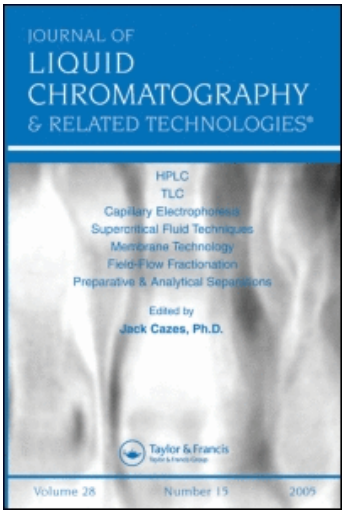

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

EFFICIENT EXTRACTION OF PACLITAXEL AND RELATED TAXOIDS FROM LEAF TISSUE OF *TAXUS* USING A POTABLE SOLVENT SYSTEM

Raymond E. B. Ketchum[a]; Judy V. Luong[b]; Donna M. Gibson
[a] Institute of Biological Chemistry, Washington State University, Pullman, WA, U.S.A. [b] Pharmaceutical Division, Bayer Corporation, West Haven, CT, U.S.A.

Online publication date: 06 September 1999

To cite this Article Ketchum, Raymond E. B. , Luong, Judy V. and Gibson, Donna M.(1999) 'EFFICIENT EXTRACTION OF PACLITAXEL AND RELATED TAXOIDS FROM LEAF TISSUE OF *TAXUS* USING A POTABLE SOLVENT SYSTEM', Journal of Liquid Chromatography & Related Technologies, 22: 11, 1715 — 1732
To link to this Article: DOI: 10.1081/JLC-100101762
URL: http://dx.doi.org/10.1081/JLC-100101762

J. LIQ. CHROM. & REL. TECHNOL., 22(11), 1715–1732 (1999)

EFFICIENT EXTRACTION OF PACLITAXEL AND RELATED TAXOIDS FROM LEAF TISSUE OF *TAXUS* USING A POTABLE SOLVENT SYSTEM

Raymond E. B. Ketchum,*,† Judy V. Luong,‡ Donna M. Gibson

USDA, ARS
U. S. Plant, Soil, and Nutrition Laboratory
Tower Road
Ithaca, NY 14853, USA

ABSTRACT

Leaves of *Taxus x media* and *Taxus brevifolia* were examined for their potential use as a source of paclitaxel and related taxoids. Various solvent systems were compared for their efficacy in the extraction of paclitaxel, as well as the extraction of contaminating residue. A method of extraction has been developed that enriches paclitaxel by over 500-fold using only potable solvents in combination with solid phase extraction, thus avoiding the use of chlorinated hydrocarbon solvent systems. This solvent system can be used on fresh leaf material, eliminating the need for drying material before extraction, and preventing degradation of the taxoids during the drying process. Using these methods, paclitaxel constituted 0.01 to 0.02% of the dry weight of the leaf tissue from *Taxus x media*, and 0.04% from dried leaves of *Taxus brevifolia*.

INTRODUCTION

Paclitaxel is a diterpenoid present in small amounts in nearly all parts of plants in the genus *Taxus.* Its success as a cancer treatment has significantly increased estimates for world-wide demand for the drug beyond the original 1989 estimate of 50 kg per year[1] to 500 kg per year (M. Suffness, personal communication). At the rate of 50 kg per year, it was estimated that there were enough trees in the Rocky Mountains and Pacific Northwest to meet demand until the year 1999. By most estimates, the demand for paclitaxel will far exceed its natural availability (from bark of *Taxus brevifolia)* within 5 to 10 years.[2]

Three methods show promise as alternatives to extraction of paclitaxel from bark. All methods use renewable resources, and are not subject to elimination of the source material through destructive harvest. Cell culture is a particularly intriguing option in that it offers the advantages of a sustainable system,[3-6] a system amenable to molecular and biochemical characterization,[7-8] and a system in which extraction of the taxoids from an aqueous medium is relatively simple.[9-10]

Semi-synthetic production of paclitaxel appears to be the source of the drug for the foreseeable future. Bristol-Myers-Squibb stated that current supplies of paclitaxel precursors, and the semi-synthetic production of paclitaxel, will eliminate the need of any yew harvests and halted harvests on federal land in 1993.[11] However, the precursors necessary for semi-synthetic production of paclitaxel are natural taxoids obtained from European and Himalayan sources that have also been seriously depleted in recent years.[12] In addition to a renewable source of taxoids, particularly 10-deacetylbaccatin III, the leaves of *Taxus* contain paclitaxel in concentrations comparable to levels found in the bark.[13-14]

Most reports of the purification of taxoids from cells or bark of *Taxus* rely on the use of chlorinated hydrocarbons, such as methylene chloride or chloroform.[15-18] Because taxoids are present at low concentrations, the volume of solvent used for extraction is very large. Published procedures have used a hexane cleanup step, followed by dichloromethane:water (1:1) partitioning. The organic phase contains the taxoids which can be further purified by use of a variety of organic solvents to remove contaminants and by use of various chromatography stationary phases. The solvents employed include toluene, dichloromethane, ethyl ether, ethyl acetate, and methanol or ethanol. Finding a more efficient, economical, protocol using less solvent, or more environmentally sensitive solvents, would be a significant breakthrough for taxoid extraction.

