Application Note

HPLC analysis of taxoids in plant and plant cell tissue cultures

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PACLITAXEL, A COMPLEX diterpene amide derived from the Pacific yew tree, has been called by the U.S. National Cancer Institute (NCI), "the most important anti-cancer agent for the past 15 years." The interest in paclitaxel dates back to 1962, when crude extracts of *Taxus brevifolia* bark were tested in a large-scale exploratory plant screening program of the NCI. Paclitaxel was later identified as the active constituent of the bark extracts by Wani and Wall.[1] Its clinical development, however, was delayed because of its toxicity; difficulties in formulation; and mainly its scarcity, which hampered a reliable supply for clinical use.

The interest in paclitaxel increased dramatically in the late 1970s, when scientists at Albert Einstein Medical College (New York, NY) described the unique mechanism of its cytotoxic action as a promoter of microtubule assembly. Mitosis is, in fact, inhibited through the enhancement of tubulin's polymerization and the consequent stabilization of microtubules. In 1983, NCI commenced phase I clinical trials, and six years later the John Hopkins Oncology Centre (Baltimore, MD) reported striking clinical results for the treatment of ovarian cancer. The same year, **Bristol-Myers Squibb** (Princeton, NJ) was selected by NCI as the partner to commercialize the drug and was granted the patent for Taxol®. Taxol received FDAapproval for the treatment of ovarian cancer in December 1992, and metastatic breast cancer in April 1994, and is now awaiting approval for the treatment of lung cancer. Numerous other pharmaceutical companies put tremendous efforts into the search for new anticancer agents of *Taxus* origin. **Yew Tree Pharmaceuticals** (Haarlem, The Netherlands) developed Yewtaxan®, a paclitaxel formulation; **Napro** (Boulder, CO) launched its own paclitaxel in Australia in 1995; while **Rhone Poulenc** (Paris, France) has been granted approval in more than 33 countries for Taxotere®, a semisynthetic taxane derivative with the generic name docetaxel. Lately, paclitaxel has shown promising results for HIVand multiple sclerosis.

Paclitaxel is now considered a prototype for a new class of chemotherapeutic agents, and research is proceeding at record pace. The fact that three books[2–4] devoted entirely to paclitaxel have been published proves the excitement the compound has raised. Until recently, *Taxus brevifolia* was the sole source of paclitaxel, despite its low content (100 mg/kg of dry bark). Shortage in supply and increasing demands for clinical use necessitated the investigation for alternative sources: other plant species or tree parts, cell culture, fungi, and synthetic approaches.

The toxicity of *Taxus* plants has long been known (reports date as far back as Julius Caesar) and was attributed to taxine, a complex mixture, which was first isolated from leaves in 1856. Taxine was later shown to be a mixture of at least seven alkaloids, with taxine A and taxine B as the main constituents. Phytochemical work on *Taxus* during the 1940s and 1950s was focused on taxines and taxinines (their cinnamate analogues), but paclitaxel's discovery overshadowed any interest in taxines. However, taxines are relatively abundant in plants and they can serve as an alternative starting material for semisynthetic production of paclitaxel derivatives.

There are significant structural resemblances between taxine B and taxanes (see *Figure 1*). Both

