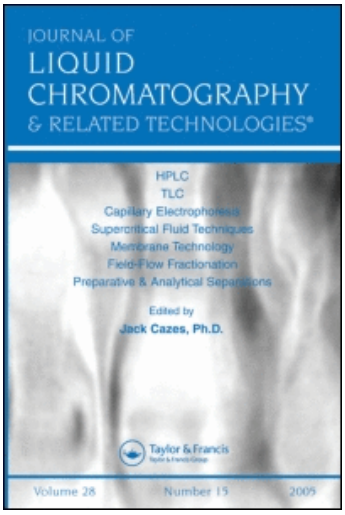

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Novel Method of Isolating Taxanes from Cell Suspension Cultures of Yew (*Taxus* Spp.)

R. E. B. Ketchum[a]; D. M. Gibson[a]
[a] USDA Agricultural Research Service Plant Protection Research Unit US Plant Soil, and Nutrition Laboratory Tower Road Ithaca, New York

To cite this Article Ketchum, R. E. B. and Gibson, D. M.(1995) 'A Novel Method of Isolating Taxanes from Cell Suspension Cultures of Yew (*Taxus* Spp.)', Journal of Liquid Chromatography & Related Technologies, 18: 6, 1093 — 1111
To link to this Article: DOI: 10.1080/10826079508009278
URL: http://dx.doi.org/10.1080/10826079508009278

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(6), 1093-1111 (1995)

A NOVEL METHOD OF ISOLATING TAXANES FROM CELL SUSPENSION CULTURES OF YEW (*TAXUS* SPP.)

RAYMOND E. B. KETCHUM AND DONNA M. GIBSON
USDA, Agricultural Research Service
Plant Protection Research Unit
US Plant, Soil, and Nutrition Laboratory
Tower Road
Ithaca, New York 14882

ABSTRACT

A method is described to isolate a mixture of taxanes from cell suspension cultures of *Taxus* species. The aqueous suspension medium is pre-filtered and centrifuged to remove cellular debris, and then passed through either nylon or PVDF membranes. Contaminants are washed from the membranes and the taxanes are eluted with appropriate solvents. This method provides a rapid, efficient, and inexpensive means of extracting taxanes from cell suspension medium, as well as a significant reduction in the total volume of solvents used.

INTRODUCTION

One possible alternative to the extraction of taxol and related taxanes from bark or needles is the use of continuous, sustainable cultures of cells of *Taxus* to produce these complex diterpenoids *in vitro* (2-7). Taxol and other taxanes are excreted from the cell into the aqueous culture medium and can be isolated

without contamination by chlorophyll or other pigments and compounds associated with isolation of taxanes from needles or bark (2,3).

Most reports of the purification of taxanes from cells or bark of *Taxus* rely on the use of chlorinated hydrocarbons, such as methylene chloride or chloroform (4,6,8,9). The feasibility of isolating taxanes from suspension cell cultures with a minimal amount of solvent, and without the use of any chlorinated hydrocarbons, would have important environmental implications. The unique ability of various *Taxus* cell lines to grow in an aqueous medium, and to excrete taxanes into that medium, eliminates the need to do solvent:solvent partitioning to extract taxanes from these cultures. In this paper we describe a novel method to isolate taxanes from *Taxus* cell suspension medium using a technique developed in this laboratory.

MATERIALS

Chromatographic Conditions

Authentic samples of taxanes were very generously provided by Dr. David T. Bailey, Hauser Chemical Research, Inc., Boulder, CO, and by Dr. Kenneth M. Snader, NCI, Bethesda, MD. All presumptive taxanes in suspension cultures of *Taxus cuspidata* cell line P991 were identified and quantified with HPLC analysis by comparison to retention times and absorption spectra with a standard mixture consisting of 10-deacetyl baccatin III [**1**], 7-epi-10-deacetyl baccatin III [**2**], baccatin III [**3**], 10-deacetyl-7-xylosyl taxol C [**4**], 10-deacetyl-7-xylosyl taxol [**5**], 10-deacetyl-7-xylosyl taxol B [**6**], 10-deacetyl taxol [7], cephalomannine [**8**], 7-epi-10-deacetyl taxol [**9**], taxol [**10**], taxol C [**11**] (not quantified), and 7-epi taxol [**12**].

Taxane concentrations were determined by comparison to an external standard curve over the range of 5 to 50 μg/mL. A method for rapid isocratic analysis of taxanes has been developed in our laboratory (7). Crude taxane

mixtures in methanol were separated on a Phenomenex 4 μm, 250 x 4.6 mm, Curosil G column with an Upchurch ODS disposable guard cartridge (1). Mobile phase was CH_3CN:H_2O (52.5:47.5), 1 mL/min flow rate, with UV detection at 228 nm and diode array scans of each peak from 200 to 300 nm. All solvents and samples were filtered through 0.2 μm Nylon 66 or PVDF filters (Gelman) prior to use.

