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Separation and Purification of High Value Phytochemicals from Plant Biomass

Nicholas Chan

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and Doctoral Studies in partial fulfillment of the
requirements for the degree of

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Abstract

Phytochemicals are the individual chemicals from which plants are made and are alternatively referred to as natural products, which have been used since ancient times as drugs. In modern times, natural products are still used as drugs; however, more importantly they are used as a source of new leads into new bioactive compounds. Paclitaxel and betulinic acid, in particular are prized for their characteristics as anti-cancer compounds among other traits.

The following work examines the extraction of paclitaxel from *Taxus Canadensis* on a pilot scale set-up using Dynamic Pressurized Liquid Extraction (DPLE). The results are then compared to the conventional solvent extraction method in order to compare DPLE to current industrial operations. The analysis and batch separation of betulinic acid from birch bark was also examined.

A paclitaxel purity of 1.1 % with 90 % recovery was achieved using DPLE. As well, ethanol was found to be the best solvent for betulinic acid extraction under conventional solvent extraction methods. DPLE is deemed to be worthy of further study in commercial processes.

Résumé

Les substances phytochimiques ou produits naturels sont les composés qui forment les plantes. Depuis l'antiquité, ainsi que de nos jours, ces produits sont utilisés comme phytomédicaments, mais ils représentent actuellement une source très importante de nouveaux développements des composés bioactifs. Notamment, le paclitaxel et l'acide bétulinique sont des exemples particulièrement notables en raison de leurs propriétés anticancéreuses parmi bien d'autres.

Le travail qui suit examine l'extraction de paclitaxel du *Taxus Canadensis*, également connu sous l'appellation sapin traînard ou if du Canada, établi sous une échelle préindustrielle en utilisant le l'extraction sous pression par solvant. Par la suite, les résultats sont comparés à la méthode conventionnelle d'extraction par solvants afin d'établir la comparaison entre l'extraction sous pression par solvant et les procédés d'exploitation industrielle présentement utilisés. La recherche examine également l'analyse et la séparation des lots d'acide bétulinique de l'écorce du bouleau.

Finalement, l'extraction sous pression par solvant s'est avérée plus efficace que les techniques traditionnelles dans le cas de l'exploitation du taxane. Des niveaux de 1.1% pureté et de 90% récupération du produit ont résulté de l'utilisation de l'extraction sous pression par solvant. Le rendement du procédé s'améliore davantage avec l'usage de particules de biomasse d'une moindre grandeur. De plus, on constate que l'éthanol est le meilleur solvant pour les techniques conventionnelles d'extraction par solvants.

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I would like to thank my supervisor Dr. Jason Zhang for the opportunity to pursue my master degree. In this time, I have come to highly prize his support and advice.

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Abbreviations

9-DAB	9-Dihydro-13-Acetal-Baccatin III
10-DAB	10-Deacetyl Baccatin III
ASE	Accelerated Solvent Extraction
BAC	Baccatin III
CBL	Chatham Biotec Ltd.
CEPH	Cephalomannine
CSE	Conventional Solvent Extraction
DPLE	Dynamic Pressurized Liquid Extraction
USDA	United States Department of Agriculture
LLE	Liquid-Liquid Extraction
MAE	Microwave Assisted Extraction
NCI	National Cancer Institure
NPC	Normal Phase Chromatography
PAC	Paclitaxel
PLE	Pressurized Liquid Extraction
RPC	Reverse Phase Chromatography
SFE	Supercritical Fluid Extraction
USGS	United States Geological Survey
WFPP	Waterloo Fast Pyrolysis Process

1.0 Introduction

Phytochemicals are the individual chemicals from which plants are made and are alternatively referred to as natural products. There are various uses of natural products such as in cosmetics and fragrances; they have also been used since ancient times as drugs. The oldest information about plants used as drugs originates from the Sumerians and Akkadians dating back to around 3000 BC. These phytochemicals are currently being used as antibacterials, antifungals, molluscicidal, trypanocidal, allelopathic, and anti-inflammatories among other uses in the modern world (Rauter et al., 2002). Even though they have been used for such a long time, it is estimated that only 5 – 15% of the approximately 250,000 species of higher plants have been systematically investigated for chemical and pharmacological compounds (Cragg et al., 2002).

Roughly 100 years ago, synthesis became the most important source for drug development after the first successful introduction of synthetic drugs such as aspirin. Synthesis was more popular than natural sources due to several reasons: it was simpler to use computational drug design for known molecular targets, long lasting bioassays, difficulties in structure elucidation of complex compounds, structural complexity for further development, need for larger amounts for in depth testing of activity, and difficulties in supply of active compounds for further drug development. Natural products were mainly screened for antitumors and antibiotics during this time. However, utilizing high throughput screening, combinatorial chemistry and other methods; natural products have moved into the spotlight as a source of new leads into bioactive compounds (Verpoorte et al., 2002).

Natural products can be generally organized by their chemical polarity. Commencing from the non-polar end of the spectrum would be where lipids are located. Lipids include simple and functionalized hydrocarbons, as well as terpenes, followed by unsaturated natural products, including polyacetylene and aromatic compounds. After these compounds, the products tend to be more hydrophilic than hydrophobic, such as sugar. Finally, the most polar compounds are those which can form salts, such as alkaloids, amino acids, and nucleosides (Kaufman et al., 1999).

1.1 Objectives

The following work had two main goals. The first was to examine the feasibility of Dynamic Pressurized Liquid Extraction (DPLE) on the extraction of paclitaxel from *Taxus Canadensis* on a pilot plant scale. Included in this goal was to examine process integration for the DPLE process. The second was to study the analysis and separation of betulinic acid from birch bark through the use of conventional solvent extraction (CSE) in a batch process.

1.2 Paclitaxel

One of the compounds discovered through plant screening was paclitaxel (Figure 1.1). It is best known under the name Taxol®, a phytochemical with a long history. Paclitaxel first garnered its reputation in 1969 after Dr. Monroe Wall and his collaborator, Dr. Mansukh C. Wani published their results on paclitaxel's potency as an anti-cancer compound. Paclitaxel is effective due to its anti-mitotic effect, which means that it halts cell division. As such, it has been used in the treatment for metastatic breast cancer, metastatic ovarian cancer, and Karposi's sarcoma. It has

also been used to treat the following: head and neck cancer, non-small cell lung cancer, small cell lung cancer, and bladder cancer.

Paclitaxel is from a family compounds known as taxanes. Taxanes are compounds containing a tricyclic ring nucleus (Figure 1.2).

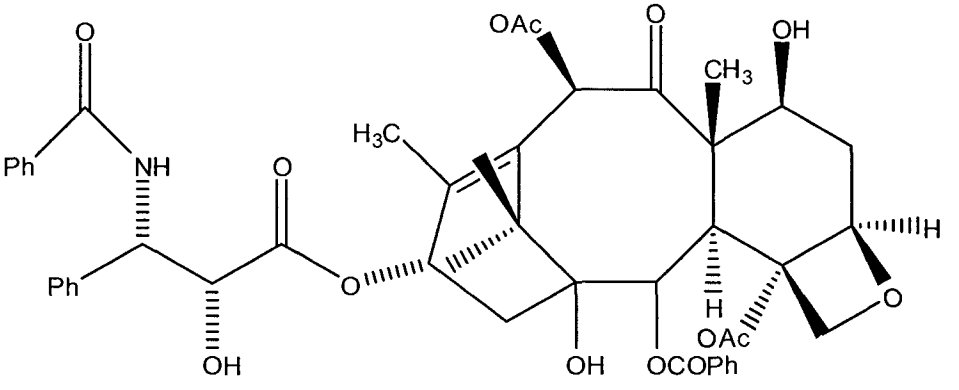

Figure 1. 1: Paclitaxel

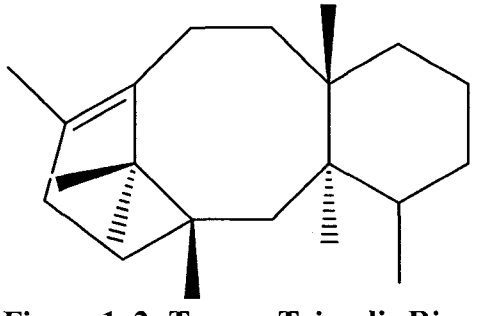

Figure 1. 2: Taxane Tricyclic Ring

It was first harvested from the bark of *Taxus Brevifolia*, or the Pacific Yew in 1962. The collection was part of an interagency plant screening program between the National Cancer Institute (NCI) and the United States Department of Agriculture (USDA).

Unfortunately, the initial isolation work from the pacific yew produced 0.5 grams from 12 kg of dried bark, resulting in a yield of 0.004%. Besides the low concentration of paclitaxel in the bark, *Taxus Brevifolia* also has other problems such as its slow growth period and its sparse growth. An average tree of 22.9 cm diameter and over 9 m tall is 125 years old. It is an understory tree that lives in dense conifer forests. The bark is extremely thin, between 3 – 6.5 mm yielding 1.3 to 2.3 kg of bark per tree. Gathering the bark results in destructive harvesting since the plant cannot continue to live without its protective layer. Although, this was not a factor for the initial work carried by Wall and associates, this comes into great consideration when planning for commercialization and production.

