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# Microwave-Assisted Extraction of Taxanes from *Taxus* Biomass

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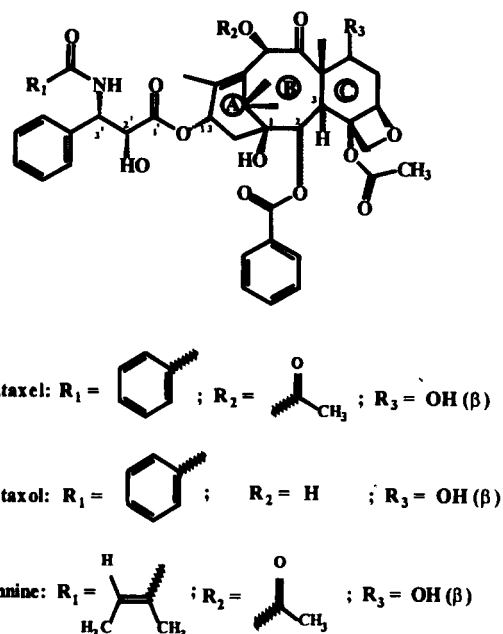
A method is presented for the extraction of the taxane class of natural products, including paclitaxel, from *Taxus* needles using microwave-assisted extraction (MAE). Various temperatures, times, and organic solvents for the MAE procedure were investigated to optimize the efficiency of the extraction. The effects of biomass to solvent ratio and the water content of the system on taxane recovery were also determined. Under appropriate MAE conditions using 95% ethanol, an extract of the needles was obtained which was equivalent to that produced by a conventional extraction method. The conventional method consisted of an overnight shake of 5 g of needles in 100 mL of methanol at ambient temperature. Thus, with optimized parameter settings, MAE was found to reduce considerably both extraction time and solvent consumption, while maintaining qualitative and quantitative taxane recovery relative to traditional solid/liquid extraction methods.

**Keywords:** Taxanes; paclitaxel; microwave-assisted extraction; *Taxus* needles

## INTRODUCTION

Study of the compound paclitaxel, a member of the taxane class of natural products, is now in its fourth decade, having begun in the mid-1960s. Taxanes are diterpenoids characterized by the tricyclic fused ring system shown in Figure 1. The taxanes are found in roots, needles, bark, and wood of the species *Taxus*. Paclitaxel is by far the best known of the taxanes. Its antimitotic effects in mammalian systems have been exploited in its clinical formulation, Taxol, approved for the treatment of ovarian and breast cancer. Excellent reviews of the development of paclitaxel are available in the literature (Borman, 1991; Huizing et al., 1995; Georg et al., 1995). While the total synthesis of paclitaxel has been reported by two laboratories (Nicolaou et al., 1994; Holton et al., 1994), the large number of steps required in both syntheses renders their commercialization problematical. Therefore, *Taxus* biomass remains the principal source of paclitaxel and other naturally produced taxanes. However, the destructive harvest of *Taxus brevifolia* to accomplish the extraction of the taxanes from the bark of the Pacific yew tree has been replaced by their extraction from alternative *Taxus* sources, mainly *Taxus* needles. The harvest of *Taxus* needles from commercial plantings of ornamental yew shrubs is both environmentally sound and sustainable.

Since 1991 our laboratory has been interested in taxanes extracted from the needles of *Taxus* cultivars grown commercially for landscape use. This research focuses primarily on the interaction of a partially purified, mixed taxane fraction, extracted from *Taxus* needles, with nonmammalian systems. Such studies may eventually lead to the development of taxanes as agrochemicals for their pesticidal qualities. These studies require the extraction and partial purification of substantial quantities of the mixed taxane fraction. A few years ago we reported the successful use of solid phase extraction (SPE) membrane technology for the scaled-up purification of the crude methanolic extract



**Figure 1.** Structures of some naturally produced taxanes.

of the needles (Mattina and MacEachern, 1994). The extraction process begins with a 16 h shake at ambient temperature of 5 g of *Taxus* needles with 100 mL of methanol.