Although the commercial process currently used by Hauser Chemical, Inc., is proprietary, a company spokesperson listed solvent recycle as a major commercial hurdle to overcome.[19] Also, since taxoids are present at relatively low levels, the quantity of excess, extracted biomass is significant.

We report on the efficiency of extraction of paclitaxel and other related taxoids from leaves of *T. x media* and *T. brevifolia*, and present a method for extracting paclitaxel from leaves to 511-fold purity using only potable solvents and a single solid phase extraction final step.

EXPERIMENTAL

General Experimental Procedures

Leaf samples were freeze-dried on a Labconco Lyophilizer.[20] Extracts were dried on a Savant Speed Vac centrifugal concentrator, or Büchi Rotovapor. HPLC instrumentation consisted of a Waters Millenium System with a Waters model 510 HPLC pump, model 717 autosampler, and model 996 photodiode array detector. Data acquisition, analysis, and reporting, was done with Waters Millenium Software, version 2.15. A Metachem Taxsil 5 µm column (4.6 mm x 250 mm) with guard column was used for HPLC analysis. All solvents were HPLC or Optima™ grade and purchased from Fisher Scientific Co.

Plant Material

Leaves and stems of an indeterminate cultivar of *Taxus x media* were harvested and collected in June 1995 from the shrubs bordering the south side of the USDA/ARS US Plant, Soil, and Nutrition Laboratory at Cornell University in Ithaca, NY. These shrubs were exposed to constant open shade under a red oak (*Quercus rubra*) canopy. Samples have been deposited at the Bailey Hortorium on the Cornell University campus. Leaves of authenticated *Taxus brevifolia* were harvested from trees cultivated in a greenhouse on the Washington State University campus, Pullman, WA.

Freeze-dried plant material was placed immediately in large lyophilizer jars and placed on a Labconco lyophilizer and dried for at least four days to constant weight. Oven-dried leaves were dried to constant weight at 45°C. Drying time typically took four days. After the leaf tissue was dried, it was stripped from the stems and ground to a fine powder (particle size <500 µm) in a Sunbeam coffee mill. Any material that was not immediately dried was kept frozen at -20°C.

Taxoid Extraction from Leaf Tissue

For all experiments a general extraction method was employed. Dried or fresh samples of leaves were placed in test tubes, centrifuge tubes, or microcentrifuge tubes (depending on the amount of sample) and extracted in a 10x volume of solvent (v/w) for 30 min in a Fisher FS-30 ultrasonic bath. Supernatant was removed following centrifugation at 2500 x g, for the extracts in centrifuge tubes or test tubes, or 16000 x g for experiments conducted in microcentrifuge tubes. Leaves were extracted three times, and extracts combined. Solvents were evaporated *in vacuo*, either on a Savant Speed-Vac or a Büchi Rotavapor.

Solid Phase Extraction of Crude Extracts

Crude residue, containing paclitaxel and other taxoids, was dissolved in a minimum amount of 95% ethanol to effect complete dissolution, and then diluted to a final concentration of 40% ethanol with water. This solution was loaded on a 200 mg, 3cc, Waters C_{18} solid phase extraction (SPE) column, according to the manufacturers instructions. The column was washed with 40% ethanol and taxoids were eluted with 95% ethanol. Eluate was dried, and residue was dissolved in 100% methanol prior to HPLC analysis. Experimentally, it was determined that breakthrough of the taxoids would occur if the total amount of residue loaded onto the column was 6% to 8% (w/w) of the column packing material. Thus, for routine SPE of taxoids, residue was loaded on the column equal to 5% of the weight of the column packing material, e.g. 10 mg crude residue on a 200 mg column.

HPLC Analysis of Taxoids

Paclitaxel and other taxoids were extracted, analyzed, and verified using HPLC methods developed in this laboratory and described elsewhere.[9-10] Taxoids were monitored at 228 nm with a diode array detector, and verified by matching absorption spectra from 200-300 nm and retention times with authentic taxoid standards. Concentrations were determined by comparison with an external standard curve over the range of 1 to 100 $mg \cdot l^{-1}$.

Statistical Analyses

Statistics were calculated using Minitab software, release 11 (State College, PA). Results were analyzed by one-way ANOVA. Means were compared by Tukey's multiple comparison test with a family error rate of 0.05. The individual 95% confidence intervals for each treatment, based on the pooled

standard deviation of an experiment, were used as the error bars in the graphical representation of data. In the text, all discussion of experimental differences is statistically significant unless noted otherwise. Where appropriate, values in the text are reported with standard error and sample number.

RESULTS AND DISCUSSION

Solvent Selection

There was a strong positive correlation (r^2=0.71) between the amount of residue extracted with a given solvent, and the amount of paclitaxel extracted from the leaf tissue (Figure 1). This trend followed the polarity of an organic solvent, with the least polar solvents extracting the least amount of taxoids and other compounds, and the most polar solvents extracting the most taxoids as well as the greatest amount of contaminating material.