Since its initial discovery, taxanes have been found in several varieties of the *Taxus* genus, documented in various journals. These are namely *Taxus Baccata*, which is found in Europe; *Taxus Canadensis*, also known as the Canada Yew, which is found in Northeastern North America; *Taxus Floridana*, also known as the Florida Yew; *Taxus Globosa*, also known as the Mexican Yew; *Taxus Cuspidate*, also known as the Japanese Yew; *Taxus Chinensis*, also known as Hicksii and a variation known as Mairei, which is normally found in southeastern Asia; and *Taxus hunnewellian*, which is a cross between *Cuspidate* and *Canadensis*. Although there are several different species of *Taxus*; *Baccata, Canadensis, and Chinensis* are the main species used for production purposes due to their larger quantities. For *Taxus Canadensis*, the pruning cycle is 5 years. From data, pruning of 6 to 8 inch material from the tree allows for maximum regrowth of the plant within 4 to 5 years (Smith, 2006). All known species of *Taxus* are listed in the table below:

Table 1.1: Location of Different Species of Taxus.

North and Central America	Europe	Asia
T. brevifolia	*T. baccata*	*T. yunnanensis*
T. globosa		*T. cuspidata*
T. canadensis		*T. media*
T. floridana		*T. chinensis*
T. baccata		*T. wallichianna*
		T. himalayansis

The current *Taxus* genus that is used as a source of biomass for paclitaxel in North America, is *Taxus Canadensis*, also known as ground hemlock. This tree is found in the northeastern corner of North America (Figure 1.3).

1.2.1 Economics

Table 1. 1: Price and Volume Estimates for *Taxus Canadensis* Biomass Produced by Various Means (Cameron et al., 2005).

	Target: 100kg of paclitaxel per year from:		
	Woodland harvested biomass	Non-elite cultivated crop	Elite cultivar crop: 2x paclitaxel content
Biomass needed(kg dry wt)	1.0 million	1.0 million	0.5 million
Harvest or cultivation and drying costs	$8.8 million	$22.1 million	$5.7 million
Cost per kg of biomass	$8.80	$22.10	$14.30
Cost per kg paclitaxel	$25,100	$63,100	$20,400

This cost of biomass is very significant as it can account for 30 ~ 50 % of total production costs (Cameron et al., 2005). With the use of elite cultivars, with a conservative estimate of twice the paclitaxel content, it is expected that the biomass component of production costs can be decreased by at least 20 %.

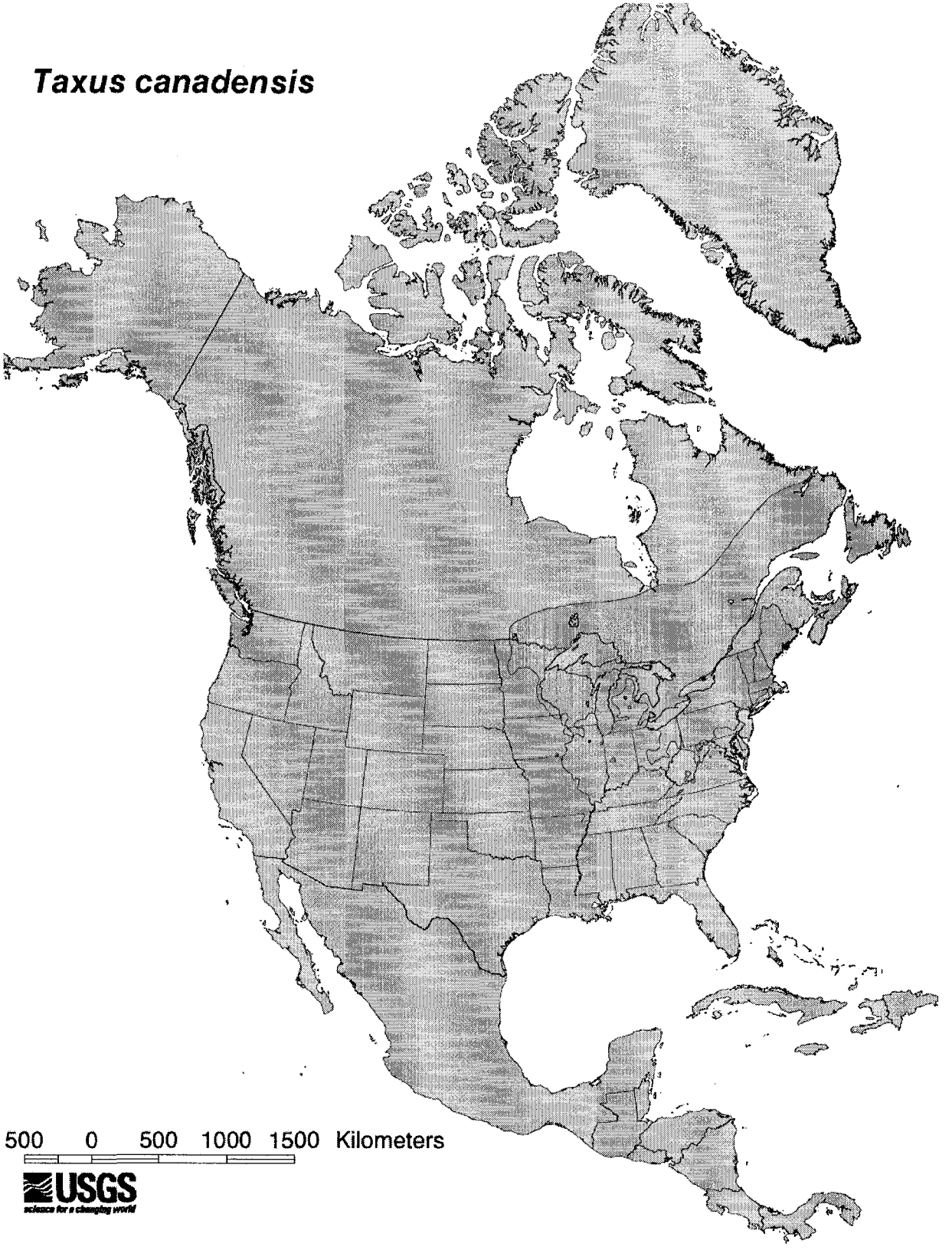

Figure 1. 3: Location of *Taxus Canadensis* in North America (Schumann, 2006).

The cost of the biomass for woodland harvested biomass can be broken down further into Table 1.2

Table 1. 2: Cost of Dried Woodland *Taxus Canadensis* Harvested by Chatham Biotec Ltd (Smith, 2006).

Company Financial Breakdown	Cost ($ / lbs)
Landowner	$0.10
Pruner	$0.70
Marketing and Development	$0.10
Training and Management	$0.10
Transportation	$0.07
Total Green Weight Cost	$1.07
Total Green Weight Cost after Drying (Using 3:1 green to dried ratio)	$3.21
Drying Cost	$0.60
Packaging Cost	$0.10
Research Funding and Administration	$0.09
Total Dried Cost	**$4.00**

Also listed on the Chatham Biotech Ltd website, the current market value of paclitaxel at $115 - $150/g is stated. However, the price of paclitaxel can be sold as high as $200,000 or more at 99% purity per kilogram (Cameron et al., 2005).

One of the main reasons for the high price is related to process inefficiencies. In conventional methanol extraction, the methanol causes the biomass to swell and in so doing becomes trapped. The recovery of this solvent is very energy intensive and can also be time consuming.

Another very important cause of the inefficiencies is the number of process steps required to obtain the desired product purity. There are two aspects which increase the costs in this regard. The first is that the overall recovery of the product will be reduced, resulting in wasted efforts to

produce a smaller quantity of material compared to the ideal amount. The second is the cost of each process step. If either adsorption or chromatography is used, resins would be necessary. Resins lose their effectiveness with repeated use, due to impurities, resulting in replacement costs. To generate the required separation for an API quality product, generally a large quantity of expensive resins will have to be used.

1.3 Betulinic Acid

Betulinic acid (Figure 1.4), a member of the family of compounds known as pentacyclic triterpenes, is found in birch trees or the tree genus *Betula*. Although there are several varieties of birch in North America, the most prominent is the *Betula papyrifera*, better known as the white birch tree. Long before the components of the birch tree's components were studied; the sap, bark, leaves, wood, twigs, and roots were used for food, construction materials, medicinal treatments, lubricants, and other practical applications.