As in our method, the majority of the solid/liquid extraction processes reported in the literature have made use of methanol as the extraction solvent at ambient temperature. However, there are also reports of refluxing with methanol (Glowniak et al., 1996); extraction using 1:1 methanol/chloroform at ambient temperature (Fang et al., 1995); percolation using ethanol (McLaughlin et al., 1981); and 95% ethanol extraction at ambient temperature (Kopycki et al., 1994). Supercritical  $CO_2$  extraction of *Taxus* needles both with and without added cosolvents has been reported as well (Chun et al., 1996).

Microwave digestion, using aqueous mineral acids, of matrices for their eventual elemental analysis has been

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Table 1. Teflon Liner Adhesion Study

solution	paclitaxel (ng/ $\mu$ L)		recovery (%)
	expected	observed	
1	2.75	2.82	102.5
2	2.06	1.94	94.2

routine for several years (Kingston and Jassie, 1988). Recently, microwave-assisted extraction (MAE) equipment for extraction of analytes from matrices using organic solvents has been commercialized. Some environmental applications of MAE for organic contaminants in soils have appeared in the literature (Lopez-Avila et al., 1994).

We investigated the suitability of microwave assistance for the extraction of the taxanes from *Taxus* needles for several reasons: (1) to scale up the preparation of the mixed taxane fraction; (2) to reduce the solvent costs associated with our conventional methanolic extraction of *Taxus* needles; (3) to reduce extraction time; and (4) to improve extraction efficiency, if possible. We report here the results of our study.

## MATERIALS AND METHODS

**Plant Material.** All *Taxus* biomass was grown by us either in the field or in the greenhouse. Specific cultivars are indicated at the appropriate location in the text. Stems were cut from the plant, and mature needles were stripped manually from the stem and then chopped in a laboratory blender such that the average size was <2 mm. Storage conditions for the blended needles are described in the following text. The moisture content of the biomass used in each experiment was determined to express all taxane concentrations on a dry weight basis.

**Standard Operating Procedures.** Our conventional solid/liquid extraction method, clean up of the crude alcoholic extract, and analysis by liquid chromatography (HPLC) have been reported previously (Mattina and MacEachern, 1994). In brief, 5 g of the ground needles is shaken overnight at ambient temperature with 100 mL of methanol. The solvent is removed under rotary evaporation, the crude residue is reconstituted with 10:1 methanol/water, and a small portion of the reconstituted extract is transferred to an SPE C<sub>18</sub> cartridge. The cartridge is then washed with water, 20% methanol/80% water, and 40% methanol/60% water. The taxanes are finally eluted from the SPE cartridge with 80% methanol/20% water. This partially purified extract is analyzed using HPLC on a Taxsil specialty column (MetaChem Technologies Inc., Torrance, CA) with UV diode array detection. Quantitation is by external standard. HPLC details are provided elsewhere (MacEachern-Keith et al., 1997).

HPLC solvents were of Omnisolv grade (EM Science, Gibbstown, NJ). The methanol and chloroform used for extraction were of HPLC grade (J. T. Baker, Phillipsburg, NJ), and the 95% ethanol was 190 proof pure (Millennium Petrochemicals, Cincinnati, OH). All water used was distilled, followed by deionization in a NANOpure II four-cartridge system (Barnstead/Thermolyne Corp., Dubuque, IA). Taxane standards were obtained from the National Cancer Institute, Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program (Bethesda, MD).

**Taxane Adhesion Study.** A known amount of paclitaxel stock solution in methanol was spiked into 10 mL of methanol contained in the Teflon PFA liner from a microwave extraction vessel. The vessel was covered, shaken vigorously, and then allowed to sit for 15 min at ambient temperature. A portion of this solution, designated solution 1 in Table 1, was removed and analyzed by HPLC. The remaining solution 1 was poured out of the Teflon liner, and the liner was thoroughly rinsed with methanol. The methanol rinsate was combined with the remaining solution 1, and the diluted solution was designated solution 2 (see Table 1) and then analyzed by HPLC.