Instrumentation consisted of a Beckman Model 126 binary pump and gradient mixer, Beckman Model 168 Diode Array Detector, and Beckman Model 507 autosampler. Data acquisition, processing, integration, and instrument control was with a Gateway 2000 computer with 80486 microprocessor running Beckman System Gold v. 6.07 software.

Tissue Culture Reagents

All chemicals used for tissue culture of *Taxus* plant cells were obtained from Sigma Chemical Company (St. Louis, MO, USA). Pre-packaged media mixes were not used.

METHODS

Experimental Design and Statistical Analysis

All treatments within an experiment were triplicated. Data is presented as the mean of the triplicates and error bars represent the standard error of the mean. Because amounts of taxanes varied between cell suspension cultures, only data from one randomly selected set of experiments is presented, although all experiments were done at least three times.

Statistical analysis, where appropriate, was performed with Minitab v. 8 software (Addison-Wesley). Treatments were analyzed with a one-way ANOVA

and differences between means tested with a Tukey multiple comparison test with a 0.05 family error rate.

Cell Line Initiation and Maintenance

Cell line P991 is a *Taxus cuspidata* cell line that has been growing in suspension cell culture since it was first isolated and established in our laboratory in September 1991. Every two weeks it was subcultured by aliquot transfer of 10 mL of cells and suspension media into 40 mL of fresh B5NB medium (Gamborg B5 liquid medium with 2% sucrose, 2.7 μM NAA, 0.01 μM BA, 0.25 mM ascorbic acid, 2 mM glutamine, pH 5.50). Cultures were grown in 125 mL Erlenmeyer flasks capped with Bellco ™ silicone/foam caps, in the dark at 25 °C and 125 rpm. For each experiment, medium was used from a single flask of P991 cells that were harvested after 21 days of growth. The culture was filtered through a single layer of Miracloth (Calbiochem) and the filtrate was used for subsequent extraction experiments.

Analysis of Taxanes in Suspension Medium

Taxanes were routinely analyzed from aqueous suspension medium by taking 1.0 mL samples of the cell-free suspension medium, and evaporating to 100 μbar dryness on a Savant Speed-Vac (Savant Instruments, Farmingdale, NY). The dried pellet was extracted with 0.2 mL acidified methanol (0.01 % glacial acetic acid added for stabilization of taxanes), agitated in an ultrasonic bath for 60 min, centrifuged at 16,000 x g for 15 min, and filtered through a 0.2 μm PVDF syringe filter (Gelman Sciences). This 5X concentrated methanol extract was then analyzed via HPLC against an external standard curve containing a mixture of 5,10, 25, and 50 μg/mL taxanes. All peaks in the suspension extracts were scanned with a diode array, and scans were compared to those of authentic taxane standards.

Modifications to this method have included extraction in 0.1 mL acidified methanol for a 10X concentration, or 0.5 mL of suspension extracted with 0.1 mL for samples where suspension medium volumes are limited.

Centrifugation Studies on the Precipitation of Taxanes in Aqueous Solution

Suspension medium was filtered through Miracloth, and 1.5 mL samples were transferred to 1.5 mL microcentrifuge tubes. Samples were centrifuged at 1000, 2000, 4000, 8000, or 16000 x g for 30 min. After centrifugation, 0.75 mL was removed from the upper portion of the medium, and transferred to a separate centrifuge tube. The separate upper and lower fractions of each tube that had been centrifuged were then prepared by drying and extracting in 0.2 mL of acidified methanol (3.75X concentration), as outlined above.

Taxane Adsorption and Selective Elution Experiments

Suspension medium was prefiltered through Miracloth. A 1.0 mL aliquot of this medium was filtered through a 0.2 μm nylon or PVDF, 13 mm syringe filter. The filtrate was dried in the same manner as the control, and residue extracted in 0.2 mL acidified methanol (5X concentration). The filter was then washed with 0.2 mL of acidified methanol (5X concentration). Samples that were prepared by adsorption to membrane filters were compared to unfiltered controls prepared in the conventional manner (see above). All samples were then analyzed via HPLC.

For selective elution experiments, 1.0 mL of suspension medium was filtered through a 0.2 μm nylon or PVDF filter. Filter was then washed with 0.2 mL of solvents of decreasing polarity starting with 10% solvent in water, and increasing the solvent concentration by 10% until reaching 100% solvent. Solvents that were tested were methanol, ethanol, isopropyl alcohol, and acetone.

Samples were dried, redissolved in 0.2 mL acidified methanol, and filtered through a 0.2 μm filter prior to HPLC analysis.