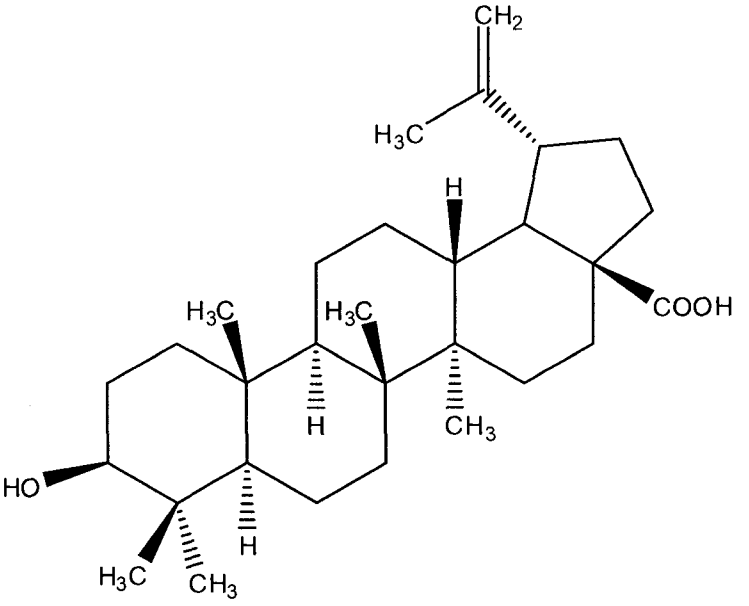

Figure 1. 4: Betulinic Acid.

Betulinic acid possesses several biological properties: antiviral, anticancer, anti-inflammatory, antiseptic, antimicrobial, antimalarial, antileishmanial, antihelmintic, antifeedent, and spermicidal activities (Czuk et al., 2006). The importance of betulinic acid to modern pharmaceuticals became apparent in the mid-1990s when its high selectivity towards melanoma cancer cells and its activity against HIV-1 were discovered. Aside from these two, betulinic acid has been found to have activity against Herpes (Flekhter et al., 2003). It is also being used as a food supplement, since birch bark contains different fatty acids bound with natural polymer suberin. It also has cosmetic applications as a skin moisturizer and for odor treatment. Finally, it has even been found in use as an animal husbandry sedative (Smith, 2005).

Betulinic acid is effectivive due to prevention of topoisomerase I-DNA interaction as a result of which the 'cleavable complex' is not formed. In consequence, it also acts as an antagonist to camptothecin-mediated cleavage (Chowdhury et al., 2002). In other words, betulinic acid inhibits division, which halts the replication of the target DNA.

Birch bark contains an average of 10-30% of betulin and 0.05 ~ 2% betulinic acid (Smith, 2005). Although betulinic acid is in the bark in relatively small quantities, but not nearly as low concentrations of paclitaxel in ground hemlock, there are large quantities of betulin (Figure 1.5) present. This is significant since there are well documented synthetic routes of achieving betulinic acid through betulin, such as the route described by Czuk et al. in 2006. As can be seen from the figure below, betulin has an almost identical form to that of betulinic acid.

Figure 1. 5: Distribution of *Betula Papyrifera* in North America (Schumann, 2006)

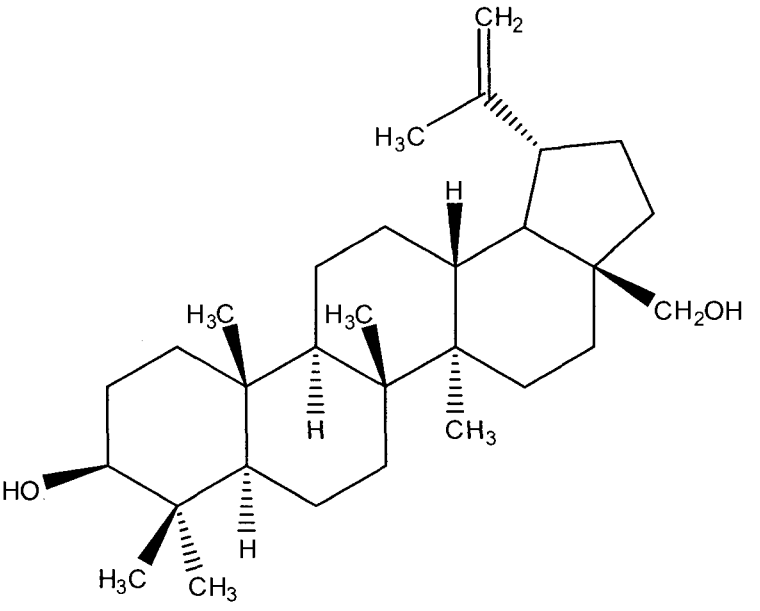

Figure 1. 6: Betulin Molecule

Availability of birch bark is quite abundant (Figure 1.6). Aside from the large coverage of birch trees in North America, birch bark is a waste product generated from pulp and paper mills. In New Brunswick alone, 50,000 kg of material is available daily, thus making supply issues non-existent in a commercial product (Smith, 2005).

2.0 Literature Review

In order to effectively use these compounds in pharmaceutical products, we must first be able to isolate the individual compounds responsible for a desired result. The steps used to obtain these compounds are known as bioseparations. Bioseparations is described as the "sequence of recovery and separation steps that maximize the purity of the bioproduct while minimizing the processing time, yield losses, and costs" (Ladisch, 2001).

As mentioned earlier, natural products can be categorized by their polarities; however, with respect to bioseparations, it is easier to classify them into the following:

- whole cells
- intracellular macromolecules
- extracellular products

To achieve the desired product, these general processing steps are followed. First, solid/liquid separation is performed and then the product is processed through initial product recovery and concentration. After recovery, there may be another round of solid/liquid separation and ending with a purification step. The major bioseparation processes are listed in the following table including their approximate yields (Table 2.1).

Table 2. 1: Single Step Yields for Common Unit Operations Used in Bioseparations (Ladisch, 2001).

Unit Operation	Separation Level	Max Yield	Min Yield
Centrifugation	Macroscopic		
	Solid	0.95	0.85
	Liquid	0.95	0.9
Filtration	Macroscopic		
	Solid	0.99	0.95
	Liquid	0.99	0.9
Microfiltration	Macroscopic		
	Solid	0.9	0.8
	Liquid	0.95	0.95
Ultrafiltration	Molecular		
	Retentate	0.99	0.5
	Permeate	0.95	0.5
Reverse Osmosis	Molecular		
	Retentate	0.95	0.5
	Permeate	0.9	0.5
Crystallization	Macroscopic	0.9	0.5
Extraction	Molecular	0.95	0.5
Adsorption	Molecular	0.99	0.5
desorption	Molecular	0.95	0.5
Chromatography	Molecular	0.99	0.5
Affinity Chrom.	Molecular	0.95	0.5

2.1 Methods of Extraction

In the following section, we will cover the processes used for the initial solid/liquid separation step that has been mentioned in literature.

2.1.1 Conventional Solvent Extraction

Conventional solvent extraction (CSE) involves wetting of the biomass in a solvent for a period of time, sometimes using agitation to increase the mass transfer.

The extraction times (retention time in extractor) for CSE can range from 3 hours or up to 72 hours for paclitaxel, with an average time of 12 hours. This extraction time is a factor of many things. The particle size of the yew biomass has a large effect, since larger particles requires more time for the paclitaxel to diffuse out into the bulk solvent. Another factor is the solvent to biomass ratio that is used since the concentration gradient is what drives the leaching process. Moisture content of the biomass can also contribute to long extraction times. The added moisture can be enough to change concentration of the bulk solvent and affect polarity resulting in reduced overall efficiency of the process. The moisture content of freshly harvested material is normally around 55-60% wt. In the Chatham Biotec Ltd (CBL) drying process, the twigs and needles are dried to less than 10% moisture content. Through the use of CSE with methanol, the purity of the extract can only reach less than 0.1% wt paclitaxel.

Due to the long process time, studies have been performed on other methods of extraction with hopes of accelerating this process or to provide a more environmentally sound solution since toxic or hazardous solvents are normally used. The following sections examine the different methodologies mentioned in literature.

2.1.2 Soxhlet Extraction

This method is used strictly for analysis due to impractical equipment sizes on larger scales. A soxhlet extraction uses the following equipment: soxhlet glassware, a condenser and heating unit. During a soxhlet extraction, the solvent is heated until it percolates evaporates, condenses above the biomass and leaches the solutes before returning to the solvent flask. Pure solvent is percolated while the extractables are collected in the collection flask, which results in complete extraction of extractables from the biomass. The biomass is held in a cellulose fibre thimble that allows solvent to pass through it. This extraction methodology offers the ability to obtain all the extractables given the correct solvent and an appropriate extraction time.

2.1.3 Supercritical Fluid Extraction

Supercritical carbon dioxide for paclitaxel extraction use was first published in 1996 by Chun and co-workers. Supercritical fluid extraction (SFE) offers the possibility of being the most environmentally friendly method of extraction, since no hazardous solvents or materials are used. Scientists and engineers have been aware of SFE for more than one hundred years, but it is only in the last thirty years that supercritical fluid solvents have been the focus of active research and development programs. SFE is "based on the experimental observation that many gases exhibit enhanced solute extraction when compressed to conditions above the critical point" (McHugh et al., 1986). There are several motivations behind the increased interest in SFE. First, there has been a sharp increase in the cost of energy resulting in the cost of energy intensive separation technologies, such as distillation, to increase significantly. As a result of increased governmental

regulations, the use of toxic solvents has become undesirable. Former profit must instead be spent for treatment, disposal and clean-up.