**Microwave Extraction.** All microwave extractions were performed using the MES-1000 microwave solvent extraction system from CEM Corp. (Matthews, NC). The MES-1000 was programmed at 100% power in a time-to-temperature mode as follows: 2:20 min ramp to the desired temperature  $x$ ; 6:00 min hold; removal of selected vessels; 0:30 min ramp to temperature  $x'$ ; 3:10 min hold; removal of selected vessels; 0:30 min ramp to temperature  $x''$ ; 3:10 min hold. Depending on the experiment being conducted,  $x$ ,  $x'$ , and  $x''$  may or may not have the same value. A parallel printer was connected to the unit to maintain a permanent record of time vs temperature and time vs pressure for each run. The biomass weight and solvent volume used in each extraction are noted at the appropriate point in the text below.

**Spiked Recovery Study.** This study was conducted at 85 °C and 9:10 min. Each of four vessels was charged with 5 g of biomass and 10 mL of methanol as the extracting solvent. Appropriate amounts of standard solutions of paclitaxel and 10-deacetylaxol in methanol were then spiked into two of the vessels prior to extraction.

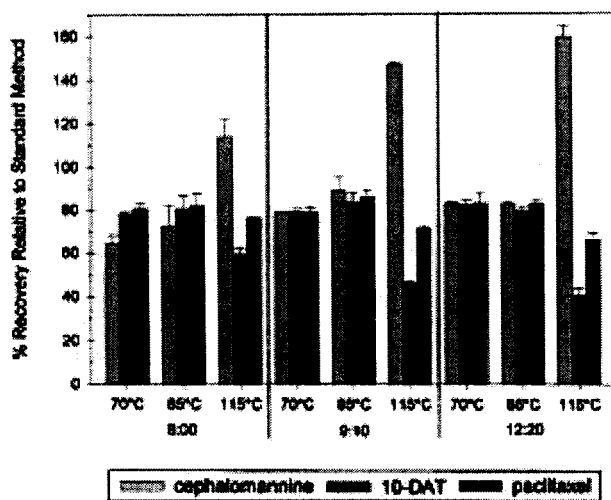
## RESULTS AND DISCUSSION

**Taxane Adhesion Study.** The unexpected adhesion of paclitaxel to fluorinated polymer surfaces has been observed. For example, in the course of investigating the degradation kinetics of paclitaxel in solution, we noted significant loss of paclitaxel when methanolic solutions of the compound came into contact with standard PTFE tape (Fisher Scientific catalog no. 14-831-300A; M. J. I. Mattina, unpublished results). Adsorption of several taxanes, including paclitaxel, to poly(vinylidene) fluoride (PVDF) syringe filters (Gelman Sciences, Ann Arbor, MI) has been reported in the literature (Ketchum and Gibson, 1995). For these reasons it was important to demonstrate that the recovery of taxanes from *Taxus* needles would not be reduced by adhesion to the Teflon liner of the MAE vessel.

The data from the adhesion study are summarized in Table 1. It is apparent from the column labeled "recovery" that paclitaxel recovery is not reduced by adhesion of the compound to the Teflon liner.

**Microwave Extraction Suitability Study.** Since our conventional extraction method utilized methanol as the extracting solvent, we began this study of the suitability of MAE of taxanes with methanol. Cuttings from 3-year-old *Taxus cuspidata* Greenwave plantings at Lockwood Farm, The Connecticut Agricultural Experiment Station's experimental facility in Hamden, CT, were collected in August 1996. The needles were stripped from the twigs and blended in a laboratory blender. Blended needles were stored frozen in airtight plastic bags. Moisture content of freshly harvested needles shows seasonal variation and must be measured just prior to the extraction. Five grams of the blended needles was measured into each extraction vessel, followed by the addition of 10 mL of methanol to the vessel. The charged vessel is allowed to sit for 10 min prior to the start of MAE. Duplicate extractions were performed for 6:00, 9:10, and 12:20 min at each of three selected temperatures: 70, 85, and 115 °C. Conventional extraction was also performed on the same biomass in order for the MAE recoveries to be expressed as a percent recovery relative to the conventional method. Average MAE recoveries for three taxanes are presented in Figure 2. With paclitaxel recoveries approaching 90%, these data indicate that MAE can provide advantages over the conventional extraction method.