RESULTS AND DISCUSSION

Cell Line Initiation and Maintenance

A *T. cuspidata* cell line, P991, has been in culture for nearly three years and continues to produce taxol [**10**] by excretion into the culture medium. While taxol [**10**] concentrations vary considerably, these cultures have produced as much as 25 mg/L taxol [**10**] with typical production of 10-15 mg/L by day 28 of culture. For these experiments, cells were harvested at day 21 to minimize the presence of any competing or interfering compounds from older cells, such as phenolics, xanthophylls, and metabolic degradation products.

Cells were viable at the time of harvest and had a light beige to white coloration. The cell-free suspension medium was colorless with a very slight shimmering or opalescent appearance.

Analysis of Taxanes in Suspension Medium

Our method for analyzing taxanes from suspension medium was developed to give the most complete and accurate quantification of taxanes with the minimum amount of interfering compounds, and minimum loss of sample during preparation. Drying samples and then extracting the residue in acidified methanol gave us higher yields for taxol and other taxanes than solvent:solvent partitioning methods that we tried with methylene chloride, chloroform, or ethyl acetate. This method also eliminates any methanol-insoluble compounds from contaminating samples to be analyzed. Cell line P991 typically produces a specific taxane profile consisting of taxanes in roughly equivalent proportion, regardless of actual concentration

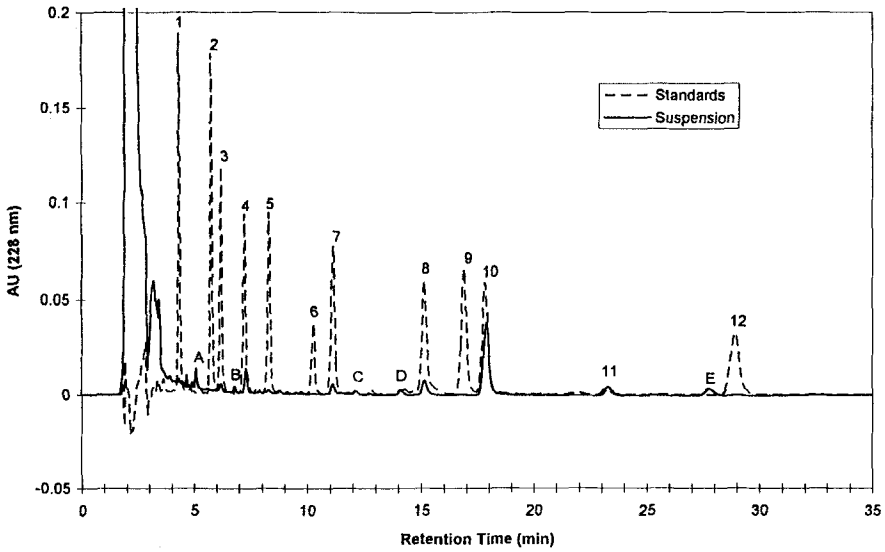

FIGURE 1. Comparison of a mixture of taxane standards, each at a concentration of 50 µg/mL, to a methanol extract of *T. cuspidata* suspension media. The suspension extract is a 5X concentration of a 1mL dried aqueous sample, redissolved in 0.2 mL acidified methanol. For each chromatogram, 10 µL samples were injected. **1** = 10-deacetyl baccatin III; **2** = 7-epi-10-deacetyl baccatin III; **3** = baccatin III; **4** = 10-deacetyl-7-xylosyl taxol C; **5** = 10-deacetyl-7-xylosyl taxol; **6** = 10-deacetyl-7-xylosyl taxol B; **7** = 10-deacetyl taxol; **8** = cephalomannine; **9** = 7-epi-10-deacetyl taxol; **10** = taxol; **11** = taxol C (*not* quantified); **12** = 7-epi taxol. Peaks A-D are unidentified taxanes, based on their absorption spectra. Peak E is not a taxane or taxinine.

(Fig. 1). In this 21-day old culture, the medium contained 10-deacetyl baccatin III [**2**] (0.004 mg/L), baccatin III [**3**] (0.19 mg/L), 10-deacetyl -7-xylosyl taxol [**5**] (1.11 mg/L); 10-deacetyl taxol [**7**] (0.38 mg/L), cephalomannine [**8**] (1.01 mg/L), taxol [**10**] (6.6 mg/L), and taxol c [**11**]. There are 4 additional unidentified taxanes that are produced by the cell lines, according to their UV absorption spectra. These unknown taxanes are not found in bark or needle samples of *Taxus brevifolia* (J. Yeggie, Hauser Chemical, personal communication). At least one of

the unknown taxanes, "A," is often the second most abundant taxane in some of the P991 cultures, next to taxol [**10**].

