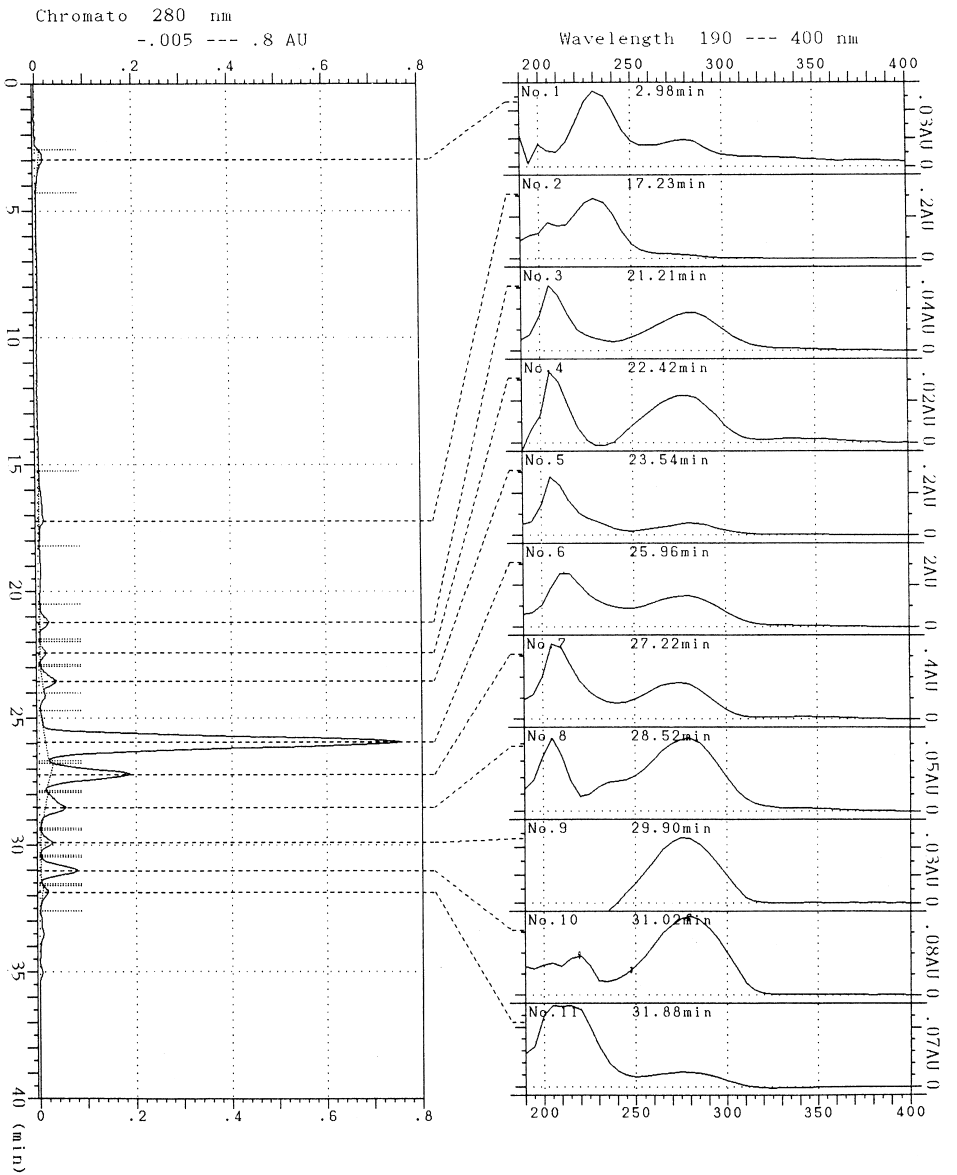


Figure 2. HPLC analysis of the semi-purified taxine extract. Conditions: Column, Ultracarb C_{18}, eluted with gradient program 1 (Table 1); Injection volume, 20 μL; Peak identities in Table 2.