The spiked recovery studies were performed with needles collected in June 1996 from 3-year-old *Taxus*



**Figure 2.** MAE suitability study: recoveries of taxanes relative to conventional extraction method from MAE of 5 g of 66% moisture Greenwave needles extracted with 10 mL of methanol at a series of times and temperatures ( $n = 2$  for each time/temperature combination).

× *media* Hicksii plants grown at Lockwood Farm. Table 2 summarizes the recoveries of paclitaxel and 10-deacetyltaxol. Since quantitative recoveries are observed, degradation under MAE conditions does not appear to be an issue at 85 °C for 9:10 min.

**Selection of Microwave Extraction Times and Temperatures.** From the recoveries summarized in Figure 2 and examination of the LC traces in Figure 3, 85 °C and 9:10 min provide the most favorable MAE conditions from the time and temperature combinations included in this study. Recoveries of 10-deacetyltaxol, cephalomannine, and paclitaxel drop below 80% at 70 °C. At 115 °C paclitaxel recovery falls to <75% and cephalomannine recovery ranges from 110% to 160%. The high cephalomannine recovery is due to coelution

**Table 2.** Spiked Recoveries of Taxanes from MAE of *Taxus* Needles

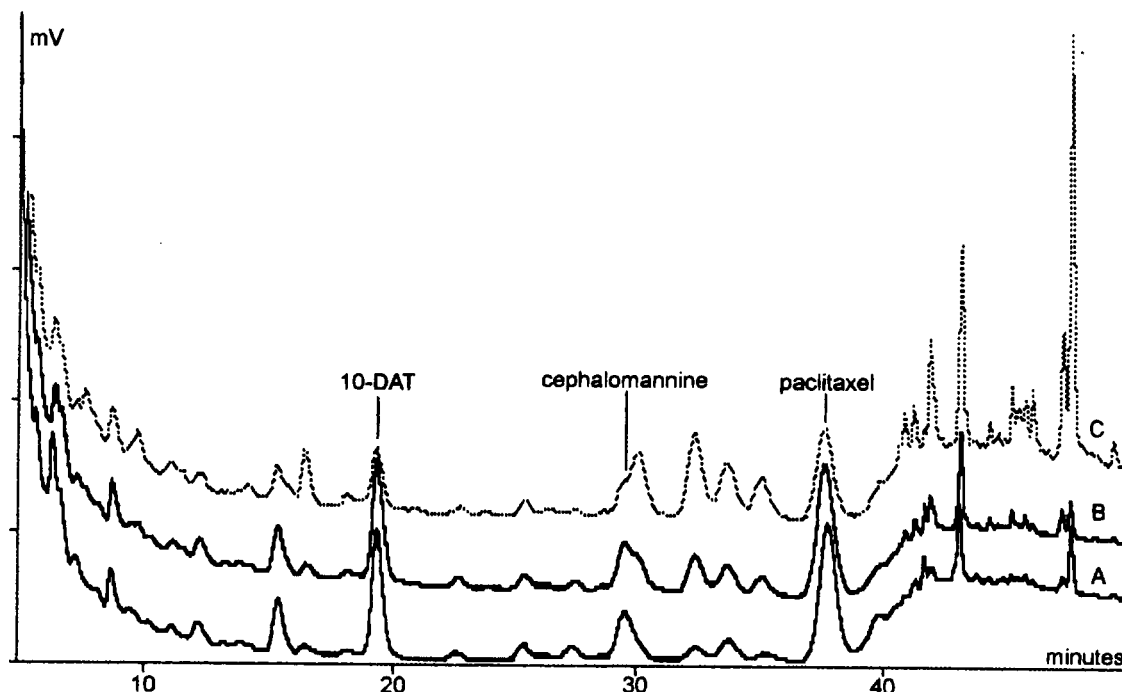
vessel no.	paclitaxel ( $\mu$ g)		recovery (%)	10-DAT ( $\mu$ g)		recovery (%)
	expected	observed		expected	observed	
1	768	771	100	542	532	98
2	788	842	107	492	516	105