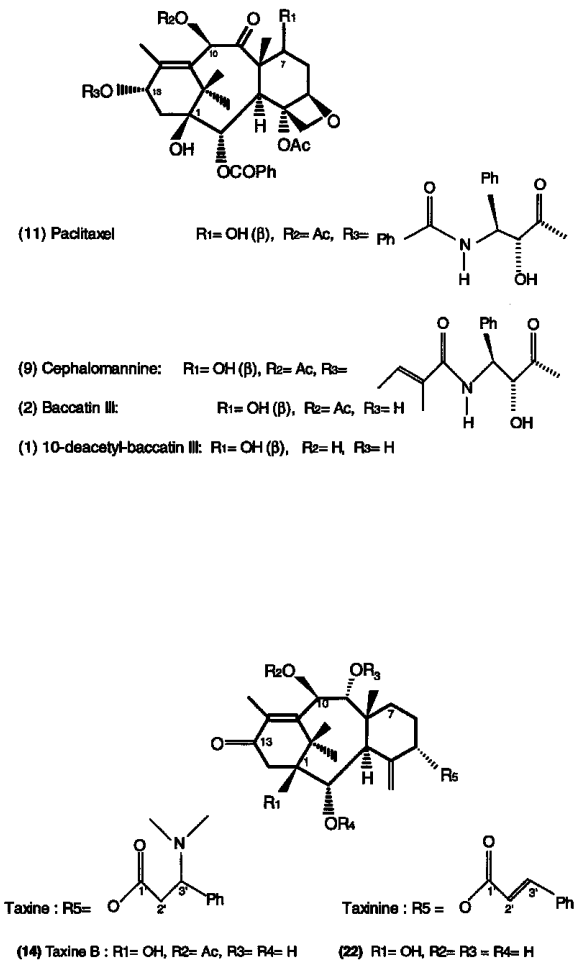

Figure 1 *Molecular structures of the taxoids of interest.*

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 demonstrated that taxine B can be converted into a baccatin V derivative.[2] Taxines do not demonstrate antitumor activity, whereas the cardiotoxicity of taxol is lower than that of taxines, but is nevertheless undesirable for clinical administration. Hence, it was assumed that the structural features necessary for the cardiotoxicity are different from those necessary for the antitumor activity. An understanding of the factors responsible for the toxicity of taxines could lead to the design of taxol analogues with diminished cardiotoxicity.[2] Unfortunately, research on taxines was hampered by their instability.

The authors' work at Leiden University (Leiden, The Netherlands) was directed toward the search for the biosynthetic pathway in plant and plant cell cultures.[5,6] They therefore had to develop analytical techniques for the determination of taxoids in plant and cell cultures. Analysis of paclitaxel and related compounds in plant material is performed exclusively by reversed-phase high-performance liquid chromatography (RP-HPLC),[7] and often represents a challenge due to interferences from the complex matrix. To tackle these problems, column manufacturers have developed specialty "taxane" columns: Curosil™ (**Phenomenex,** Torrance, CA), TAC 1 (**Whatman,** Springfield Mill, U.K.), Taxil™ (**Metachem,** Torrance, CA),

Table 1

Results of the LC-MS analysis of the taxane standard mixture and the *T. baccata* bark extract; chromatographic conditions in *Table 2,* program 1

Compound	*m/z*	Peak	MW	t_R*	Bark
1) 10-DAB III	562	$[M + NH_4]^+$	544	4.00	+
2) Baccatin III	604	$[M + NH_4]^+$	586	7.00	+
3) 7-Xylosyl-10-10-deacetyltaxol B	922	$[M + H]^+$	921	11.00	+
4) Taxinine M	704	$[M + H]^+$	703	11:30	+
5) 7-Xylosyl-10-deacetyltaxol	944	$[M + H]^+$	943	13:00	+
6) 7-Xylosyl-10 deacetyltaxol C	938	$[M + H]^+$	937	14.30	+
7) 10-Deacetyltaxol	812	$[M + H]^+$	811	15.00	+
8) 7-Xylosyl-taxol	986	$[M + H]^+$	985	16.00	+
9) Cephalomannine	832	$[M + H]^+$	831	18.00	+
10) 7-Epi-10-deacetyltaxol	812	$[M + H]^+$	811	19.30	–
11) Paclitaxel	854	$[M + H]^+$	853	20.00	+
12) Taxol C	848	$[M + H]^+$	847	22.00	+
13) 7-Epi-taxol	854	$[M + H]^+$	853	25.00	–

*t_R: Retention time, (+): detection of the compound, (–): no detection.

and Zorbax SW-Taxane (**BTR Separations,** Wilmington, DE). HPLC analysis was optimized with regard to both mobile and stationary phases.[8] For the identification of the analytes (the biggest problem in real sample analysis) diode array detection (DAD) and mass spectrometry coupled to HPLC were employed. Sample pretreatment was studied utilizing solid-phase extraction (SPE). Several SPE cartridges and conditions were tested in the search for the best cleanup and the highest recovery.[9] Gradient elution schemes were employed as a means of selective elution and enhanced interference removal.