Filtration and Adsorption of Taxanes to Membrane Filters

Early experiments to analyze taxanes directly from suspension media resulted in failure to detect any taxanes, even though other methods to extract taxanes from the suspension media had demonstrated that the cultures were producing taxanes. An experiment was designed to see if taxanes were being filtered out of solution by a 0.2 μm filter when the suspensions were prepared for HPLC analysis (Fig. 2). Indeed, all of the 10-deacetyl taxol [**7**], significant amounts of cephalomannine [**8**], and virtually all of the taxol [**10**] was missing from the filtrate after suspension media was passed through a nylon membrane filter. The missing taxanes were then recovered from the nylon filter after washing with 0.2 mL methanol. One of the interesting features of the experiment was that not all of the taxanes were retained by the membrane filter. One of the possible explanations of the selective nature of the filter is that taxanes exist in the suspension medium as insoluble aggregates, either smaller or larger than the 0.2 μm pore size of the membrane. Another possible explanation is that the membrane itself was selectively retaining taxanes.

When the experiment was repeated with a 0.2 μm PVDF filter membrane, all of the taxanes were retained by the filter (Fig. 2). Thus, differences in retention of taxanes is not a result of the 0.2 μm pore size but, more likely, a selective affinity for some of the taxanes to the nylon membrane. That affinity also does not appear likely to be based solely on the polarity of individual taxanes. While taxanes of similar polarity (10-deacetyl taxol [**7**], cephalomannine [**8**], and taxol [**10**]) are preferentially retained by the nylon membrane in comparison to more polar taxanes (baccatin III [**3**], and 10-deacetyl-7-xylosyl taxol [**5**]) the less polar

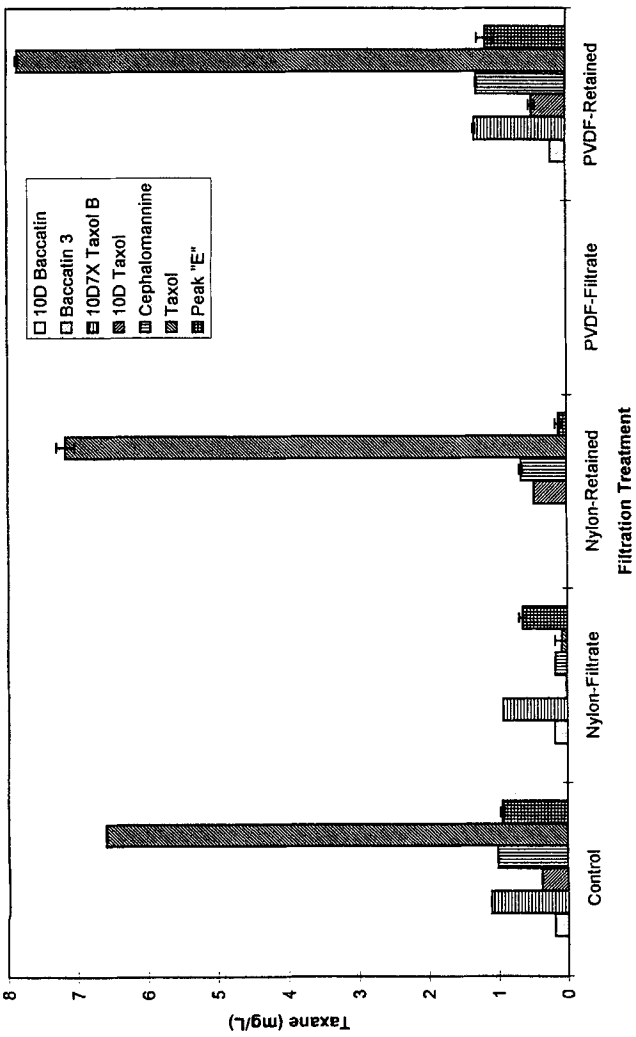

FIGURE 2. Comparison of retention of taxanes on membrane filters with 0.2 μm pore size. Control was a 1 mL suspension sample, dried and redissolved in 0.2 mL acidified methanol. Filtrates were prepared the same way as the control. Retained taxanes were washed from the filters with 0.2 mL acidified methanol. Taxane values are actual concentrations present in the suspension medium.

peak "E" is only partially retained by the membrane. These experiments demonstrated that taxanes in the medium must be dissolved in a less polar solvent than water prior to filtration. Also, the results from these experiments suggested that it might be possible to exploit filtration as a selective method for isolating certain taxanes.

Centrifugation of Taxanes in Aqueous Solution

If the taxanes present in the aqueous medium were present as small, insoluble particles, or as an insoluble suspension, it might be possible to centrifuge them out of solution (Fig. 3). When comparisons are made between different speeds of centrifugation on the amount of taxol in the upper or lower fractions of the centrifuged suspension medium, there are almost no significant differences. The only significant difference in taxol [**10**] concentration between upper and lower fractions of the centrifuged suspension media was in the 4000 x g treatment, with 9% more taxol found in the lower phase. Differences between the control (no centrifugation) and the centrifugation treatments were significant in all lower fractions except the 16,000 x g treatment. These results may be more of a reflection of differences between the analysis of a 1.0 mL sample at a 5X concentration (control) and a 0.75 mL at a 3.75X concentration (centrifuged samples), rather than real differences. Thus, it is possible to centrifuge the aqueous media at 16,000 x g for 30 minutes without causing a pelleting of taxanes. This means that relatively high speed centrifugation can be used as a simple and efficient means of removing cells, cell wall material, and other insoluble debris from the medium prior to extraction of taxanes.