with increased amounts of interfering taxanes, most likely cinnamyls. This is seen in the comparison of the HPLC trace recorded at  $\lambda = 230$  nm of the partially purified mixed taxane fraction from MAE at 85 °C (trace B) vs that at 115 °C (trace C) as shown in Figure 3. Indeed, from our study of paclitaxel degradation kinetics in methanol (MacEachern-Keith et al., 1997), the degradation half-life of paclitaxel in methanol,  $t_{1/2}$ , is calculated to be 1:42 min at 115 °C and 47 min at 85 °C. Obviously, degradation of the taxanes in methanol is a serious concern at 115 °C with MAE both in theory and in practice.

#### Selection of Microwave Extraction Solvents.

Having shown that MAE of taxanes is feasible with  $1/10$  the volume of methanol used in our conventional extraction method, we next examined the suitability of 95% ethanol and chloroform as alternative MAE solvents. Cuttings from the *T. cuspidata* Greenwave plantings at Lockwood Farm were collected in March 1997 and prepared as described above. In this set of experiments 5 g of freshly harvested, blended needles was mixed in the extraction vessel with 20 mL of methanol, 95% ethanol, or chloroform and extracted at 85 °C for 9:10 min. The data are presented in Figure 4. While recoveries for the three taxanes are comparable with methanol or 95% ethanol, recoveries with chloroform are significantly lower.

A cost comparison of the use of various solvents is presented in Table 3. Since MAE with 95% ethanol would represent a cost saving over methanol, without significant reduction of taxane recoveries, we decided to optimize the MAE of taxanes from *Taxus* needles using 95% ethanol, 85 °C, and 9:10 min.



**Figure 3.** Comparison of HPLC trace at  $\lambda = 230$  nm of 66% moisture Greenwave needles extracted using methanol via conventional 16 h shake at ambient temperature (trace A), MAE at 85 °C for 9:10 min (trace B), and MAE at 115 °C for 9:10 min (trace C).

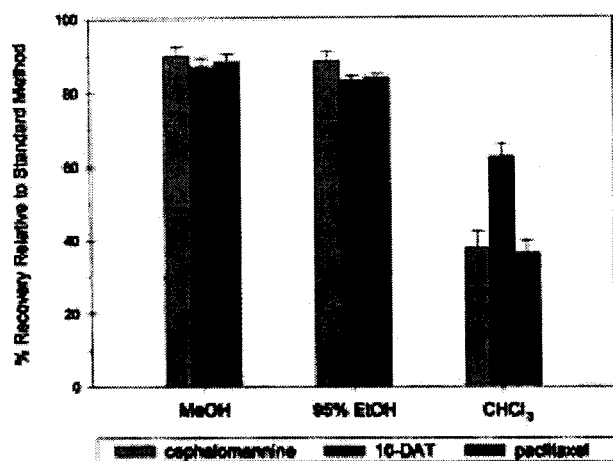


Figure 4. MAE solvent study: MAE of 5 g of 55% moisture Greenwave needles at 85 °C for 9:10 min with 20 mL of methanol ( $n = 3$ ), 20 mL of 95% ethanol ( $n = 4$ ), and 20 mL of chloroform ( $n = 2$ ).

Table 3. Comparison of Microwave Extraction Solvent Costs

	conventional with methanol	MAE with ethanol	MAE with 95% ethanol	MAE with chloroform
volume (mL)/g of dry biomass <sup>a</sup>	50	10	10	10
solvent	190	40	10	70
cost (\$)/kg of dry biomass <sup>a</sup>				
paclitaxel	100	87	85	37
recovery (%)				
solvent	380	92	24	378
cost (\$)/g of paclitaxel <sup>b</sup>				

<sup>a</sup> Assuming 60% moisture in needles. <sup>b</sup> Assuming 500 ppm of paclitaxel in needles.