Experimental

Chemicals

Paclitaxel and 10-deacetyl baccatin III (10-DAB III) standards were obtained from **Sigma** (St. Louis, MO). A mixture of 12 taxanes and one taxinine (*Table 1*) was generously provided by Dr. Steven Richheimer (**Hauser Chemical Research, Inc.,** Boulder, CO). A semipurified taxine extract with taxine B as the main component was kindly offered from Dr. E. van Rozendaal (Wagenigen University, The Netherlands). The mixture was expected to contain at least six taxines and some taxinines as products of taxine degradation.[10]All solvents were distilled in house prior to use and filtered through a 0.45-µm RC 55 membrane filter (**Schleicher & Schuell,** Dassel, Germany). All chemicals used were of analytical grade and water was of **Millipore** (Bedford, MA) quality.

Apparatus and chromatographic conditions

HPLC analysis was performed in a **Waters** gradient LC system (Milford, MA) consisting of a 600 E pump, WISP 712 autosampler, 991 photodiode array detector, and 5200 printer/plotter. LC-MS analysis was performed on a TSQ-70 custom-made electrospray interface (ESI) (**Finnigan MAT,** San Jose, CA). Splitting

Table 2
Selected isocratic and gradient elution programs*

Isocratic elution system (Ultracarb column)
Mobile phase—acetonitrile:water:0.05 *M* ammonium acetate 54:4:42 vol/vol

Gradient 1 (Novapak Phenyl column)
A:0.05 *M* CH_3COONH_4:CH_3CN 7:3 vol/vol
B:0.05 *M* CH_3COONH_4:CH_3CN 1:9 vol/vol

t (min)	A (%)	B (%)
0	100	0
30	66	34
32	100	0

Gradient 2 1 (Ultracarb column)
A:0.05 *M* CH_3COOHN_4:CH_3CN 7:3 vol/vol
B: H_2O:CH_3CN 1:9 vol/vol

t (min)	A (%)	B (%)
0	80	20
30	0	100
32	0	100
34	80	20

*Table reproduced with permission from Ref. 8.

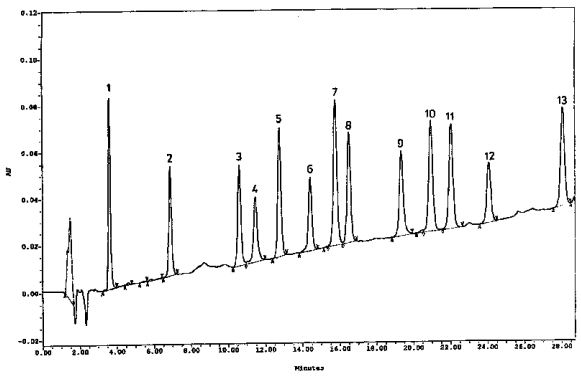

Figure 2 *HPLC analysis of the taxane reference mixture. Conditions: gradient 1, Table 2;results: Table 1.Figure repro - duced with permission from Ref. 8.*