No solvent could efficiently extract all of the taxoids with one single extraction (Figure 1A) and all solvents tested continued to extract taxoids even after three extractions (Figure 1B). There were no significant differences in paclitaxel content following a single extraction, between methanol, ethanol, or acetone. However, methanol extracted a significantly larger amount of non-taxoid material than did any other solvent after one extraction (Figure 1A), and significantly greater paclitaxel and non-taxoid residue after three extractions (Figure 1B). Methanol was the solvent of choice for extraction of the maximum amount of paclitaxel from leaves. However, up to 55% of the dry weight of *Taxus* leaves are methanol-soluble (38 ± 10 %, n=12), so the use of methanol requires substantial purification to extract the taxoids from the resulting tar-like residue.

The next best solvent in our experiments was acetone. After three extractions, acetone removed about 80% of the paclitaxel, compared to methanol, but produced only about 25% of the total residue as methanol. The resulting acetone extract was therefore about 4 times as enriched in paclitaxel as was the methanol extract. The use of acetone as an extraction solvent also has the advantage of preventing degradation of paclitaxel to its tetraol by methanolysis.[21]

Witherup and co-workers[18] described a method of isolating taxoids from leaves that has been cited by a number of groups. Briefly, leaves percolate for 16 h in CH_2Cl_2:CH_3OH (1:1), the organic extract is evaporated and partitioned between CH_2Cl_2 and H_2O, the organic phase is removed and dried to give the CH_2Cl_2 soluble residue.

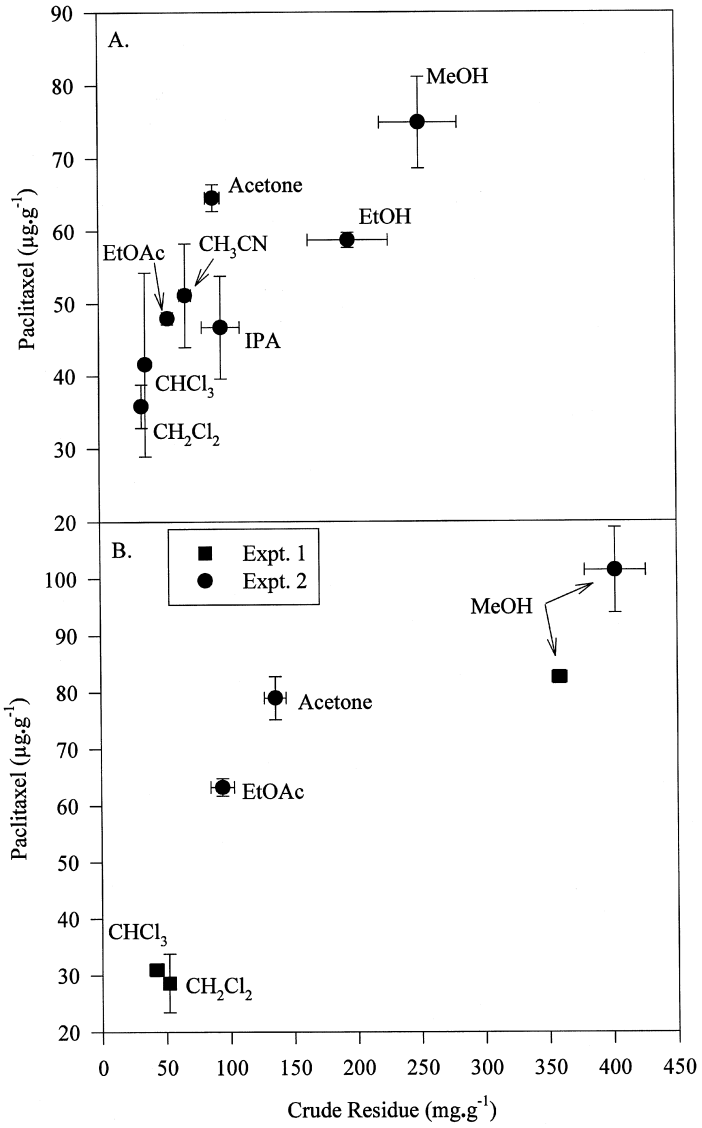

Figure 1. Comparison of extracted paclitaxel and total crude residue from *Taxus x media* leaves. Three one-gram replicates of lyophilized leaves were extracted (A) one time or (B) three times in the given solvent. Error bars represent the standard deviation of three replicate extracts. Two separate experiments are combined in (B).

Table 1

Comparison of Extraction Methods, of Leaves of *Taxus x media*, with the Method of Witherup, et al.[18],*

Treatment	N	Paclitaxel ($\mu g.g^{-1}$ Dry Leaves)	Std Err	Crude Residue ($mg.g^{-1}$ Dry Leaves)	Std Err
Witherup Method	3	20.1[a]	1.14	57.8[a]	3.70
100% MeOH Extract/20% MeOH Wash	3	27.9[b]	0.83	325[b]	6.91
100% MeOH Extract/Hexane Wash	3	30.4[b]	1.13	303[b]	11.3

* Means with the same letter are not significantly different (Tukey's multiple comparison test, $p \leq 0.05$).