**Effects of Biomass to Solvent Ratio and System Moisture Content on Microwave Extraction Efficiency.** As the information in Table 3 indicates, MAE of 5 g of *Taxus* needles containing ~60% moisture with 20 mL of 95% ethanol provides a significant cost saving over MAE with methanol. In an attempt to achieve additional savings, we reduced the volume of 95% ethanol to 10 mL for the same amount of biomass. The data from these studies are presented in Figure 5. Paclitaxel recovery drops from 84% (trial A) to 80% (trial B) of the level from the conventional method.

In an attempt to improve the MAE recovery of paclitaxel from the 55% moisture needles, we began to examine the influence of water content on MAE efficiency. Soaking 5 g of needles in 2.5 mL of water for 10 min prior to addition of 20 mL of 95% ethanol barely improved paclitaxel recovery from 84% (trial A) to 87% (trial C) of the level from the conventional method. However, presoaking 5 g of 55% moisture needles in the extraction vessel with 5 mL of water for 10 min prior to addition of 10 mL of 95% ethanol improved MAE paclitaxel recovery from 80% without water addition (trial B) to 90% (trial D) of the level from the conventional method. Similar improvement in recovery (trial E) is noted when the water and 95% ethanol are premixed in a 1:2 ratio before addition to the needles in the extraction vessel. Thus, moisture content would seem to impact microwave extraction efficiency.

We examined the influence of moisture on microwave extraction efficiency through several extractions of freeze-dried needles. Several years ago we determined

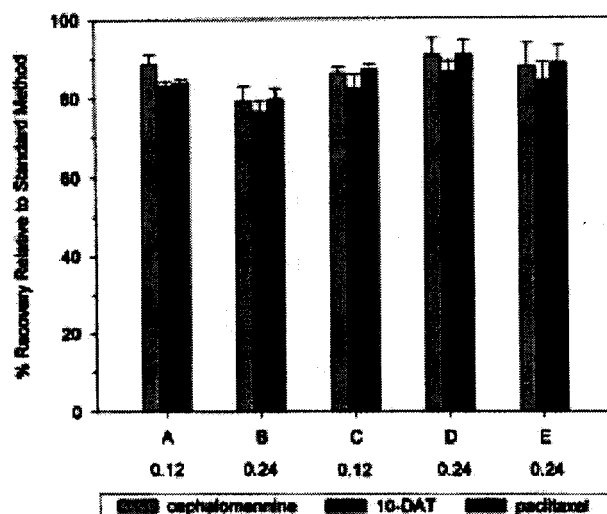
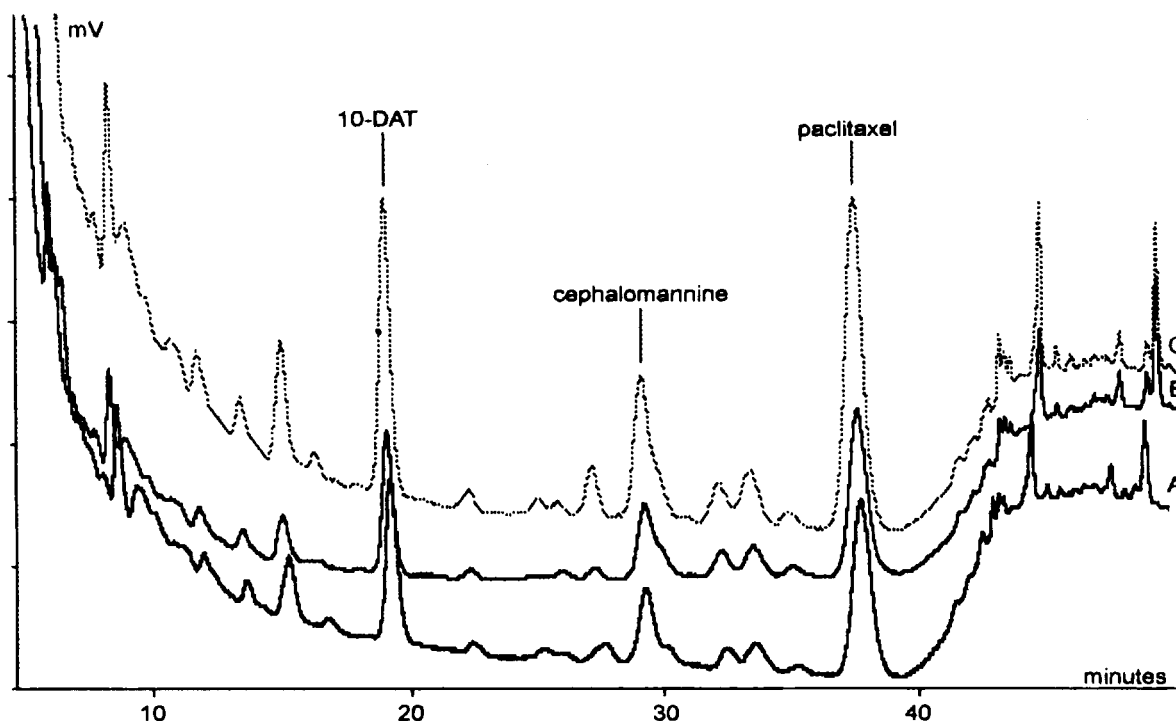