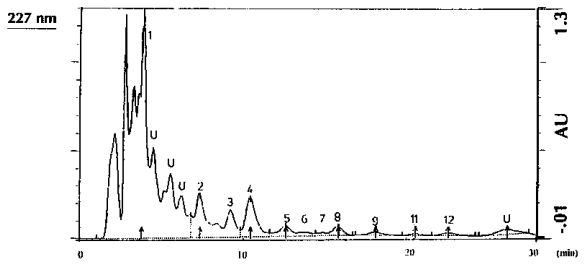

Figure 3 *HPLC analysis of a* Taxus *bark extract (gradient 1, Table 2).*

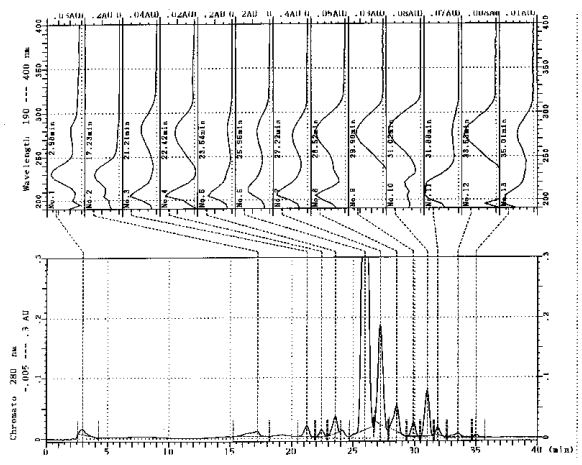

Figure 4 *HPLC analysis of the taxine mixture (gradient 2, Table 2).Figure reproduced with permission from Ref. 8.*

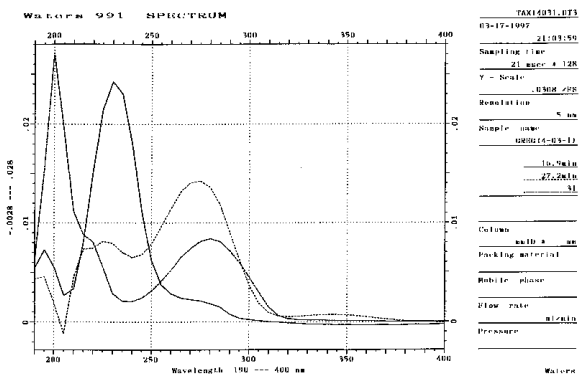

Figure 5 *Typical UV spectrum of a taxine, taxinine, and tax - ane obtained on the DAD.*

the flow at a ratio of 19:1 before the ESI probe introduced 50 µL/min into the mass spectrometer.

The following HPLC columns were evaluated: 1) Ultracarb C_{18}, 5 µm, 60 Å, 150 × 4.6 mm (**Phenomenex**); 2) Phenyl, 4 µm, 60 Å, 150 × 3.9 mm (**Waters**); 3) Novapak Adsorbospher HS C_{18}, 3 µm, 80 Å, 150 × 4.6 mm (**Alltech,** Deerfield, IL); and 4) Zorbax SW-Taxane, 10 µm, 60 Å, 250 × 4.6 mm (**BTR Separations**). All columns were protected by a 20 × 4.6 mm pre-column, filled in house with Lichrosorb RP-18, 10-µm material (**Merck,** Darmstadt, Germany). The flow rate was 0.8 mL/min and the injection volume was 10 µLthroughout the study.

The taxoids of interest can be divided into the following groups: paclitaxel, cephalomannine, 10-DAB, taxines, and taxinines. All groups possess the main taxane ring; paclitaxel and cephalomannine differ in the substitution of the 3′-N atom of the C-13 side chain, whereas 10-DAB III lacks the C-13 side chain (Figure 1). These differences affect molecular cytotoxic activity, polarity, and chromatographic behavior. Spectral data were collected over the 190–400 nm range and chromatograms were plotted at 215, 227, 245, and 280 nm to enable taxoid's differentiation. Linearity of the detector response was determined with triplicate analysis of 10-DAB III and paclitaxel standard solutions of 12 concentration points ranging from 0.46 to 166 and 0.83 to 240 µg/mL, respectively.

Cell line initiation, maintenance, and conditions

Callus cultures originated from explants derived from a *T. baccata* tree were maintained in a modified Gamborg's B5 medium supplemented with 100 mg/L mesoinositol, 10 mg/L thiamine di-HCl, 1 mg/L pyridoxin HCl, 1.86 mg/L naphthalene acetic acid, and 10 g/L agar. Cell suspension cultures were initiated from callus culture. They were grown in the same medium as callus cultures with the omission of agar. Cells were grown in 2-L flasks containing 500 mL of medium with an inoculum size of 50 g. The medium was refreshed every two weeks and cells were subcultured every third week. Flasks were placed on a rotary shaker set at 80 rpm under continuous light at 25 °C.