In this laboratory, extraction of dried *Taxus* leaves by the method of Witherup, et al.[18] produced nearly one sixth the residue compared to leaves extracted in 100% methanol and washed in 20% aqueous methanol, or hexane (Table 1). However, both methanol methods extracted significantly greater amounts of paclitaxel than did the Witherup method. In the case of methanol followed by a hexane wash, 50% more paclitaxel was extracted (Table 1). There was no significant difference in paclitaxel yield if the methanol pellet was washed with hexane or 20% aqueous methanol (Table 1).

Comparison of Extraction from Fresh, Air-Dried, and Freeze-Dried Tissues

We examined the effects of treatment of leaf tissue on paclitaxel yield, prior to extraction with 40% aqueous ethanol (Table 2). Paclitaxel concentrations are presented both on a dry weight basis as well as on a fresh weight basis, so that comparisons could be made to the extraction of fresh tissue. There was no significant difference in paclitaxel extracted from fresh leaves, or from leaves dried at 45°C for 4 days.

However, extraction of freeze-dried leaves produced 107.4 $\mu g g^{-1}$ (or 0.011%), more than twice as much as either of the other two methods (Table 2). Our results agree with those of ElSohly et al.[22] who found that fresh *T. x media* leaves contained equal amounts of paclitaxel (0.009%) as leaves stripped from

Table 2

Comparison of Extraction of Fresh, Air-Dried, and Freeze-Dried Leaves of *Taxus x media

Treatment	N	Paclitaxel ($\mu g.g^{-1}$ Fresh Leaves)	Std Err	Crude Residue ($mg.g^{-1}$ Dry Leaves)	Std Err
Dried (45°C)	3	14.7[b]	1.09	46.2[b]	2.67
Fresh	3	11.6[b]	2.61	n/a	n/a
Freeze-Dried	2	34.0[a]	1.97	107[a]	7.90

* Means with the same letter are not significantly different (Tukey's multiple comparison test, $p \leq 0.05$).

Table 3

Comparison of Methanol and Ethanol Extraction of Fresh and Dried Leaves of *Taxus x media

Tissue	Solvent	N	Paclitaxel ($\mu g.g^{-1}$ Tissue)	StdErr	Crude Residue ($\mu g.g^{-1}$ Tissue)	StdErr
Freeze-Dried	95% EtOH	3	53.8[b]	4.61	0.32[b]	0.02
Fresh	95% EtOH	3	20.2[c]	1.20	0.11[c]	0.01
Freeze-Dried	100% MeOH	3	83.8[a]	5.87	0.36[a]	0.00
Fresh	100% MeOH	3	26.1[c]	7.58	0.07[d]	0.01

* Means with the same letter are not significantly different (Tukey's multiple comparison test, $p \leq 0.05$).

the stem and dried at 32-40°C for 3 days (0.009%). However, those investigators found that leaves dried on the stem at 32-40°C for 3 days contained the greatest amounts of paclitaxel of all the treatments (0.014%), an amount similar to the paclitaxel concentration found in this study.

Table 4

Methanol Purification of Paclitaxel from Dried Leaves of *Taxus x media

Treatment	Weight (g)	% Paclitaxel	Purification Factor
Fresh Leaves	0.309	0.005	1.00
Freeze-Dried Leaves	0.109	0.015	2.82
100% MeOH	0.053	0.031	5.75
50% MeOH	0.039	0.042	7.80
C18 SPE	0.005	0.324	60.6
Total Paclitaxel (µg)	16.37		

* Data are the means of three replicates. At each step in the purification, the final paclitaxel is expressed as a percentage of the residue at that step, as well as on both a fresh and dry weight basis.

Comparison of Methanol and Ethanol Extraction of Fresh and Dried Leaves

Extraction of fresh leaves with either 100% methanol or 95% ethanol did not result in a significant difference in the paclitaxel yield (Table 3). However, if leaves were freeze-dried prior to extraction with either alcohol, a significantly greater amount of paclitaxel was recovered than if the tissue was fresh. On a dry weight basis, treatment of freeze-dried leaves with 100% methanol resulted in the recovery of 83.8 $\mu g g^{-1}$ paclitaxel, significantly greater than 53.8 $\mu g g^{-1}$ extracted with 95% ethanol (Table 3).

Solid Phase Extraction (SPE) of Alcohol Extracts of *Taxus* Dried Leaves

From freeze-dried *T. x media* leaves, an initial methanol extraction followed by fractionation in 50% methanol and purification on a C_{18} SPE column, resulted in greater than a 60-fold increase in purification of paclitaxel compared to the concentration in fresh leaves. The paclitaxel yield using this method was 0.015%, on a dry weight basis (Table 4). Extraction of freeze-dried leaves in 95% ethanol, with further purification in 40% ethanol, followed by purification on a C_{18} SPE column, resulted in a much smaller proportion of crude residue (2 $mg \cdot g^{-1}$ vs. 5 $mg \cdot 0.3 g^{-1}$). While the paclitaxel yield from ethanol was not significantly less than that of the methanol method (0.011% vs. 0.015%), the total purification was greater than 510-fold (Table 5).