Figure 5. Optimization of MAE of 5 g of 55% moisture Greenwave needles using 95% ethanol at 85 °C for 9:10 min. Number under each trial indicates the grams of dry biomass to milliliters of ethanol ratio. Trial A: 20 mL of solvent ( $n = 4$ ). Trial B: 10 mL of solvent ( $n = 2$ ). Trial C: 2.5 mL of H<sub>2</sub>O soak followed by 20 mL solvent ( $n = 2$ ). Trial D: 5 mL of H<sub>2</sub>O soak followed by 10 mL of solvent ( $n = 2$ ). Trial E: 15 mL of 2:1 95% ethanol/water solvent ( $n = 2$ ).

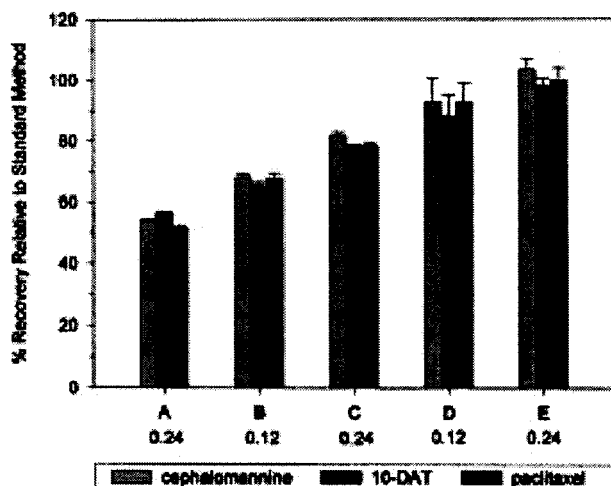
that vacuum freeze-drying, which reduced the moisture content of *Taxus* needles from 62% to 11% overnight, produced no qualitative changes in the partially purified extract derived by conventional extraction of freeze-dried needles compared with freshly harvested needles (M. J. I. Mattina, unpublished results). Furthermore, the recovery of paclitaxel from freeze-dried needles was the same as that from fresh needles and was not altered by addition of up to 10 mL of water to the 100 mL of methanol extraction solvent. Thus, freeze-drying has no qualitative or quantitative effects on taxane extraction via the conventional method.

When the ground Greenwave needles from the March 1997 harvest were stored in a paper rather than a plastic bag in a frost-free freezer, the moisture content dropped from 55% to ~7% in 7 weeks. The partially purified extract of 9.5% moisture needles produced using the conventional method (Figure 6, trace C) once again showed neither qualitative nor significant quantitative changes when compared with the conventional extract from the original 55% moisture needles (Figure 6, trace A).