Sample pretreatment

The first effort was the development of a fast, convenient, robust, multipurpose extraction protocol. The extraction of several plant and cell culture materials was studied. The chosen methods for each of the materials studied are given below.

Dried *T. baccata* bark was ground in a blender and 20 g were extracted twice with 50 mLMeOH with the assistance of sonication and shaking (110 rpm for 30 min). The methanolic fraction was evaporated to dryness in a rotary evaporator (40–45 °C). The residue was reconstituted with 5 mL of methanol, 0.5 mL of which was applied to a Supelclean™ LC-18 SPE cartridge (**Supelco,** Bellefonte, PA) following the SPE protocol developed.

Prior to loading, the cartridge was conditioned by appropriate washing. After loading, it was washed with 2 × 2 mLof water, 2 mLof 20% methanol in water, and 2 mL of 50% methanol in water. The compounds of interest were eluted with 2 mLof methanol and the collected fraction was evaporated to dryness in a SpeedVac (**Savant,** Farmingdale, NY). The residue was reconstituted in solution with 2 × 100 µL of acetonitrile. Ten microliters of the resulting solution was analyzed by HPLC.

Concerning *Taxus* needles and clippings, pretreatment was as follows: 3 g of *T. canadensis* clippings were ground in the blender and extracted with 30 mL of methanol. The extract was washed with 30 mL of *n*-hexane and the hexane layer was discarded. The methanolic fraction was evaporated to dryness in a rotary evaporator (40–45 °C) and the residue was reconstituted in 5 mL of methanol. An aliquot of 0.5 mL was subjected to SPE, as above.[8] The same methodology was applied to the extraction of 5 g of *T. baccata* seeds.

Fifty milligrams of dried cell suspension material was extracted twice with 20 mL MeOH, and the methanolic fractions were filtered and evaporated to dryness. The residue was reconstituted in 3 × 1 mL of MeOH, with the help of vortexing and ultrasonication. Finally, 0.5 mL of the resulting solution was brought to 10% MeOH in water and loaded on an SPE cartridge.[9]

Cell suspension medium was first filtered through a glass filter, to separate the cell debris, and subsequently through a double paper filter. Next, 50 mL of the medium was loaded on the SPE cartridge and extracted, as above.[9]

Quantitative studies included standard addition experiments for the construction of calibration curves. The two diterpenoids were added in amounts to give six final concentrations: 13.25–220 mg mL^{-1} for 10-DAB III, and 23.13–370 mg mL^{-1} for taxol. HPLC analyses were performed in triplicate. SPE of the above-mentioned plant materials was always tested in all of the SPE cartridges.

Results and discussion

Separation method

With regard to the separation method used, isocratic elution (conditions in *Table 2*) was able to separate up to 11 of the 13 taxanes present in the standard mixture. Variations in the nature and content of the organic modifier and salt concentration of the mobile phase did not alter the elution order and affected band spacing only. Baseline separation of all 13 taxanes was achieved with a gradient elution program of acetonitrile over ammonium acetate solution (gradient 1, Table 2), run with the Novapak Phenyl column within 30 min (*Figure 2*). This system, compared to other systems published, performed better in terms of resolution and efficiency. Furthermore, it allowed coupling to MS and employed a low-cost column without the need of specialty columns.[8] A typical chromatogram of a bark extract is given in *Figure 3*.

Retention and quantitation reproducibility were evaluated with repeated analysis for 12 consecutive days and were found to be satisfactory. After months of use and numerous injections of plant and cell culture extracts, the system performed acceptably. Refilling the pre-column after 300–400 injections (depending on the nature of the samples analyzed) was sufficient. The Zorbax SW-Taxane column showed increased retention for the polar taxanes and a different fingerprint compared to the other stationary phases. For some reason, the column showed a significant loss of efficiency after a few months. The use of this column was therefore omitted.