Table 5

Ethanol Purification of Paclitaxel from Dried Leaves of *Taxus x media

Treatment	Weight (g)	% Paclitaxel	Purification Factor
Fresh Leaves	1.056	0.003	1.00
Freeze-Dried Leaves	0.282	0.011	3.74
95% EtOH	0.096	0.033	10.9
40% EtOH-C18 SPE	0.002	1.543	511
Total Pacllitaxel (μg)	31.90		

* Data are the means of three replicates. At each step in the purification, the final paclitaxel is expressed as a percentage of the residue at that step, as well as on both a fresh and dry weight basis.

An SPE method, based on C_{18} reversed-phase chemistry, was employed to further purify the crude 40% ethanol extracts. While it was possible to go directly from the crude extract to purification on the C_{18} SPE column, most experiments included a drying step to determine the weight of the crude residue, followed by dissolution in a minimum amount of 95% ethanol and adjustment of the ethanol concentration to 40% with deionized water. This helped to minimize the volume of extract that needed to be loaded on the column. Figure 2 shows the effect of purification of a crude leaf extract by SPE.

A large amount of polar material is removed from the sample by this method of purification, as can be seen by comparison of the amount of material eluting from the column in the more aqueous fractions with the chromatogram corresponding to the 60:40 fraction (Figure. 3). The use of a highly polar solvent, such as 40% ethanol, prevents the extraction of many of the lipids and waxes that are present in leaves of *Taxus*. Thus, the need for a hexane or other non-polar extraction is not necessary. The C_{18} SPE further purifies the extract by removal of the most polar contaminants.

Summary of Alcohol-Based Extraction Methods

A summary of alcohol-based extraction methods from fresh leaves, as well as freeze-dried and heat-dried leaves, is presented in Table 6. For all solvent systems, the greatest amount of paclitaxel was extracted from freeze-dried leaves, while no significant difference was noted between fresh leaves and leaves dried at 45°C for four days.

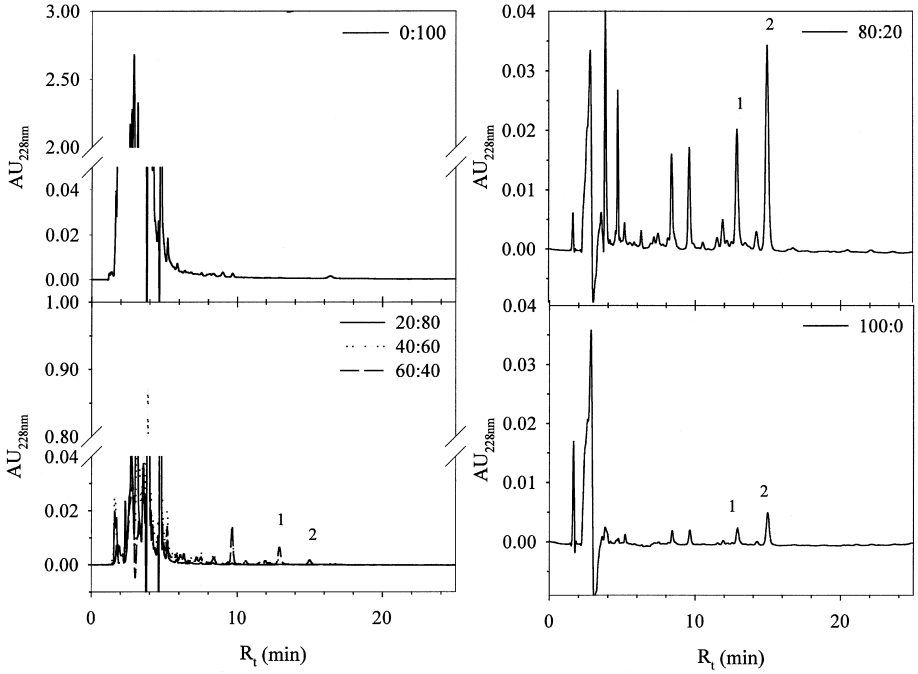

Figure 2. HPLC chromatograms of 40% ethanol extract of freeze-dried *T. x media* leaves and following purification by C_{18} SPE. The absorbance axis is split at AU 0.05 to show the change in the relative concentration of taxoids in each fraction. Each fraction was dried, following SPE in the given eluant of methanol and water, dissolved in 1 mL of 100% MeOH, and 10 µL of this extract was injected. The concentration of paclitaxel in the 80% MeOH fraction is 25 $\mu g mL^{-1}$. Mobile phase was acetonitrile:water (47.5:52.5). Peak identity: 1) cephalomannine 2) paclitaxel.

There was no significant difference in paclitaxel recovered from freeze-dried leaves extracted with 100% methanol (130 $\mu g g^{-1}$) or 40% ethanol (129 $\mu g g^{-1}$), however, both of these solvents are significantly better than extraction with 95% ethanol. While the efficacy of 40% ethanol as a solvent for extracting taxoids is surprising, it matches the trend seen in Figure 1. A positive correlation is observed between solvents of increasing polarity and the recovery of paclitaxel; 40% ethanol would be even more polar than methanol, the most polar solvent in that particular experiment. The 40% ethanol concentration was the lowest concentration of organic solvent that could be effectively used and still recover the majority of the taxanes.