However, the data summarized in Figure 7 show that a very different quantitative situation is observed with MAE of freeze-dried needles. For this set of experiments when 2.5 g of 9.5% moisture needles was extracted for 9:10 min at 85 °C using 10 mL of 95% ethanol, paclitaxel recovery dropped to ~50% (trial A) of that observed with the conventional method. Increasing the 95% ethanol volume to 20 mL improves recovery somewhat (trial B). However, if the low-moisture needles are soaked with 1 mL of water for 10 min prior to the addition of 10 mL of 95% ethanol to the MAE vessel, paclitaxel recovery improves to almost 80% (trial C). Similarly, soaking 2.5 g of freeze-dried needles with 2.5 mL of water prior to MAE with 20 mL of 95% ethanol improves recovery (trial D) over trial B. If the freeze-dried needles are soaked with 5 mL of water for 10 min prior to the addition of 10 mL of 95% ethanol to the MAE vessel, paclitaxel recovery improves to 100%, cephalomannine recovery to 104%, and 10-



**Figure 6.** Comparison of HPLC trace at  $\lambda = 230$  nm of (trace A) 55% moisture Greenwave needles extracted using conventional method with methanol, (trace B) 9.5% moisture Greenwave needles extracted using MAE with 5 mL of  $H_2O$  soak followed by 10 mL of 95% ethanol, and (trace C) 9.5% moisture Greenwave needles extracted using conventional method with methanol



**Figure 7.** MAE of 2.5 g of freeze-dried Greenwave needles at 85 °C for 9:10 min. Number under each trial indicates the grams of dry biomass to milliliters of ethanol ratio. Trial A: 9.5% moisture needles with 10 mL of 95% ethanol ( $n = 2$ ). Trial B: 7% moisture needles with 20 mL of 95% ethanol ( $n = 4$ ). Trial C: 9.5% moisture needles with 1 mL of  $H_2O$  soak followed by 10 mL of 95% ethanol ( $n = 2$ ). Trial D: 7% moisture needles with 2.5 mL of  $H_2O$  soak followed by 20 mL of 95% ethanol ( $n = 2$ ). Trial E: 9.5% moisture needles with 5 mL of  $H_2O$  soak followed by 10 mL of 95% ethanol ( $n = 6$ ).

deacetyltaxol to 98% of the conventional method (trial E). As expected, at the 95% confidence level, recoveries for all three taxanes from trials D and E are significantly higher than those from trial A. The lyophilization of the *Taxus* biomass appears to make the taxanes more accessible to MAE provided that the system contains sufficient water. Qualitatively, the mixed taxane extract from trial E (Figure 6, trace B) is equivalent to that from the conventional extraction of the fresh needles (Figure 6, trace A).

**Conclusions.** Parameters for the MAE of taxanes from *Taxus* needles have been determined which result in distinct advantages of MAE over conventional solid/liquid extraction. The methanol used in the conventional method has been replaced with less expensive 95% ethanol, and the solvent consumption has been considerably reduced. When 5 g of freshly harvested needles, having a moisture content of 55–65%, is presoaked with 5 mL of water prior to MAE with 10 mL of 95% ethanol at 85 °C for 9:10 min,  $\approx 90\%$  of the taxanes are recovered. This ratio of grams of dry biomass to milliliters of ethanol,  $<0.25$ , should be maintained, as well as the presoaking with sufficient water, to achieve these recovery levels. Taxane recoveries can be improved to  $\approx 100\%$  of the conventional method if the biomass is freeze-dried to  $<10\%$  moisture, the 0.25 ratio is adhered to, and the biomass is presoaked with a volume of water equal to half the volume of 95% ethanol to be used in the extraction. After partial purification of the taxane extract with SPE, the microwave assisted extract is quantitatively and qualitatively equivalent to that from the conventional extraction method.

It should be noted that using the above MAE parameters, taxane recovery equal to, but not significantly greater than, that observed using the conventional method may be achieved. It is, therefore, reasonable to assume that concentration levels obtained via the conventional method represent quantitative extraction of the taxanes from the *Taxus* needle biomass.

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