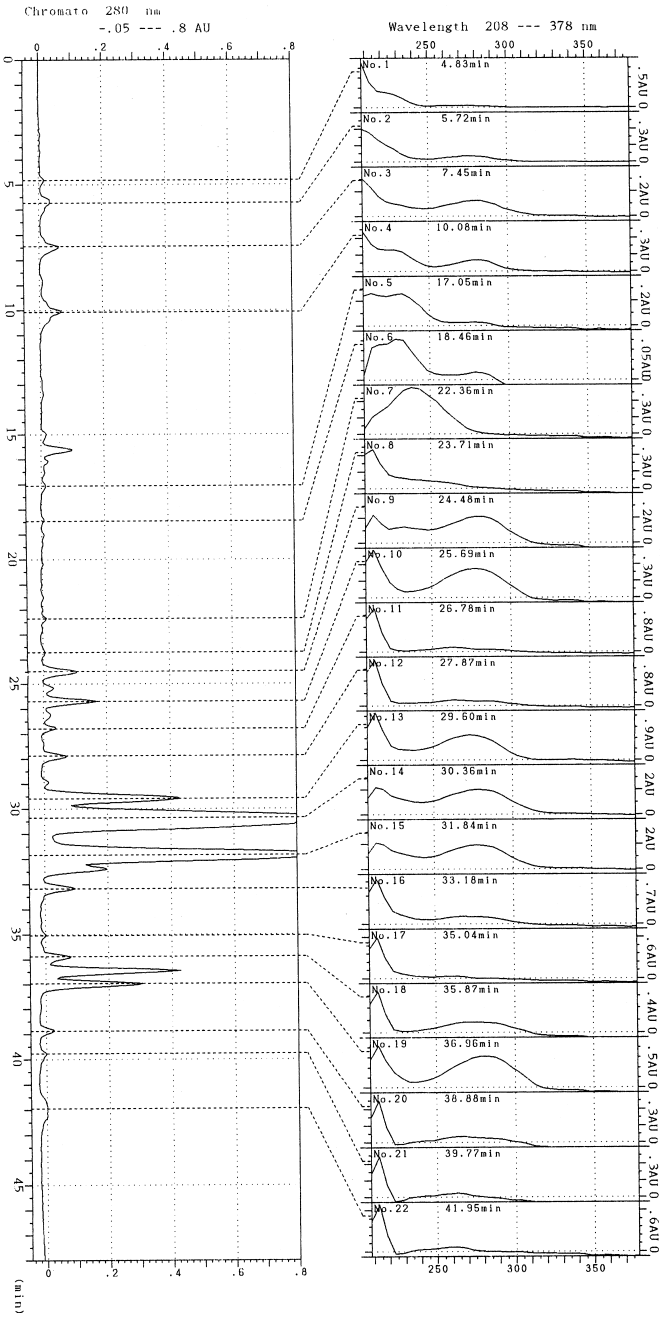


Figure 3. HPLC analysis of the semi-purified taxine extract. Conditions: Column, Luna C_{18}, eluted with gradient program 3 (Table 1); Injection volume, 20 µL. Peak identities in Table 3.