continued

Table 3

LC-DAD and LC-MS analysis of the purified taxine mixture

Compound	t_R* (min)	A227/A249	A227/A280	A249/A280	*m/z*	Seeds	Needles
10-DAB III	17.14	3.397	15.22	4.564	545	–	–
Unknown	18.50	4.426	16.875	3.813	N/D	–	–
Unknown	19.02	3.608	12.068	3.88	N/D	–	–
Unknown	19.24	3.273	10.207	3.345	N/D	–	–
23	19.95	8.867	7.025	N/D	496	–	–
16	21.21	1.512	0.522	0.320	584	+	+
20	22.42	0.619	0.211	0.339	542	–	–
15	23.54	3.533	1.249	0.352	568	–	–
17	24.22	0.774	0.410	0.53	626	+	–
Unknown	25.02	3.341	7.069	2.116	N/D	–	–
14	26.05	1.689	1.006	0.597	584	+	+
18	27.27	1.366	0.732	0.532	568	+	+
19	28.56	1.359	0.622	0.459	626	+	–
Unknown	29.40	N/D	N/D	N/D	N/D	–	–
21	31.02	0.929	0.319	0.345	668	+	+
Unknown	32.5	3.720	7.72	2.500	N/D	–	–
22	35.1	1.422	0.806	0.571	652	+	–

*t_R: Retention time, N/D: not determined, (+): detection of the compound, (–): no detection.

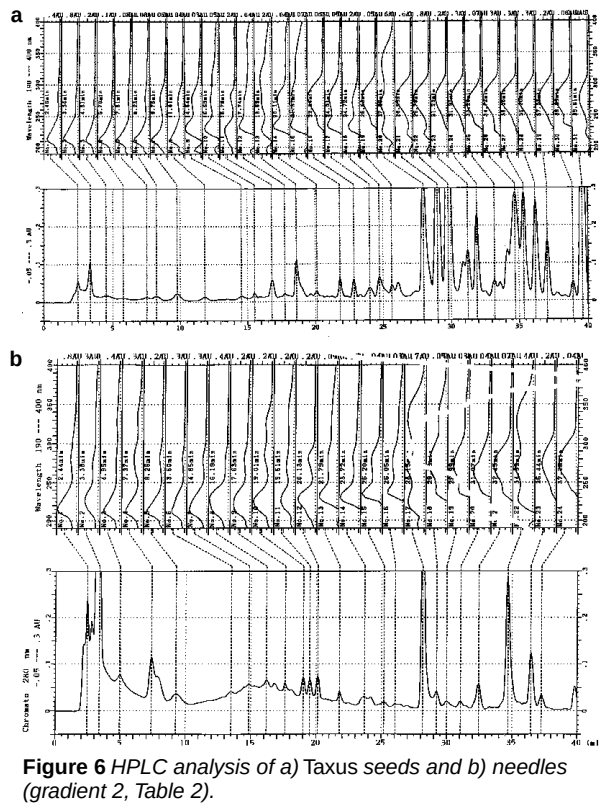

Figure 6 *HPLC analysis of a)* Taxus *seeds and b) needles (gradient 2, Table 2).*

For the analysis of taxines, the Ultracarb column with the mobile phases used for taxane analysis gave the best resolution. Slight modifications of the gradient elution program and the flow rate (gradient 2, Table 2) gave a baseline resolution of the taxines and taxinines within 40 min,[10] as can be seen in *Figure 4*. The authors'goal was to devise a versatile scheme for the analysis of both taxane and taxine extracts in the same experimental setup. Quantitation of the peaks in the selected wavelengths (215, 227, 249, and 280 nm) was satisfactory. The linearity of the method was evaluated in the range of 21–210 µg of purified taxine mixture for detection at 280 nm, giving R^2 values ranging from 0.9976 to 0.9998.

LC-DAD and LC-MS as identification tools

Taxanes were identified as the protonated molecules, except for 10-DAB III and baccatin III, which gave the $[M + NH_4]^+$ as the base peak. Another interesting phenomenon was the very high signal/response of taxines and taxinines in MS. These compounds gave a 15–20 fold higher signal compared to the taxanes. LC-MS analysis of a bark extract identified 11 of the 13 taxanes in the authors' database (Table 1).