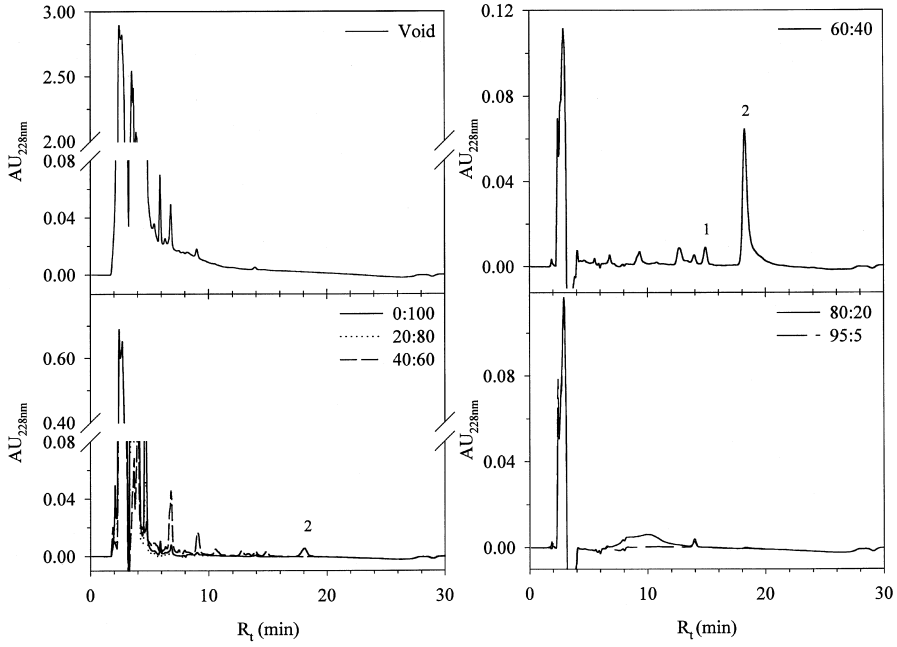

Figure 3. HPLC chromatograms of 40% ethanol extract of freeze-dried *T. x media* leaves and following purification by C_{18} SPE. Each fraction was dried, following SPE in the given eluant of ethanol and water, dissolved in 1 mL of 100% MeOH, further diluted 1:10 in methanol, and 10 µL of this extract was injected. The concentration of paclitaxel in the 60% MeOH fraction is 34 $\mu g mL^{-1}$. Total paclitaxel recovered in this experiment was 0.08% of the dry weight of the needles. Mobile phase was acetonitrile:water (46:54). Peak assignments are the same as in Fig. 2.

Further decreases in the ethanol concentration, by dilution with deionized water, resulted in the precipitation of taxoids from a standard solution containing 100 $\mu g.mL^{-1}$ each of baccatin III, cephalomannine, and paclitaxel. Depending on the solvent used for extraction, and the particular experiment, the average recovery of paclitaxel on a dry weight basis was 0.011 to 0.015% for leaves of *T. x media*. These values are comparable to some of the highest recorded paclitaxel values for leaf tissue from any species of *Taxus.* Hoke et al.[23] reported values of 0.019% for leaves of *T. canadensis*, and Rao et al.[24] reported values of 0.012 to 0.015% paclitaxel from leaves of *T. floridana* and *T. x media*, respectively, essentially identical to those obtained in these experiments. Most other investigators have reported average paclitaxel concentrations less than those obtained in these experiments for *T. x media* leaf tissue.[14,18,23]

Table 6

Comparison of the Efficacy of Extraction Solvent on Recovery of Paclitaxel from Leaves of *Taxus x media

Leaf Treatment	Solvent	N	Paclitaxel ($\mu g.g^{-1}$ Tissue)	Std Err
Fresh	95% EtOH	8	23.5	3.03
Freeze-Dried	95% EtOH	8	49.2	6.04
Dried	40% EtOH	3	46.2	2.66
Freeze-Dried	40% EtOH	9	130	9.08
Fresh	40% EtOH	3	11.6	2.71
Freeze-Dried	100% MeOH	9	129	15.9
Fresh	100% MeOH	3	20.2	1.20

* Means with the same letter are not significantly different (Tukey's multiple comparison test, $p \leq 0.05$).

Table 7

Comparison of Paclitaxel Content from Freeze-Dried Leaves of *Taxus x media* and Leaves of *Taxus brevifolia

Species	Solvent	N	Paclitaxel ($\mu g.g^{-1}$ Tissue)	Std Err
T. Brevifolia-Tree 1	40% EtOH	3	361	12.6
T. Brevifolia-Tree 2	40% EtOH	3	182	16.7
T. x media	40% EtOH	12	120	8.78

* Means with the same letter are not significantly different (Tukey's multiple comparison test, $p \leq 0.05$).