Taxane Adsorption and Selective Elution Experiments

Attempts were made to see if taxanes retained by either nylon or PVDF membrane filters could be selectively eluted from the filter with solvents of

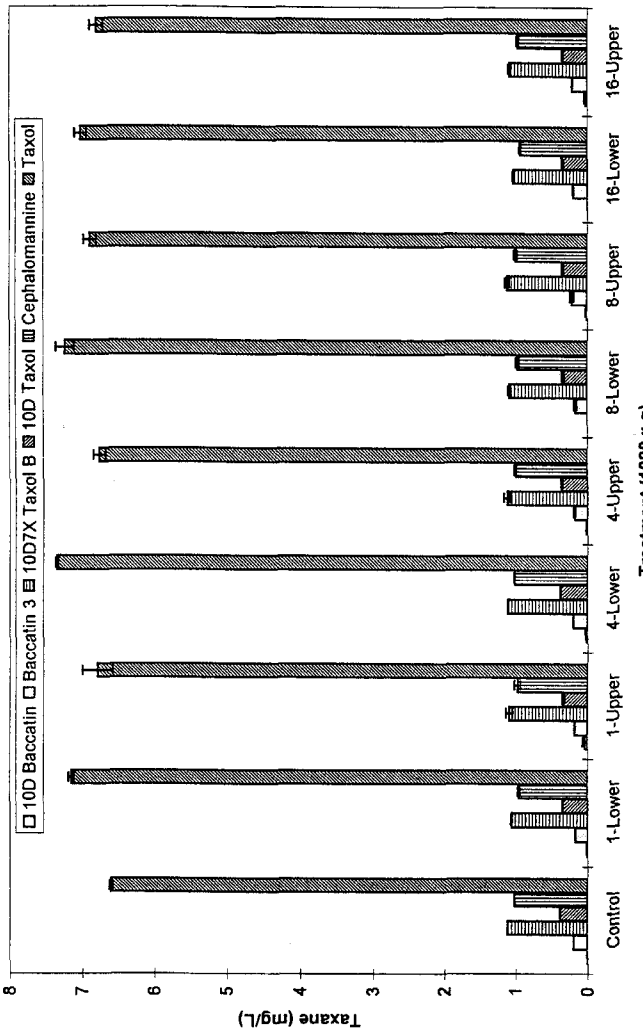

FIGURE 3. Effect of centrifugation on pelleting of taxanes in suspension medium produced by P991 cultures. Control consisted of 1 mL suspension medium dried, and extracted with 0.2 mL acidified methanol. A 1.5 mL sample of suspension medium was centrifuged for 30 minutes, split into separate 0.75 mL samples, dried, and each fraction extracted with 0.2 mL acidified methanol. Taxane values are actual concentrations present in the suspension medium.

gradually decreasing polarity (Fig. 4). When retained by a nylon membrane, no taxanes were eluted by washing with 10% or 20% aqueous methanol (Fig. 4a). The majority of taxol [**10**] comes off in three different eluates: 30%, 40%, and 60% methanol with lesser amounts coming off in the 50% fraction.

A similar pattern of elution occurs when ethanol is used instead of methanol (Fig 4b). Taxol [**10**] begins to elute or wash off of the filter with 10% aqueous ethanol with a peak at 20%, slightly less at 30%, and another peak of taxol [**10**] coming off in the 40% and 50% washes. Few additional taxanes remain on the filter after washing with 60% or greater aqueous ethanol. The reason for the reproducible occurrence of taxol [**10**] in two fractions, in aqueous methanol or ethanol, is not known, but may be related to the existence of two taxol [**10**] isomers or conformational variants. Magee (10) has suggested that the binding of aromatic hydrocarbons to nylon is highly sensitive to structural change, and thus the apparent elution of taxol in two predominate pools may be a result of the partial resolution of two structurally distinct forms of taxol.

This fractionation of taxol [**10**] into two separate pools that can be washed from a nylon membrane with different aqueous solvent fractions does not occur when the taxol [**10**] is bound to a PVDF membrane (Fig 5). Taxol [**10**] and all other taxanes were washed off of the PVDF filter in a fairly narrow range of polarities of aqueous solvents. Not surprisingly, the less polar the solvent, the lower the concentration of solvent in water was required to elute the taxanes. For methanol, the majority of taxanes were washed off in the 40% to 60% eluates (Fig. 5a). For ethanol, taxanes were washed off in the 30% to 50% eluates (Fig. 5b). For 2-propanol, some of the early eluting taxanes came out in the 10% and 20% washes, with cephalomannine [**8**], taxol [**10**], and taxol c [**11**] coming out almost exclusively in the 30% wash, and the remainder coming out in the 40% wash (Fig. 5c). Washes with aqueous acetone resulted in a fairly broad elution of taxanes from the PVDF filter, with taxanes coming off of the filter in the 10% to 50% eluates (Fig. 5d).