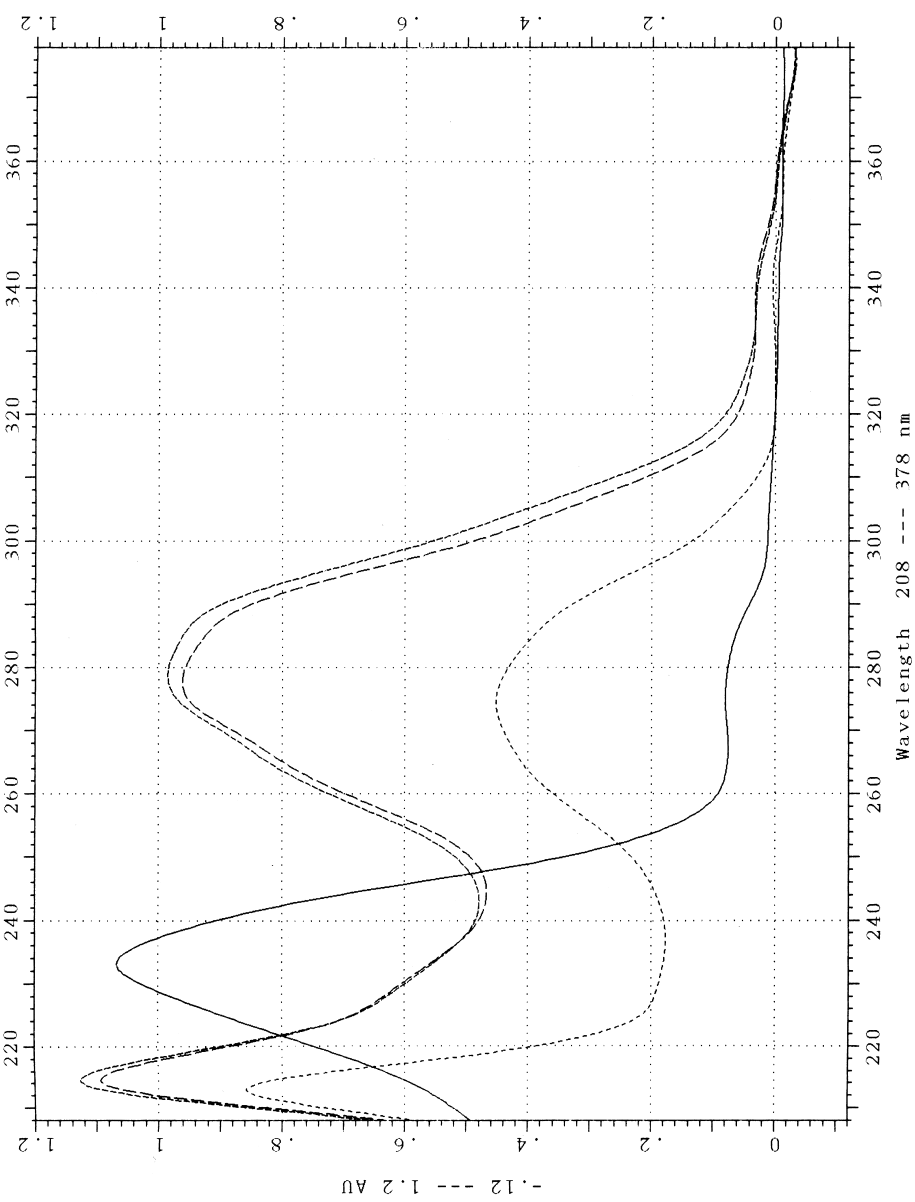


Figure 4. UV spectra of a taxane (solid line), a taxine (—–) and two cinnamates (– –).

nm, with a A280/A228 ratio of approximately 3.5. Using absorbance ratioing of these two wavelengths, they managed to overcome quantitation problems.

Analysis of the semi-purified taxine mixture in the Ultracarb column by gradient HPLC-PDA revealed the presence of 15 taxines and seven taxanes. Apart from the nine taxines reported by Jenniskens et al.,(15) two additional ones were detected. Absorbance ratioing between the three selected wavelengths proved a powerful tool for peak identification and characterisation. As it concerns the taxane peaks, only 10-DAB III was positively identified by LC-MS (ion at 545 m/z). The rest of the six taxane peaks did not exhibit an adequate signal in the mass spectrometer; thus, identification was not possible. Interestingly, there are great differences in the responses of taxane and taxine-cinnamates between the two detection modes under the tested conditions. Compared to taxines, taxanes give much stronger UV signals, while the opposite happens in the mass spectrometer.

LC-MS analysis, combined with detection at 254 nm, achieved the identification of most of the peaks observed by HPLC-PDA. Identification of the taxines of the mixture was easily achieved by multiple ion detection (MID). Analysis in full scan mode resulted in additional mass ions as can be seen in both Tables 2 & 3. For the analysis in Ultracarb (Table 2), three of the six taxine unknown peaks detected in the PDA (29.90, 31.88, and 33 min) were granted a molecular mass signal. Additionally, two other ions (565 and 670 m/z) were assigned as taxane peaks at 25.02 and 32.5 min, respectively. The rest of the taxine and taxane peaks observed could not be identified by LC-MS, due to their low content in the sample and the low response to the mass spectrometer. Utilising the Luna column, many additional taxine peaks were observed, totalling the taxine peaks to 21 (Table 3).

No matter the chromatographic system, an absolute identification of all the detected peaks was not practically feasible due to the absence of reference standards, the abundance of peaks and the rapid isomerisation of the taxines. However, after thorough UV and MS data manipulation, four of the unknown peaks were characterised as taxanes, whereas nine unknown peaks were characterised as taxines. The spectral data for some of the observed peaks was in agreement with previous reports.(8-10) This finding signifies the importance of taxines in *Taxus* biosynthesis.

Analysis of Plant Material

Analysis of needle extracts by HPLC-PDA revealed the presence of several peaks comprising the taxines and cinnamates UV spectrum. LC-MS analysis confirmed the presence of four of the known taxines: **(4)**, **(5)**, **(6)**, **(8)**. Once again, several other masses were observed which were attributed to unknown taxines.

Table 2. Chromatographic Data Obtained After the LC-PDA and LC-MS Analysis of the Purified Taxine Mixture on the Ultracarb Column (for Experimental Details, see Table 1)

Compound	t_R (min)	A227/A249	A227/A280	A249/A280	m/z
10-DAB III	17.14	3.397	15.22	4.564	545
Taxane	18.50	4.426	16.875	3.813	N/Dt
Taxane	19.02	3.608	12.068	3.88	N/Dt
Taxane	19.24	3.273	10.207	3.345	N/Dt
10	19.95	8.867	7.025	N/Dn	496
U	21.21	1.512	0.522	0.320	584
1	22.42	0.619	0.211	0.339	542
2	23.54	3.533	1.249	0.352	568
3	24.22	0.774	0.410	0.53	626
Taxane	25.02	3.341	7.069	2.116	565
4+5	26.05	1.689	1.006	0.597	584
6	27.27	1.366	0.732	0.532	568
7	28.56	1.359	0.622	0.459	626
Taxane	29.40	12.119	N/Dn	N/Dn	N/Dt
U	29.90	0.123	0.429	0.362	612
8	31.02	0.929	0.319	0.345	668
U	31.88	6.34	5.898	0.931	610
Taxane	32.5	3.720	7.72	2.500	670
U	33	1.036	1.657	1.6	654
9	35.1	1.422	0.806	0.571	652

U= Unknown, N/Dt= Not Detected, N/Dn= Not Determined.