Chun et al. tested their system, using a pressure range from 100 to 300 atm and a temperature range from 308 to 343 K with and without the addition of several co-solvents: ethylacetate, methanol, dichloromethane, and ethylether. In addition to this, the oven dried ground needles were pre-treated with n-hexane to reduce the concentration of waxes and non-polar compounds. It was found that when the biomass had been pre-treated with n-hexane and extracted using pure carbon dioxide, the purity of the compounds jumped to 0.190 and 0.671% wt for paclitaxel and Baccatin III, respectively. In comparison, 0.078 and 0.089% wt for PAC and BAC using untreated biomass and pure carbon dioxide. They found that during the first four hours of SFE that the highest purity of paclitaxel and Baccatin III was about 0.262 and 0.644% wt, respectively. Although these values are significantly higher than that of CSE, the extraction efficiency leaves much to be desired. The amount of paclitaxel and Baccatin III from their CSE standard experiments were between 150 to 250 μg/g and 150 to 300 μg/g, respectively. It was found that their product purity reduced as more of the desired products were extracted. The process was only able to extract 10% and 35% of the total amounts found within the biomass. When co-solvents were used, the extraction only noticeably increased with the use of dichloromethane. However, this result applies only to paclitaxel; Baccatin III extraction resulted in lower efficiency than those using pure carbon dioxide.

Another group, Vandana et al. in 1996, has also studied SFE for the extraction of paclitaxel and other taxanes. They studied extraction using nitrous oxide as their solvent instead of carbon

dioxide. In this paper, they used ethanol as a co-solvent, with a temperature range between 320 to 331 K and a pressure range of 100 to 380 atm. Comparisons between extractions using carbon dioxide and nitrous oxide as the solvent were made. In both cases ethanol was also added as a co-solvent. When the two solvents were compared at the same pressure, it was found that nitrous oxide was able to extract more from the biomass than carbon dioxide. It was found that the use of ethanol as a co-solvent helped to obtain a greater quantity of paclitaxel. Although there was a process difference, where 10.5% mol ethanol and 11.5% mol ethanol for carbon dioxide and nitrous oxide, respectively, were used. The use of ethanol and without ethanol along with nitrous oxide was studied. In the comparison, it was shown that ethanol indeed did have an effect on extraction; therefore, due to the difference in ethanol concentrations, it would be difficult to determine whether the effect was due to the ethanol or strictly the difference between the solubility of paclitaxel in nitrous oxide. It would appear that given enough ethanol, the yield achieved 100% efficiency in comparison to a sonicated methanol extraction.

In another paper in 1997 by the same group, Vandana et al., the solubilities of paclitaxel in both supercritical carbon dioxide and nitrous oxide were studied. In this case, carbon dioxide was measured at 311.1, 322.2 and 328.9 K and over a pressure range of 140 to 350 atm, while nitrous oxide was measured at 326.2 K and over a pressure range of 140 to 280 atm. Their results show that nitrous oxide was able to dissolve more paclitaxel than carbon dioxide; however, the solubility of paclitaxel in both solvents was very low and ranged from $1.1 \times 10^{-6} - 7.4 \times 10^{-6}$ mole fraction. When results were compared at similar temperature and pressure, the difference between the results was 10% and an accuracy of ±20 % due to the low solubility.

From these papers, although a lab scale appears to work, a larger scale system would have to be tested to validate the commercial viability of this system. It was concluded that the use of either carbon dioxide or nitrous oxide would not matter, although the use of co-solvents affected the amount of product extracted. However, the latter papers indicate that a significant amount of co-solvent would have to be used to achieve desired yields, but lower than amounts used in CSE.

2.1.4 Microwave Assisted Extraction

Microwave assisted extraction (MAE) was presented in 1997 by M.J. Incorvia Mattina et al. MAE involves subjecting solvent and biomass to heating through microwave energy. Various temperatures, times, and organic solvents for the MAE procedure were investigated to enhance taxane extraction efficiency. The effects of biomass to solvent ratio and the water content on taxane recovery were also determined. The control extraction consisted of an overnight shake of 5 g of needles in 100ml of methanol at ambient temperature. Although methanol and ethanol have similar properties, costing was taken into consideration and ethanol was decided upon. MAE was able to obtain an extract equivalent to CSE using 95% ethanol.

It was found that they could achieve ~90% recovery when they used freshly harvested needles with a moisture content of 55 – 65%. Recoveries close to 100% could also be achieved when using freeze-dried biomass with moisture content of less than ten percent. The study was performed at times of 6:00, 9:10, and 12:20 minutes with temperatures of 70 °C, 85 °C, and 115 °C. It was observed that the level of cephalomannine was more pronounced and that the detected levels of paclitaxel and 10-deacetyltaxol were greatly reduced at temperatures above 85 °C. This

effect was most likely the degradation of the other taxanes into cephalomannine. It was found that the optimum conditions for achieving maximum yield, was 5 g of needles with 10mL of 95% ethanol at 85 °C for 9:10 minutes. A solvent ratio of 1:4 grams of biomass to milliliters of ethanol was kept. To achieve these yields, the biomass was presoaked with water. The water appears necessary to maximize the use of the microwaves.

Evaluating this method, we find that there is one important advantage to this method and it is its quick extraction time. However, there are several drawbacks. The amount of solvent used is comparable to that of CSE and therefore offers no benefits. As well, the temperatures used could pose a possible hazard due to the vapours formed, in the form of fire, health, and environmental hazards.

2.1.5 Sonication

This extraction method uses ultrasound to help extract the taxanes into the solvent. One patent from the University of Laval (Stevanovic et al., 2005) describes using this method for extraction. By use of the ultrasound waves, diffusion of the taxanes into an organic solvent occurs. The ultrasound is preferentially at a frequency of 10 – 20 kHz with pulse and rest periods of 0.1 – 2 seconds. The pulse and rest period may be performed from 10 to 90 minutes. This process is performed at a non-denaturing temperature, preferentially from 20 – 40 °C. The solvents mentioned in the patent are methanol, dichloromethane, hexane, or ethyl acetate.

Unfortunately, no information is provided on the efficiency of this process, although it is mentioned that the resulting solution is comparable to an extract obtained from maceration. From the given conditions, it would seem that process could be a viable option, if more information on its yield was available. The extraction time is lower than that of CSE and is performed at relatively low temperatures and at atmospheric pressure. We can likely conclude that the yield would be similar to that of CSE. However, the solvent to biomass ratio would most likely be similar as well, resulting in similar expenditures in solvent recovery with respect to both time and money.

2.1.6 Pyrolysis

A process called the Waterloo Fast Pyrolysis Process (WFPP) was used to determine the feasibility of extracting taxanes from needles, twigs, and whole clippings of *Taxus Canadensis* by B.J. Cass et al., 2001. The various runs were performed at 359, 425, and 480 °C for dried needles: fresh needles at 433 °C, twigs at 431 °C and whole clippings at 430 °C. The average run length was 40 minutes with 42 g of biomass fed. Typically, 24 g of liquid products, 12 g of char, and 6 g of gas were produced. The pyrolysis oil used in this study was a combination of the liquor and tar fractions obtained from the condensed vapour.

One of the challenges of this process was the presence of high quantities of phenolics in the pyrolysis oil. The research group encountered a significant problem with the phenolic compounds since they interfere with the isolation of the taxanes. Overall, the use of pyrolysis recovered less than 20% of the paclitaxel that was in the original *Taxus Canadensis*, as measured

with solvent extraction. Since the recovery is so low and the phenolic content is so high, this is one of the least viable methods of isolating paclitaxel from biomass. Also, if all the taxanes were made to volatize, there would be a high probability that the taxanes would be degraded by the large amount of heat, similar to some of the results that occurred in MAE.

2.1.7 Pressurized Liquid Extraction

In Pressurized Liquid Extraction (PLE), also known as Accelerated Solvent Extraction (ASE), organic solvents are used at high pressures, and at temperatures above their boiling points. It has been found to be the most economic extraction technique in small scale sample preparation (Kikuchi et al., 1997).

PLE is typically conducted under static conditions with pressures of 30 to 207 atm and holds up for 15 minutes. Since PLE is operated under static conditions, the high pressure is used to force the solvent into the pores of the biomass that are occupied by water or air to dissolve the solutes trapped within them and to maintain the solvent in liquid form.

Pressurization of the vessel and the elevated temperature could cause a less than economical process on a larger scale. In addition, temperatures will have to be carefully monitored so as not to exceed the stability of the products.