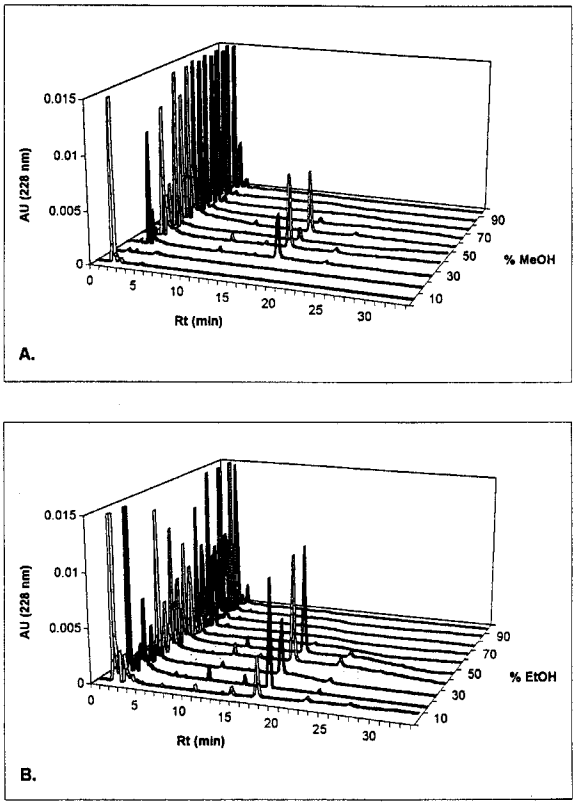

FIGURE 4. Effects of gradual elution of taxanes retained by nylon membrane filter with 0.2 μm pore size. A 1 mL sample of suspension medium was eluted with a discontinuous gradient of **A**) methanol or **B**) ethanol. Samples were analyzed directly after elution. The largest peaks are taxol; other peak identities are the same as in Fig. 1 and have been omitted for clarity.

Selective elution of taxanes from the filters was not attained, in these experiments, although it seems that it should be theoretically possible (10). Chemical modification of nylon membranes may allow the technique to be refined to more selectively elute taxol from a mixture of taxanes. Attempts to selectively elute taxol did, however, give rise to a technique that can be used to further purify the taxane fraction prior to extraction (Fig. 6). When taxanes that are bound to

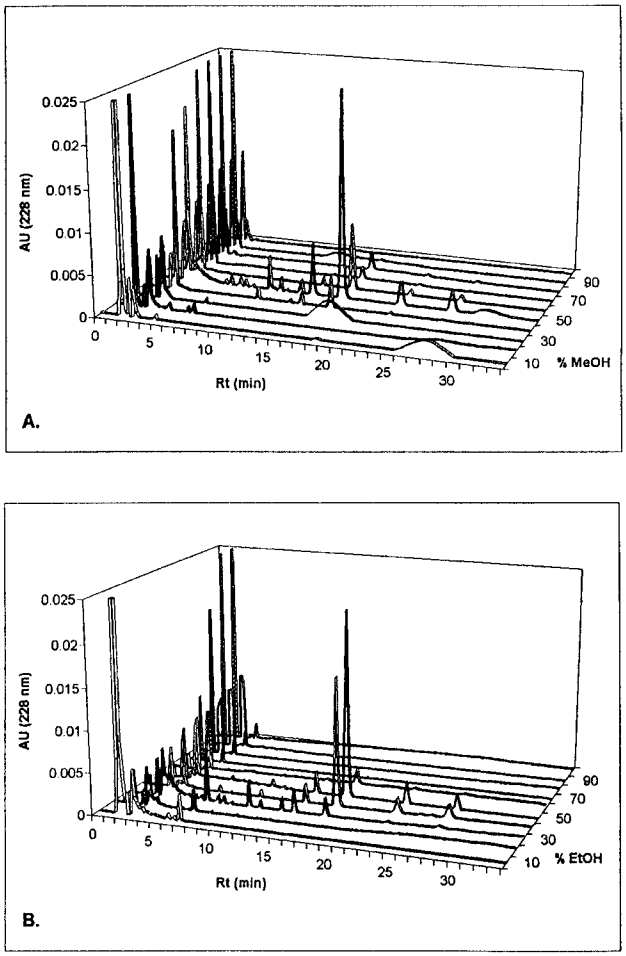

FIGURE 5. Effects of gradual elution of taxanes retained by PVDF membrane filter with 0.2 µm pore size. A 1 mL sample of suspension medium was eluted with a discontinuous gradient of solvent. Methanol and ethanol samples were analyzed directly after filtration; 2-propanol and acetone samples were first dried, then redissolved in 0.2 mL acidified methanol. **A**) methanol; **B**) ethanol; **C**) 2-propanol; **D**) acetone. The largest peaks are taxol; other peak identities are the same as in Fig. 1 and have been omitted for clarity.