The efficiency of extraction in 40% ethanol was tested on leaves of *T. brevifolia* (Table 7). *T. brevifolia* leaves contained as much as 0.036% paclitaxel, nearly three times the amount in *T. x media* leaves (Table 7), and twice the maximum level reported for *T. x media* by Rao et al.[24] This concentration of paclitaxel in *T. brevifolia* leaves is comparable to values previously reported for the bark[24-25,14] and are the highest paclitaxel amounts

reported from leaves of any species in this genus. Witherup et al.[18] reported that leaves of *T. brevifolia* contained 0.006% paclitaxel on a dry weight basis. Only the bark of *T. brevifolia* has recently been reported to have more paclitaxel than the values reported for leaf tissue in these experiments; 0.02% to 0.04%.[26] Vidensek et al.[14] reported average values of 0.015% and 0.0015% paclitaxel from *T. brevifolia* bark and leaves, respectively. Hoke and co-workers have reported a *T. brevifolia* bark paclitaxel concentration as high as 0.084%, as determined by MS/MS, although it appears that this was a measurement of only one sample.[23]

In greenhouse-grown *T. brevifolia* trees, paclitaxel concentration varied significantly (Table 7). Leaves from a second tree were found to have significantly less paclitaxel than the first tree. Each of these *T. brevifolia* trees contained significantly greater amounts of paclitaxel than the *T. x media* shrubs used for this study (Table 7). A large amount of variation in paclitaxel concentration has been reported in trees sampled in natural populations.[14,27] That variation appears to carry over into trees grown under identical, carefully controlled greenhouse conditions.

These results are also consistent with some of our earlier observations that cell cultures derived from somatically different embryos vary greatly in their ability to produce paclitaxel in culture.[3] Thus, this variation is likely to be genetic and may be exploited through breeding to obtain trees with enhanced paclitaxel concentration.

We have demonstrated that efficient extraction of paclitaxel from leaves of *T. x media* can be obtained using a combination of ethanol and ethanol:water mixtures. These solvents are renewable and generally considered non-toxic, yet recoveries of paclitaxel are comparable to more hazardous solvent systems. The methods presented here result in paclitaxel concentrations as high as any reported in the literature, and in general, are comparable to the paclitaxel concentrations found in the bark of the same species. Application of this method to leaves of *T. brevifolia* yields paclitaxel concentrations comparable to those found in the bark of this species.

The efficiency of this method with leaf material may be due to the use of a very polar solvent for the initial extraction steps, as opposed to the more typical use of a non-polar solvent. Witherup et al.[18] suggest that early CH_2Cl_2:H_2O partitioning steps used in the extraction of bark are less efficient at concentrating paclitaxel in leaf extracts because of higher amounts of waxy, non-polar constituents in the leaves. The ethanol:water extraction method that we describe eliminates the need for a hexane wash to remove the waxy, and non-polar contaminants, because these compounds are not extracted in 40% ethanol.

Rao et al.[24] recently reported a large-scale process for paclitaxel isolation from leaves of two *Taxus* species that utilized a reversed-phase column chromatography step. This method involved extraction of leaves with methanol, concentration of the extract, and partitioning with $CHCl_3$ or CH_2Cl_2. The dried residue was dissolved in acetonitrile and separated on a C_{18}-bonded silica column. One of the most time-consuming aspects of our method is drying the 40% ethanol:H_2O extract. However, we have found that rather than drying this extract, it can be loaded directly on a C_{18}-bonded SPE column, and washed and eluted to yield a highly purified taxoid extract (Figure 3). Thus, our method should be readily adaptable to a large-scale process similar to the one described by Rao et al.[24]

ACKNOWLEDGMENTS

This investigation was supported by a grant from USDA/CSRS; NRI #93-37103-9269. I (R.K.) thank K. A. Ketchum for enthusiastic support and insightful comments.

REFERENCES

†Present Address: Institute of Biological Chemistry, Washington State University, Pullman, WA 99164.

‡Present Address: Bayer Corporation, Pharmaceutical Division, 400 Morgan Lane, West Haven, CT 06516.

1. D. G. I. Kingston, "The Chemistry of Taxol," Pharmac. Ther., **52**, 1 (1992).

2. G M. Cragg, K. M. Snader, "Taxol: The Supply Issue," Cancer Cells Mon.Rev., **3**, 233 (1991).

3. R. E. B. Ketchum, D. M. Gibson, "Paclitaxel Production in Cell Suspension Cultures of *Taxus*," Plant Cell, Tissue, & Organ Cult., **46**, 9 (1996).

4. R. E. B. Ketchum, D. M. Gibson, L. G. Gallo, "Media Optimization for Maximum Biomass Production in Cell Cultures of Pacific Yew (*Taxus brevifolia* Nutt.)," Plant Cell, Tissue, & Organ Cult., **42**, 185 (1995).

5. D. M. Gibson, R. E. B. Ketchum, T. J. Hirasuna, M. L. Shuler, "Potential of Plant Cell Culture for Taxane Production,"in **Taxol: Science and Applications**, M. Suffness, ed., CRC Press, Boca Raton, 1995, p. 71.

6. D. M. Gibson, R. E. B. Ketchum, N. C. Vance, A. A. Christen, "Initiation and Growth of Cell Lines of *Taxus brevifolia* (Pacific Yew)," Plant Cell Rep., **12**, 479 (1993).