2.1.8 Dynamic Pressurized Liquid Extraction

In dynamic pressurized liquid extraction (DPLE), solvents in liquid form are continuously forced through the matrices of biomass in the extraction column. The column is operated above the pressure and temperature necessary to keep the solvent from boiling. In comparison to PLE, high pressure is not required to force the solvents into the pores of the biomass particles occupied by water or air (Wang, 2004). Water or air that block the pathway to the solutes in the pores are gradually dissolved and removed by solvents. The operating pressure of DPLE does not exceed 5 atm, which is lower than that used by other commercial unit operations.

DPLE is also performed at elevated temperatures, which is necessary for a variety of factors:

- capacity of the solvent to dissolve the solutes is increased
- improved mass transfer rate is achieved
- can disrupt the solute-matrix interactions
- decreases the viscosity of the solvent and allows for better penetration of the biomass.

DPLE in conjunction with a dual solvent system, derived from the solute distribution model (Wang, 2004), can produce an extract with a high yield similar to that from a CSE process; although, the extract should be a better quality than that obtained from CSE due to the increased selectivity. The dual solvent system means that two different solvents with respect to their polarities are used instead of just one solvent. Maximum concentration of paclitaxel can be achieved after 10 minutes of extraction when using smaller than 100 mesh needle powder when performed on a 5 g scale [Wang, 2003].

The fast extraction time and the increased selectivity give this methodology quite an advantage over the CSE method. As well, the reduced pressure demand gives a greater desirability over SFE and PLE. Unlike PLE, the temperatures are in a more suitable range, allowing for maximum extraction without product degradation.

2.2 Extraction Alternatives

All the previous extraction methodologies deal with using biomass as the source of the desired compounds. However, there are alternatives such as cell cultures, semi-synthetic and synthetic routes, which we will explore in greater detail. These alternatives could be more desirable due to the variability of taxane content, which are affected by the following factors:

- Harvest time of the plant material.
- Varying ratio of different parts of the plant
- Differences between wild variations of the Taxus species

With large variabilities in taxane content, expected output from a production plant can vary causing inefficiency as well as production scheduling issues.

2.2.1 Cell Cultures

Several papers have used cell cultures of the *Taxus* genus to obtain paclitaxel. "The cultivation of plant cells in bioreactors is a promising means for the production of commercially important

phytochemicals, particularly those derived from naturally rare and difficult to cultivate plant species" (Wu, 2003).

One such paper was published in 2003 by J. Wu et al. The researchers had grown cultures of *Taxus Chinensis*. Ultrasound and methyl jasmonate's effects were studied on these cultures. Methyl jasmonate is used as a putative chemical elicitor, which is used to prompt a plant defense response. This is significant since the desired products of plant cell cultures are mostly secondary metabolites, which are generally produced when the plant is stressed. Ultrasound treatment seemed to have stimulated the enzyme activity of secondary metabolic pathways. Dibutyl phthalate (DBP) was used for the in situ solvent extraction of taxol. Individually, the ultrasound treatment yielded an increase of 1.5 -1.8 fold, the methyl jasmonate increased production by 5 fold, and the DBP produced a 7 – 9 fold difference in volumetric paclitaxel yield. It was concluded that all three factors were partially responsible for the stimulated enzyme activity since taxol yield were 20 – 50 % higher than with each of the stimulators alone. This higher yield translated into a paclitaxel yield of 33 – 35 mg/L, which is about seventeen times more than the control at 1.9 mg/L.

Cell cultures have the advantage of producing the target compound without the amounts of impurities found in plant biomass, which can lead to fewer purification process steps. Although these results are quite promising, this methodology, in the case of taxanes, is far from commercialization. First off, the culture period before obtaining product is 4 weeks. Also, the low amount of product actually produced would require extremely large bioreactors to achieve a commercial amount of material, which is not feasible due to sterilization and contamination

issues. Therefore, this process is not commercially viable yet, unless the yield was much higher and/or the culture period was significantly reduced. Unfortunately, the production of betulinic acid from cell cultures has not been published.

2.2.2 Semi-synthesis

Since there are several different taxanes that are found in these species, chemists looked to the other taxanes as building blocks to form paclitaxel. The following are the main taxanes that are found along with paclitaxel:

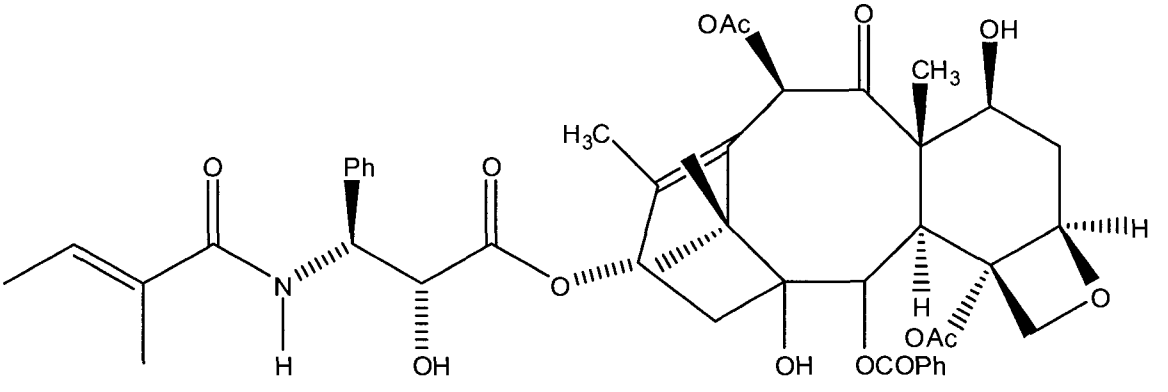

Figure 2. 1: Cephalomannine.

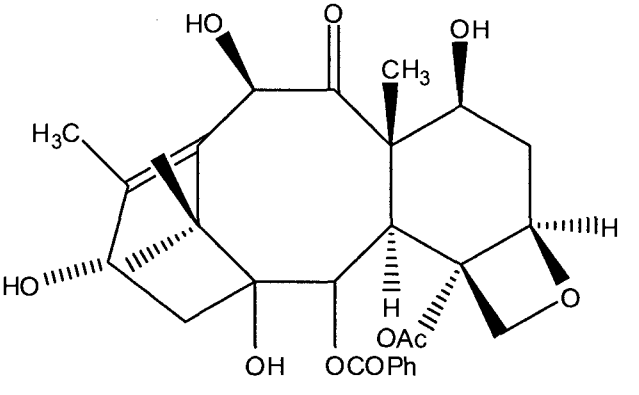

Figure 2. 2: 10-Deacetyl Baccatin III.

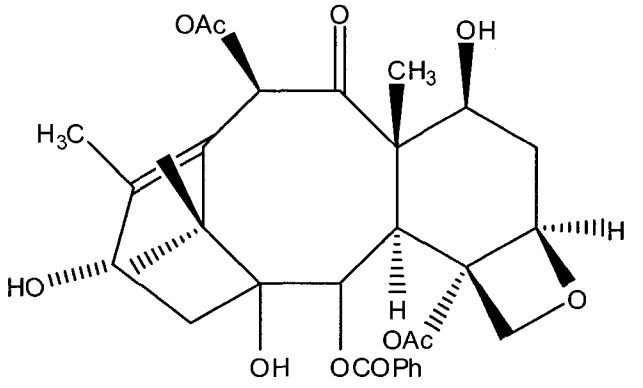

Figure 2. 3: Baccatin III.

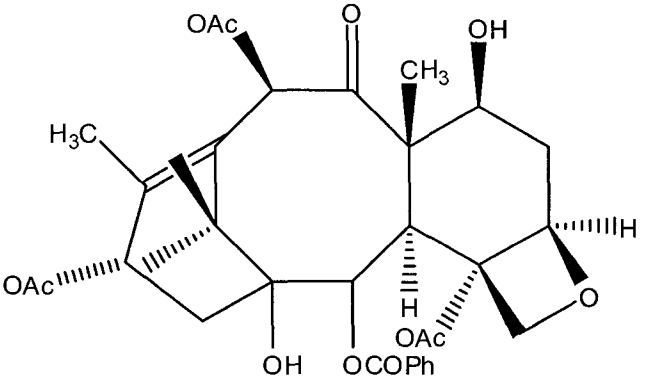

Figure 2. 4: 9-Dihydro-13-Acetal-Baccatin III.

Although Cephalomannine (Ceph) has been considered an impurity in the past, since it is very similar in structure to paclitaxel, new methods have been developed to isolate Ceph. This allows the use of Ceph as a starting point for transformation into paclitaxel and its derivatives.

The main turning point of semi-synthesis in the case of taxanes was in the early 1990s when Bristol-Myer-Squibb (BMS) had bought Bob Holton's semi-synthetic process, which converted 10-DAB to paclitaxel. BMS found a renewable source of taxanes in *Taxus Baccata*, which grows in the Himalayas and Europe, supplied by the company Indena (Goodman, 2001). Numerous papers describe other methods of semi-synthesizing paclitaxel and its analogues.

One of the analogues of paclitaxel of note is Docetaxel, which is the active pharmaceutical ingredient (API) of Taxotere®, first approved in 1996, of Aventis Pharma Inc.