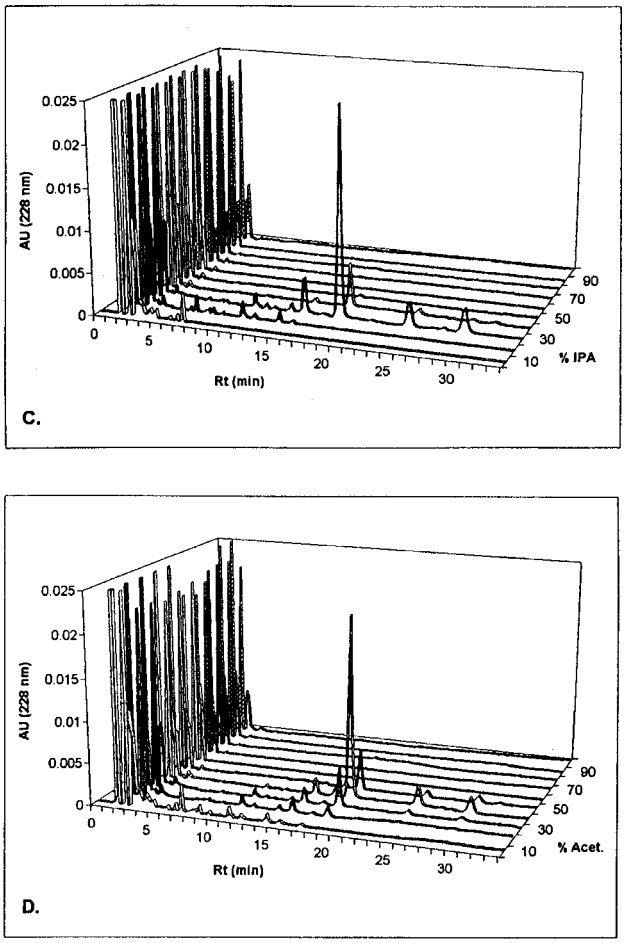

FIGURE 5 (continued).

either nylon or PVDF are eluted with 100% acidified methanol, a large number of polar, non-taxane, compounds elute with the solvent front on HPLC (Fig. 6a and 6b). When the filter is first washed with 20% aqueous methanol, which was found to be too polar to elute any taxanes from the filters (Fig. 4 and 5), the compounds eluting with the solvent front are significantly reduced. Further purification of the

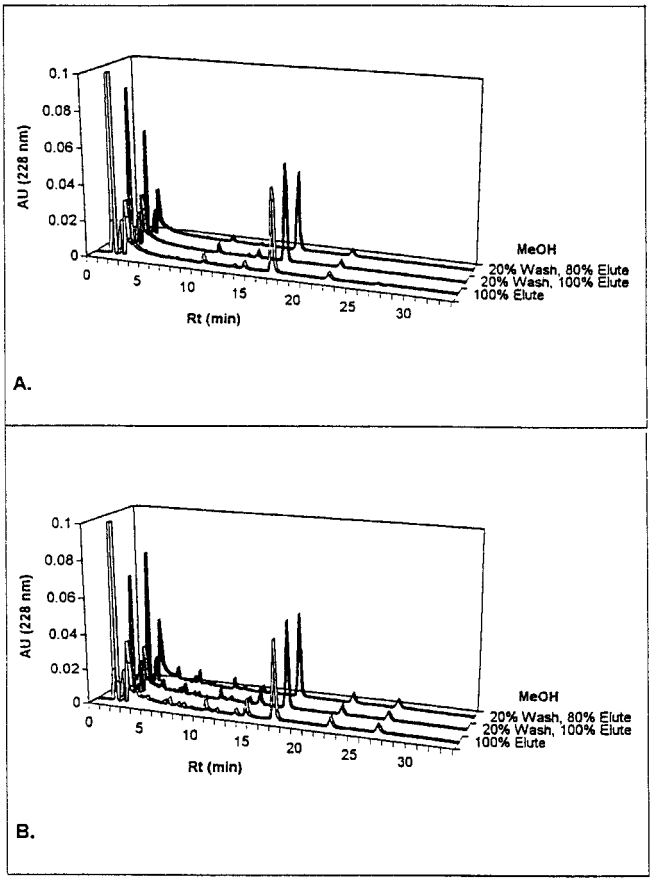

FIGURE 6. Washing of filters prior to elution of taxanes retained by **A)** nylon or **B)** PVDF membrane filters. The largest peaks are taxol; other peak identities are the same as in Fig. 1 and have been omitted for clarity.

samples is achieved by following the 20% methanol wash with elution with 80% aqueous methanol, which was found to be adequate for elution of all taxanes from either membrane (Fig. 4 and 5). This washing procedure produces no significant reduction in the number or quantity of taxanes recovered regardless of whether a nylon or PVDF membrane is used (Fig 6). It is significant that this procedure of

washing taxanes from membrane filters is remarkably similar to a procedure described by Wickremesinhe and Arteca (4) for purification of taxanes on C_{18} solid phase extraction (SPE) columns..