Numerous taxine-like peaks were found in seed extracts. Absorbance ratioing and LC-MS analysis in MID mode enabled the identification of seven taxines of the known mixture: **(1), (4)**, **(5)**, **(3)**, **(6)**, **(7)**, **(8)**, **(9)**. The rest of the peaks were characterised by retention, UV spectra, and ion mass data.

Certain unknown peaks found in the taxine mixture (but not assigned to a specific taxine) were also found in both seed and needle extracts. These peaks were eluted at retention times: 29.79, 31.23, 37.05, and 39.60 min. They were characterised as taxines because they comprised a taxine UV spectrum. Other "taxine-like" peaks not detected in the original taxine mixture were found in both seed and needle extracts: 22.84, 28.99, 34.70, and 35.35 min. Finally, an unknown taxane was detected at 16.78 min. Unfortunately, identification of the above-mentioned peaks could not be accomplished by UV and MS spectral data alone. As already mentioned, the low content of the compounds, the complexity of the sample, but most of all, the instability of the compounds, did not allow an adequate NMR study that would allow structure elucidation. However, it is evi-

Table 3. Chromatographic Data Obtained After the LC-PDA and LC-MS Analysis of the Purified Taxine Mixture on the Luna Column (for Experimental Details, see Table 1)

Compound	t_R (min)	A227/A249	A227/A280	A249/A280	m/z
Taxane	13.5	3.57	10.66	2.96	N/Dt
10 DAB III	15.5	2.53	10.44	4.12	545
Taxane	17	3.8	10.12	2.78	N/Dt
Taxane	18.5	4.17	24.2	5.8	N/Dt
Ut	18.8	N/Dn	N/Dn	N/Dn	612
U	19.2	3.26	N/Dn	N/Dn	604
Taxane	20.3	3.22	15.1	4.72	584
U	22.4	1.16	13.1	11.36	584
2	24.5	2.03	0.93	0.46	568
1	25.4	1.62	1.08	0.66	542
3	25.8	1.21	1.10	0.92	626
Ut	25.8	N/Dn	N/Dn	N/Dn	642
5	26.8	1.60	1.12	0.66	568
Ut	27.4	N/Dn	N/Dn	N/Dn	628
Ut	27.7	1.97	0.94	0.48	584
Ut	29.6	1.30	2.25	1.73	628
4	30.4	1.16	1.13	0.98	584
5	31.7	1.28	1.23	0.96	584
7	32	1.85	0.93	0.47	626
Ut	32.3	3.83	N/Dn	N/Dn	610
U	32.6	5.63	3.25	0.57	784
8	33.1	1.75	0.88	0.51	668
Ut	33.6	1.75	0.90	0.50	612
Ut	36.5	1.44	0.32	0.45	668
Ut	37	1.90	0.45	0.32	610
Ut	38.9	3.77	1.19	0.31	654
Ut	39.5	2.18	1.02	0.47	610
9	41.4	1.88	1.50	0.80	652
Ut	41.9	N/Dn	N/Dn	N/Dn	668

U=Unknown, Ut=Unknown "Taxine", N/Dt= Not Detected, N/Dn= Not Determined.

dent that what was initially called taxine contains a much greater number than 11 compounds as originally described.

As concerns pollen extracts, they were found to contain numerous unknown contaminants with only insignificant traces of taxines recognised by UV detection; however, LC-MS analysis in MID mode revealed the presence of four of the known taxines **(1), (3), (6), (7)**.

Analysis of Cell Cultures

HPLC-PDA and HPLC-MS detected up to 18 taxines in a purified *Taxus* plant cell culture extract. Peaks comprising taxine UV spectra were detected in many of the analysed cell suspension medium extracts. Such peaks were characterised by both retention and spectral data, and their presence was screened in numerous extracts of various cells. The content of the detected taxines varied greatly from sample to sample, depending on culture conditions, period of collection, etc. With the use of LC-MS and LC-MS-MS, it was possible to confirm the presence of taxine B in *T. baccata* cell culture.(24)

CONCLUSIONS

We report on the first HPLC-PDA and HPLC-MS analysis of taxines and cinnamates. With the reported methods, taxanes and taxines can be analysed with the same experimental set-up and with only minor modifications to the mobile phase. Identification of taxines in extracts was enhanced by absorbance ratioing in the PDA and MID mode in LC-MS. Using this experimental protocol, taxine was shown to be a mixture of 21 alkaloids, only nine of them being structurally characterised.

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