Figure 5 shows the UV spectrum of some of the analyzed taxines, taxinines, and taxanes. It can be seen that 227 nm is the maximum for the UV spectrum of taxanes, whereas 280 nm is the λmax of taxinines and taxines. Hence, 280 nm can be used as a selective wavelength for the detection of taxines and taxinines.[11] Absorbance ratioing between three wavelengths of quantitation was also used as a means of identification. The absorbance ratios obtained after the analysis of reference mixtures were:

For 10-DAB III:

$A_{227}/A_{215} = 1.67$, $A_{227}/A_{249} = 2.99$, $A_{215}/A_{259} = 1.78$

For taxol:

$A_{227}/A_{215} = 1.35$, $A_{227}/A_{249} = 3.51$, $A_{215}/A_{249} = 2.55$

A comparison of these values with those calculated from peaks eluting at the same retention times in the analysis of extracts is a quick way to verify the identify and check the peak purity.

Analysis of the semipurified taxine mixture by gradient HPLC-DAD revealed the presence of seven taxanes with 10-DAB III among them. All nine expected taxines were positively identified (*Table 3*). Absorbance ratioing between three selected wavelengths (227, 249, and 280 nm) proved a powerful tool for identification purposes. Taxines and taxinines were reported to cause problems in the HPLC analysis of taxanes, coeluting with the peak of paclitaxel.[7]

Several unknown taxane-like peaks and contaminants were found in high concentrations in *T. baccata* cell extracts. Some were identified as taxines. Taxine B and deoxytaxine B were identified in very low amounts using LC-MS-MS.[6] Peaks that revealed a taxine or taxinine UV spectrum were characterized by means of retention and spectral data, and their presence was screened in numerous cell suspension medium extracts. Taxine-like peaks were also found in extracts of callus and cell material. However, LC-MS analysis should be used for the confirmation of their identity.[11]

HPLC-DAD analysis of seeds and needle extracts revealed the presence of several peaks comprising taxines and taxinine's UV spectrum (*Figure 6*). Absorbance ratioing was again used, but for definite identification, the sample was analyzed by LC-MS. This confirmed the presence of seven of the known taxines in seeds and four in needles (Table 3).[11]

Sample pretreatment

As a general rule, methanol is a more potent solvent than less polar organic solvents (e.g., dichloromethane) and is capable of efficiently extracting paclitaxel as well as 10-DAB III. For bark extraction, it was found that a long agitation time with a magnetic stirrer is not as effective as homogenization combined with sonication. For SPE, C18 and C8 SPE cartridges gave the highest recovery when eluted with MeOH or EtOH. 10-DAB III was best extracted with polar solvents in nearly all of the cartridges tested.

An important goal of the SPE method used is the straightforward application of the aqueous medium on column resulting in a much faster and simpler extraction scheme. Liquid–liquid extraction of cell suspension medium led to the formation of emulsions, probably from the sugars or other constituents of the suspension medium. These emulsions are difficult to break and require centrifugation, which is time consuming and difficult when processing large volumes. Washing the cartridge with water and aqueous MeOH (up to 50% in water) resulted in the removal of many interferences.[9] Taxanes were recovered from the SPE cartridge with the elution fraction of 80% MeOH. Numerous unknown peaks were found in cell suspension medium extracts. Interestingly, new types of UV spectra (λmax at 240–260 and 360–380 nm) were revealed from many peaks. Trials were done to correlate the content of the unknown compounds with the phase of the cell culture growth and/or other factors affecting the cell. These aspects are still under investigation.

Conclusion

RP-HPLC analysis of paclitaxel and other taxoids was optimized. LC-DAD and especially LC-MS proved to be powerful identification tools in the analysis of complex plant and cell culture extracts. Sample pretreatment by SPE with gradient elution enhanced the removal of interferences and extraction recovery. The analytical methodology was applied in the characterization and study of cell lines, providing interesting results concerning the biosynthesis of taxanes.

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