In our experience, we have found that with pre-filtration through a single layer of Miracloth, it is possible to filter 3 mL of suspension medium containing a concentration of 20 mg/L taxol through a 13 mm Gelman nylon syringe filter having a filter area of 0.8 cm^2. It should, therefore, be possible with prefiltration and centrifugation, to filter 7.5 L of suspension medium through readily available commercial filtration capsules that contain 2000 cm^2 0.2 μm nylon membranes. This would give a yield of 187.5 mg of taxol [**10**], in addition to other taxanes, from a 7.5 L suspension culture of one of our higher production cell lines (25 mg/L). Additional investigations on pore size may reveal that much larger volumes of media can be passed through filters without affecting ability of the membranes to retain the taxanes. Also, customization of membrane filters would undoubtedly enable this method to be scaled to handle volumes of media that would be necessary for commercial production of taxol [**10**] from cell suspension cultures.

We have described a relatively inexpensive and efficient means of separating a mixture of taxanes from aqueous cell suspension media. The method relies on the inability to pellet taxanes produced by *T. cuspidata* cell cultures, from their culture broth, by centrifugal force of 16,000 x g for 30 min. When the cells are filtered through 0.2 μm nylon or PVDF membranes, the taxanes are retained by the filters. These taxanes can then be washed and eluted to produce a relatively clean mixture of taxanes. This technique eliminates the need for a solvent partitioning step to extract the crude taxane mixture, as well as eliminating the need for chlorinated hydrocarbons typically used in these extractions. A relatively small volume of methanol or ethanol is all the solvent that is needed to elute the taxanes from the filter membranes, greatly reducing the environmental impact of this method.

ACKNOWLEDGMENTS

The authors wish to thank Judy Luong for her excellent technical assistance. We thank Dr. Tom Hirasuna for his thoughtful and thorough review of the manuscript and Dr. Venkatesh Srinivasan his review and stimulating discussions. I (R.K.) also thank Christopher David Ketchum for his enthusiastic comments and support.

This investigation was partially supported by the National Cancer Institute, grant #CA55138-02.

REFERENCES

1. 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.

2. Christen, A.A., Bland, J., and Gibson, D.M., Cell Culture as a Means to Produce Taxol, Proc. Am. Ass. Cancer Res., 30, 566, 1989.

3. Christen, A.A., Gibson, D.M., and Bland, J., Production Of Taxol Or Taxol-Like Compounds In Cell Culture, US Patent 5,019,504, 1991.

4. Wickremesinhe, E.R. and Arteca, R.N., *Taxus* Callus Cultures: Initiation, Growth Optimization, Characterization, and Taxol Production, Plant Cell, Tissue, & Organ Culture 35, 181, 1993.

5. Fett-Neto, A.G., DiCosmo, F., Reynolds, W.F., and Sakata, K., Cell Culture Of *Taxus* As A Source Of The Neoplastic Drug Taxol And Related Taxanes, Biotechnology, 10, 1572, 1993.

6. Fett-Neto, A.G., Melanson, S.J., Sakata, K., and DiCosmo, F., Improved Growth And Taxol Yield In Developing Calli Of *Taxus cuspidata* By Medium Composition, Biotechnology, 11, 731, 1993.

7. Ketchum, R.E.B., and Gibson, D.M., Rapid Isocratic Reversed Phase HPLC Of Taxanes On New Columns Developed Specifically For Taxol Analysis, J. Liq. Chromatog. 16, 2519, 1993.

8. Witherup, K.M., Look, S.A., Stasko, M.W., Ghiorzi, T.J., and Muschik, G.M., Taxus Spp. Needles Contain Amounts of Taxol Comparable to the Bark of *Taxus brevifolia*: Analysis and Isolation, J. Nat. Prod., 53, 1249, 1990.

9. Cardellina, J.H., HPLC Separation of Taxol and Cephalomannine, J. Liq. Chromatogr., 14, 659, 1991.

10. Magee, P.S., Analysis of Binding Energetics in Paper and Thin-Layer Chromatography, Quant. Struct.-Act. Relat., 5, 158, 1986.

Received: September 10, 1994
Accepted: September 29, 1994