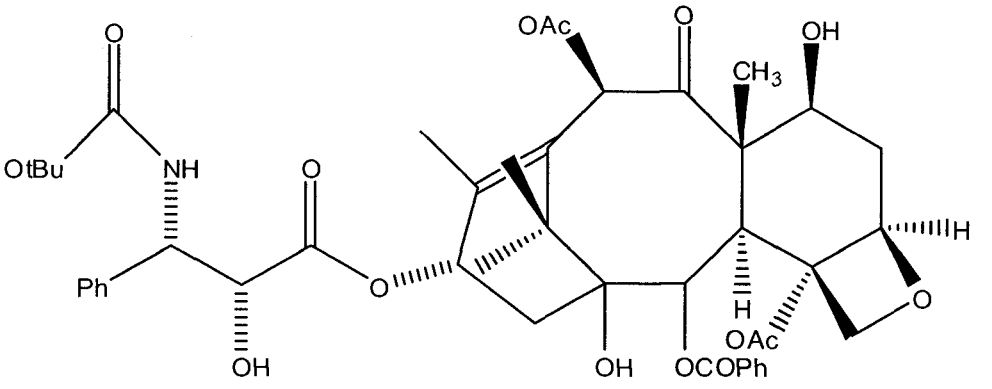

Figure 2. 5: Docetaxel.

Semi-synthesis is often performed in order to maximize profits. In *Taxus Canadensis*, 10-DAB levels are normally four times as much as paclitaxel and 9-DHB levels are normally the same as paclitaxel levels. Dried biomass of *Taxus Canadensis*, with moisture less than 10%, can have around 300 parts per million of paclitaxel. Therefore, in the case of paclitaxel, the theoretical output of paclitaxel and its derivatives can be six-fold or higher; thereby supplementing the low level of paclitaxel present.

Semi-synthesis is very important in regards to betulinic acid. Although the amount of betulinic acid is relatively low in birch, there is an ample amount of betulin. In fact, the amount of betulin found can be more than 10 times that of betulinic acid. The process of converting betulin to betulinic acid has been documented in several patents such as Hughes et al. in 2006, where the

betulin is subjected to two oxidation steps. Czuk et al. (2006) claimed a process where the conversion of betulin to betulinic acid required only one step.

However, semi-synthesis still requires the molecular backbones of the desired compounds. Although semi-synthesis cannot be a standalone process, semi-synthesis of the other taxanes and betulin is desirable to maximize profit potential and to ensure a high and stable production output.

2.2.3 Synthesis

Nicolaou et al. were the first to complete a total synthesis of paclitaxel. Unfortunately, the yields from their methodology were very low. Their results were published in February 17, 1994, in the journal of *Nature*. Although other groups have managed to synthesize paclitaxel through other synthetic routes, none of the methods have been deemed commercially viable (Goodman, 2001).

Since synthesis is incapable of matching the yields and production levels of semi-synthesis and separation from taxane biomass, total synthesis is not used for making paclitaxel. However, synthesis research has provided new methods to obtain new taxane analogues (Nicolaou, 1994).

The synthesis of betulinic acid is not mentioned. This is most likely due to the abundance of betulin found in birch bark available for semi-synthesis of betulinic acid and its derivatives.

2.2.4 Drawbacks of the Alternatives

Since the production output of alternatives is lower, there will be a continued reliance on biomass to obtain our taxanes. The use of semi-synthesis in addition to the various extraction methods allows increased efficiency for the production of large amounts of paclitaxel for the pharmaceutical industry. In the future, elite cultivars with taxanes content of twice or more of those in current use (Cameron et al., 2005) will increase the yields obtained from extraction. Cultivar use will result in a larger gap for alternative methods to bridge before becoming a competitive technology.

2.3 Refinement Techniques

2.3.1 Liquid – Liquid Separation

Liquid–Liquid partitioning is used mostly for the separation of either very polar or very non-polar compounds prior to liquid chromatography. The Waterloo pyrolysis process, for example, employed this process to clean up the pyrolysis oil; a hexane wash was used to remove high molecular weight non-polar components. Each volume of pyrolysis oil was contacted with five equal volumes of hexane. Then, the volume of pyrolysis oil was topped off with methanol. The methanolic pyrolysis oil was then mixed with dichloromethane, The mixture is then partitioned with deionized water, with small aliquots of methanol added to clarify the phase interface.

Liquid–Liquid extraction (LLE) is a very unattractive method of separation due to several factors. One is the use of several different solvents. Most of the solvents used in these types of

processes are generally toxic and environmentally harmful. A large amount of solvent is required to perform these operations, with respect to the desired product, especially with taxanes due to their low solubility in most solvents. Another reason is the difficulty in distinguishing the phase interface, either through mixing or precipitation. This makes plant scale liquid – liquid separation extremely difficult. In addition, the phases rarely separate immediately from each other. Most times, a settling time will be required after mixing, before the separate phases become distinguishable. In the above case, emulsions were observed, which were broken using methanol and octanol. Precipitation, which occurs frequently between phases, can cause added problems for distinguishing the phase interface, as well as, post-operation equipment clean-up.

Continuous testing may be required to ensure that all the products are kept, since the phase distinction may be unclear and lead to a loss of product. Unclear phases can therefore lead to reprocessing to ensure maximum yield. For the above stated reasons, commercial scale operations using liquid-liquid extractions for taxanes may be unpopular.

2.3.2 Membrane

This form of refinement uses either the concentration gradient or a high pressure to drive the solute through the membrane. Although this could theoretically work, the choice of membrane would be difficult, since the range of taxanes span a large range of polarities. Impurities in taxane extracts tend to bond irreversibly to resins, thereby reducing resin life. In the case with membranes, these same impurities could cause blockages of the membrane pores. Also, another difficulty is the small concentration of taxanes that are found in a crude extract, where normally

the concentration is less than 1%. With the lower concentration, the driving force gradient is not very high, which may result in very poor separation. Another factor to consider is that a crude extract is normally composed of several different compounds in addition to taxanes. These compounds may cause fouling of the membrane, further reducing the efficiency of the separation.

2.3.3 Solid Phase Extraction

Solid phase extraction (SPE) is an extraction method that uses a solid phase and a liquid phase to isolate one, or one type, of analyte from a solution. It is usually used for sample preparation before using a chromatographic or other analytical method. Typically, a solution is loaded onto the solid phase, the undesired compounds are washed away, and then the desired compounds are washed off with another solvent.

This method has many advantages over the more traditional LLE. First of all, there is a significant improvement in selectivity. There are generally improved recoveries. Toxic and flammable solvent use is reduced. Solvent miscibility and emulsion issues are eliminated. As well, greater reproducibility is observed.

Unfortunately, SPE can only be run in batch mode. However, the selectivity of paclitaxel can be quite high given the correct conditions. Also, since the other taxanes have different hydrophobic and hydrophilic tendencies, it would be theoretically possible to use multiple washes to

selectively choose the desired taxanes to be removed from the column. To achieve this kind of separation, several different solvents with different polarities would be required.

2.3.4 Normal Phase Chromatography

Normal phase chromatography (NPC) is described as where the stationary bed is strongly polar in nature, such as silica gel, and the mobile phase is nonpolar, such as hexane. Polar samples are retained on the polar surface of the column packing longer than less polar materials.This results in the different elution times of different compounds.

In the *Journal of Chromatography A*, 1995, Dauh-Rurng Wu et al. studied various types of columns in normal phase operation. They compared a Zorbax SW-Taxane column, an alkyl phenyl column, and a perfluorophenyl (PFP) column.

The Zorbax column was eluted with a mobile phase of heptane-ethanol in 50:50 ratio. The alkyl phenyl column was eluted using a heptane-ethanol mobile phase in a 96:4 ratio. The PFP column was eluted with heptane-ethanol in 93:7 ratio. They were all measured at a UV absorbance wavelength of 227 nm.

Using the Zorbax column in normal phase gave a separation of all the taxanes; however, the elution is in a relatively tight band and is eluted in a span of six minutes. The other two columns give much poorer separation, where the peaks overlap and co-elute. Although the Zorbax gives an acceptable separation, other methods greatly improve the separation of the desired taxanes, since large scale separation with close eluting bands is more difficult.

A study performed at the Chinese Academy of science, using an alkaline Al_2O_3 chromatography showed that it is possible to increase the yield of paclitaxel above 100%, in comparison to other columns packed with silica gel, neutral Al_2O_3, and acidic Al_2O_3. From their results, it was concluded that 7-epi-taxol was isomerized into paclitaxel.