7. M. Hezari, R. E. Ketchum, D. M. Gibson, R. Croteau, "Taxol Production and Taxadiene Synthase Activity in *Taxus canadensis* Cell Suspension Cultures," Arch. Biochem. Biophys., **337**, 185 (1995).

8. J. Hefner, S. M. Rubenstein, R. E. B. Ketchum, D. M. Gibson, R. M. Williams, R. Croteau, "Cytochrome P450-Catalyzed Hydroxylation of Taxa-4(5),11(12)-diene To Taxa-4(20),11(12)-dien-5α-ol: The First Oxygenation Step in Taxol Biosynthesis," Chem. Biol., **3**, 479 (1996).

9. R. E. B. Ketchum, D. M. Gibson, "A Novel Method of Isolating Taxanes From Cell Suspension Cultures of Yew (*Taxus*)," J. Liq. Chromatogr., **18**, 1093 (1995).

10. R. E. B. Ketchum, D. M. Gibson, "Rapid Isocratic Reversed-Phase HPLC of Taxanes on New Columns Developed Specifically for Taxol Analysis," J. Liq. Chromatogr., **16**, 2519 (1993).

11. M. D. DeFuria, Z. Horovitz, "Taxol Commercial Supply Strategy," Monogr. Natl. Cancer Inst., **15**, 195 (1993).

12. D. Liu, G. Y. Zhang, X. Wang, Z. B. Hu, "A Survey of *Taxus* Resources and Taxol Production," J. Plant Res. Env., **6**, 48 (1997).

13. S. D. Harvey, J. A. Campbell, R. G. Kelsey, N. C. Vance, "Separation of Taxol from Related Taxanes in *Taxus brevifolia* by Isocratic Elution Reversed-Phase Microcolumn High-Performance Liquid Chromatography," J. Chromatogr., **587**, 300 (1991).

14. N. Vidensek, P. Lim, A. Campbell, C. Carlson, "Taxol Content in Bark, Wood, Root, Leaf, Twig, and Seedling from Several *Taxus* Species," J. Nat. Prod., **53**, 1609 (1990).

15. E. R. Wickremesinhe, R. N. Arteca, "*Taxus* Callus Cultures: Initiation, Growth Optimization, Characterization, and Taxol Production," Plant Cell, Tissue, & Organ Cult., **35**, 181 (1994).

16. A. G. Fett-Neto, S. J. Melanson, K. Sakata, F. DiCosmo, "Improved Growth and Taxol Yield in Developing Calli of *Taxus cuspidata* by Medium Composition," Biotechnology, **11**, 731 (1993).

17. J. H. Cardellina, "HPLC Separation of Taxol and Cephalomannine," J. Liq. Chromatogr., **14**, 659 (1991).

18. K. M. Witherup, S. A. Look, M. W. Stasko, T. J. Ghiorzi, G. M. Muschik, "*Taxus spp.* Leaves Contain Amounts of Taxol Comparable to the Bark of *Taxus brevifolia:* Analysis and Isolation," J. Nat. Prod., **53**, 1249 (1990).

19. D. P. Stull, "Current Taxol Production from Yew Bark and Future Production Strategies," in **Proceedings of the Second National Cancer Institute Workshop on Taxol and Taxus**, Alexandria, VA, NCI, Sept. 23-24th, 1992.

20. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

21. M. Suffness, M. E. Wall, "Discovery and Development of Taxol," in **Taxol: Science and Applications**, M. Suffness, ed., CRC Press, Boca Raton, 1995, p. 3.

22. H. N. ElSohly, El-Kashoury, E. M. Croom, Jr., M. A. ElSohly, K. D. Cochran, J. D. McChesney, "Effect of Drying *Taxus* Leaves on Their Taxol Content: The Impact of Drying Intact Clippings," Planta Med., **61**, 290 (1995).

23. S. H. Hoke, J. M. Wood, R. G. Cooks, X-H. Li, C. Chang, "Rapid Screening for Taxanes by Tandem Mass Spectrometry," Anal. Chem., **64**, 2313 (1992).

24. K. V. Rao, R. S. Bhakuni, J. Juchum, R. M. Davies, "A Large Scale Process for Paclitaxel and Other Taxanes from the Leaves of *Taxus x media* Hicksii and *Taxus Floridana* Using Reverse Phase Column Chromatography," J. Liq. Chrom. & Rel. Technol., **19**, 427 (1996).

25. N. C. Vance, R. G. Kelsey, T. E. Sabin, "Seasonal and Tissue Variation in Taxane Concentrations of *Taxus brevifolia*," Phytochemistry, **36**, 1241 (1994).

26. K. V. Rao, "Taxol and Related Taxanes. I. Taxanes of *Taxus brevifolia* Bark," Pharm. Res., **10**, 521 (1993).

27. N. C. Wheeler, K. Jech, S. Masters, S. W. Brobst, A. B. Alvarado, A. J. Hoover, K. M. Snader, "Effects of Genetic, Epigenetic, and Environmental Factors on Taxol Content in *Taxus brevifolia* and Related Species," J. Nat. Prod., **55**, 432 (1992).

Received September 15, 1998
Accepted October 18, 1998
Manuscript 4923

Request Permission or Order Reprints Instantly!