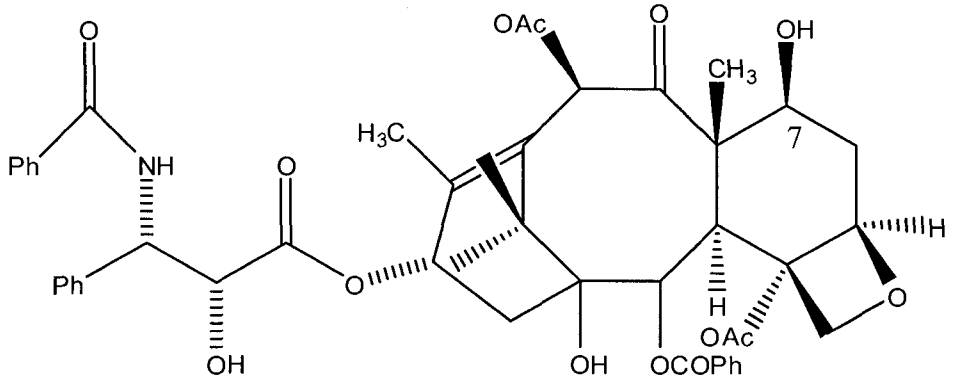

Figure 2. 6: 7-epi-taxol.

It was found that using this type of column gave a higher purification factor as well, as up to 170% paclitaxel was recovered. Using this method, the researchers were able to obtain a paclitaxel content of 29% from an initial extract with less than 1% paclitaxel, although these results were only obtained when both the alumina and the extract had been dehydrated. When dehydration of both was not performed, the results were similar to that of normal NPC.

2.3.5 Reversed Phase Chromatography

Reversed phase chromatography (RPC) is described as where the stationary bed is relatively non-polar in nature, while the mobile phase is more polar, containing not only water but also

polar organics, methanol, or acetonitrile. Here the more non-polar the material is, the longer it will be retained. It is referred to as reversed phase, since polar compounds are eluted first followed by the more non-polar compounds, which is the opposite of normal phase operation.

In the same paper from Wu et al., 1995, the Zorbax column was studied in reversed phase operation. The reversed phase separation of the taxane standards resulted in better peak separation.

Although the peaks are well spaced, the peak width of the later taxanes are very wide, indicating that it is not the most efficient column for separation. The study was performed on a C8 column.

In a separate paper, van Rozendaal et al., 2000, studied reversed phase high pressure liquid chromatography using a C18 column. Using a higher carbon chain phase in their column yielded higher efficiency, since it resulted in sharper, more distinct peaks.

In addition to the benefits of the well-spaced elution peaks, reversed phase chromatography also holds a key advantage. This method uses very little of organic solvents in comparison to NPC, which means low disposal costs of analytical solvents. With the well-spaced elution peaks, application of this methodology in plant-scale operations becomes easier since the fractions can be collected for further refinement. Since there is a lower amount of impurities that separate at the same time as the desired taxanes, the separation factor of the desired product is much higher. Using NPC, taxane co-elution would be more likely to occur, which would then require more stages of separation.

RPC is a necessary step in regards to betulinic acid separation. Since betulinic acid and betulin are very similar on the molecular level, RPC is used to differentiate the two compounds.

Regardless of the route of purification, the final natural product goes through a crystallization process for "polishing" of the finished product and is repeated as necessary to achieve the desired purity level.

3.0 Materials and Methods

3.1 Materials

3.1.1 Biomass

The ground hemlock biomass samples were purchased from Chatham Biotec Ltd, lot 408-708, containing on average 200 ppm by mass of paclitaxel from soxhlet extraction. The material was processed October 8, 2004 and was collected from Balmoral, NB, Canada. The material was used as provided, which had come grounded with the bulk of the material (80%) between 10 and 20 mesh (2.00 – 0.84 mm) in size with the rest being smaller particle sizes. The biomass had a moisture content of about 10%. The material was refrigerated until ready for use. To obtain smaller mesh sized ground hemlock, the material was ground up using a coffee grinder (Braun, Type 4041, and Model KSM2).

The birch bark was also purchased from CBL, which was harvested in northeastern New Brunswick. The material was ground up using a common household blender. The material was then dried overnight in a drying oven set to 60 °C to a moisture content of about 10%.

3.1.2 Solvents and Water

Optima grade acetonitrile (Fisher Chemicals, Ottawa, ON) and double de-ionized water were used for High Performance Liquid Chromatography (HPLC) analysis. The purity of ethanol was 99% ethanol/water. All other solvents were American Chemical Society (ACS) grade (Fisher Chemicals, Ottawa, ON): hexane, dichloromethane, methanol, tetrahydrofuran (THF), chloroform, isopropanol, butanol, and water.

3.1.3 Solid Phase Extraction Adsorbents

The following adsorbents were used:

- Mitsubishi Chemical Diaion HP2MG resin (Lot 1C501) with pore diameter 400 Å, pore volume 1.2 mL/g, and surface area 500 m^2/g.
- Mitsubishi Chemical Sepabeads SP207 (Lot 2H503) with pore diameter 220 Å, pore volume 1.3 mL/g, and surface area 600 m^2/g.
- Mitsubishi Chemical Sepabeads SP20SS (Lot 1D502) has a bulk particle size distribution between 63 and 75um.
- Silica Gel from Selecto Scientific (Lot 306056302) with particle size between 32-64um.

All of these adsorbents were used without any further treatment.

3.1.4 External Standards

Taxane standards were purchased from Hauser Pharmaceutical Services. Paclitaxel (99%), 10-DAB (97%), 9-DAB(96%), BAC (95%). Betulinic acid (98%) was also purchased from Hauser Pharmaceutical Services.

3.2 Equipment

The overall set-up can be seen in Figure 3.1.

A Prominent Fluid Controls ProMus117BSS1001X11E0 with a Marathon Electric ½ HP motor and a Genesis AC Motor Speed Controller was used for pumping solvents through the system.

The stainless steel extractor had an internal diameter of 10 inches and length of 20 inches with both ends capped and 60 mesh stainless steel gauze covers both openings. An adjustable Swagelok pressure relief valve, model RL3, was used for pressure control. Valves before and after the extractor were Swagelok stainless steel ball valves ¼". Omnifit PTFE 3-way valves were used for connections leading to the adsorption column. Pressure gauges were Swagelok, rated for 0 to 100 psi.

The hot water bath was a Cole-Parmer BT-15 used for heating the solvent to the desired temperature. The cooling bath was a Cole Parmer model 12108-10 temperature controlled bath set to 5 °C. The cooling bath was used reduce the volatility of the solvent.

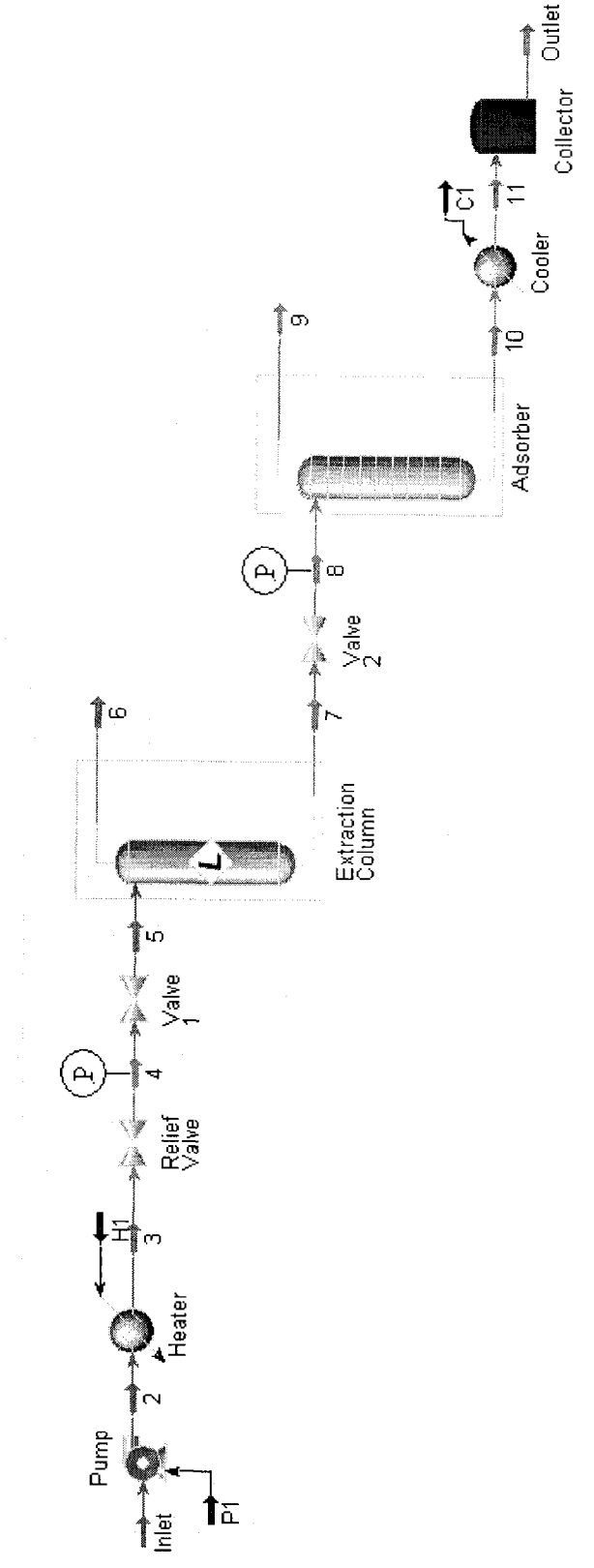

Figure 3. 1: Diagram of Equipment Setup.

The adsorption column used was a 250 mL Omnifit glass column with PTFE stopper ends. Each end was fitted with a filter to retain the resin within the column and for flow distribution. One end of the column was fitted with an adjustable screw end.

A Buchi rotary evaporator with 1L round bottom evaporation flask and 1L receiving flask were used for solvent recovery from all processes.

3.3 Extraction Procedures

3.3.1 Batch Extraction of Betulinic Acid

Roughly 1.3 g of birch bark was weighed on a weigh boat and carefully transferred into a 250 mL Erlenmayer flask. The hot plate was adjusted to the desired temperature and left to stabilize before using.

Approximately 150 mL of solvent was then measured out and transferred to the Erlenmayer flask. The top of the flask was then sealed. The stirrer/hotplate was then set for a rotation speed of approximately 200 rpm. The flask was left for 8 hours before being removed and the extract collected and analyzed.

3.3.2 Soxhlet Extraction

The flat-bottom flask was filled roughly two-thirds full with desired solvent, normally methanol. The soxhlet glassware was then inserted on top of the flask. A cellulose thimble was then filled with a known quantity of biomass, leaving about 1 cm of room at the top of the thimble. The soxhlet equipment was then assembled and the hot plate was switched on. The system was allowed to extract for 8 hours. All the solvent was collected into the collection flask and was measured in a graduated cylinder before the sample was taken to be analyzed.

3.3.3 DPLE Procedure

3.3.3.1 Hexane Extraction

The hot water bath was adjusted to the desired temperature (between 50 and 90 °C) and left to stabilize before use. Then 800 g of 20 mesh ground hemlock was placed into the stainless steel extractor.

During the hexane pre-wash step, the hexane was pumped through the system at a constant rate, 45 mL/min or 100 mL/min, depending on testing parameter, while being heated. The lower flowrate corresponded to the optimum cross-sectional flowrate as discussed by Wang (2003). The extract was collected in an Erlenmayer flask, which rested in the cold water bath. As the flask became full, the exit valve from the extractor was closed and a new flask replaced the full one before the valve was reopened. Operating pressure of the system was controlled between 4.8

and 5.2 atm, using a safety valve of variable set pressure. Samples were taken using a three-way valve connected to the exit stream into 10 mL vials. The system was then drained of the solvent after completion of the test run.

The solvent had a residence time of 15 minutes within the extractor. Time recording was started when the first drop of extract appeared from the extractor. After extraction, the extractor was allowed to cool at room temperature overnight. The biomass was then unloaded and allowed to ventilate under the fume hood overnight.

3.3.3.2 Taxane Extraction

Two operational modes were tested: (1) Hexane pre-wash, followed by dichloromethane extraction of the biomass and (2) direct dichloromethane extraction without the pre-wash step.

The extractor was loaded with 800 g of 20 mesh ground hemlock. The hot water bath was adjusted to the desired temperature and left to stabilize before use. Hexane was pumped through the system at 45 mL/min during the hexane pre-wash as described above. Afterwards, the system was drained until no more hexane exited the system. If the dichloromethane step required a different temperature, the hot water bath was adjusted at this time. After draining the hexane, the system was returned to its previous set-up.

During the dichloromethane extraction step, dichloromethane was pumped instead of hexane, at 45 mL/min. The operating pressure of the system was controlled between 4.8 and 5.2 atm, using

a safety valve of variable set pressure. The extract was collected in an Erlenmayer flask, which rested in the cold water bath. As the flask became full, the exit valve from the extractor was closed and a new flask replaced the full one before the valve was reopened. Samples were taken using a 3-way valve connected to the exit stream into 10 mL vials. The system was then emptied after the completion of the test run.

Time recording was started when the first drop of extract appeared from the extractor. After extraction, the extractor was allowed to cool at room temperature overnight. The biomass was then unloaded and allowed to ventilate under the fume hood overnight.

3.3.3.3 Adsorption Procedure

After the hexane pre-wash, the extract outlet stream from the extractor was connected to the Omnifit adsorption column with tubing, allowing the inline loading of the adsorption column. The adsorption column was loaded with 200mL of adsorbent. The outlet from the adsorption column then flowed into a collection flask, cooled by the cold water bath. Samples were collected from a 3-way valve connecting the tubing between the extractor and the adsorption column. As well, samples were collected from the exit of the adsorption column from another 3-way valve connecting the exit of the adsorption column to the collection flask.

The elution step was performed offline from the extraction system. The pump was connected to the inlet of the adsorption column while the outlet was still connected to the collection flask and

cold water bath. The elution solvent, methanol or ethanol mixtures, was then pumped at 20 mL/min. After elution, the adsorbent was regenerated by circulating 1 L of ethanol.

3.3.4 Taxane Extraction with Methanol

The pump was connected directly to the extractor without heating. Then 800 g of ground hemlock was measured into the stainless steel extractor. Methanol was pumped at 45 mL/min without pressurizing the system to simulate CSE. The extract was then collected in a collection flask. Samples were taken using a 3-way valve connected to the exit stream into 10 mL vials. The system was then emptied after the completion of the test run.

3.5 Analytical Methods

3.5.1 HPLC analysis

A Waters HPLC system was used, which included: Millenium 2010 Chromatography Manager (Millenium Software 2.0), Waters 600E Multisolvent Delivery system, Waters 717 Autosampler, and 486 tunable Absorbance Detector. High purity helium was used for online degassing. A Phenomenex Curosil-PFP, 250 x 4.6 mm column was used, along with a Phenomenex Curosil 30 x 4.6 mm guard column. The UV detector was set to a wavelength of 227 nm and the injection volume was 5 μL. The mobile phase consisted of HPLC grade acetonitrile and water (Table 3.1). O-Phosphoric acid was added to the mobile phase as 0.1% v/v.

For taxane analysis, a Phenomenex Curosil-PFP, 250x4.6 mm column was used, along with a Phenomenex Curosil 30x4.6 mm guard column. The wavelength of the UV detector was set at 227 nm. The injection volume was 5 μL.

Calibration curves were prepared by analyzing the external standards under the same analysis conditions and the results are given in Appendix A.

Table 3. 1: HPLC Gradient Table

	Time (min)	Flow (mL/min)	% acetonitrile	% water	curve no.	curve description
1	0	1	25	75	6	linear gradient
2	40	1	75	25	2	convex gradient
3	55	1	100	0	2	convex gradient
4	65	1	25	75	11	maintains start condition

For betulinic acid analysis, the same gradient table was used for the elution. The wavelength of the UV detector was set to 210 nm. The column used was a Varian Pursuit XRs 5u C18 column, 250x4.6mm in conjunction with a Varian Pursuit XRs 5u C18 guard column, 30x4.6 mm.

The concentration of the target compounds were determined by comparing the areas of the peak with the calibration curves prepared previously.

The retention times of selected taxanes and betulinic acid are given in Table 3.2.

Table 3. 2: Product Retention Times

Product	10 DAB	BAC	9 DAB	PAC	Betulinic Acid
Retention Time (min)	14.950	22.083	24.750	32.742	55.383

Examples of standard elution curves are shown in Figure 3.2 and Figure 3.3.

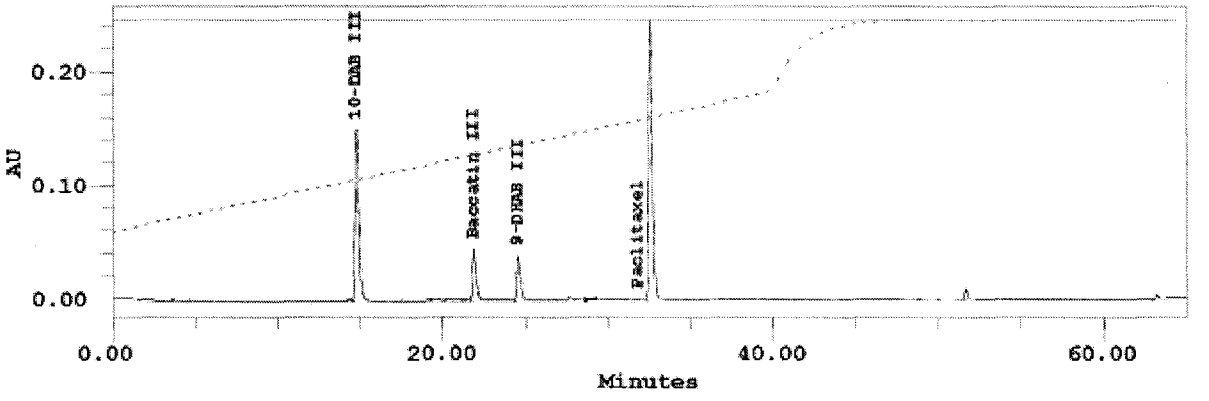

Figure 3. 2: Chromatograph of Taxane Standards.

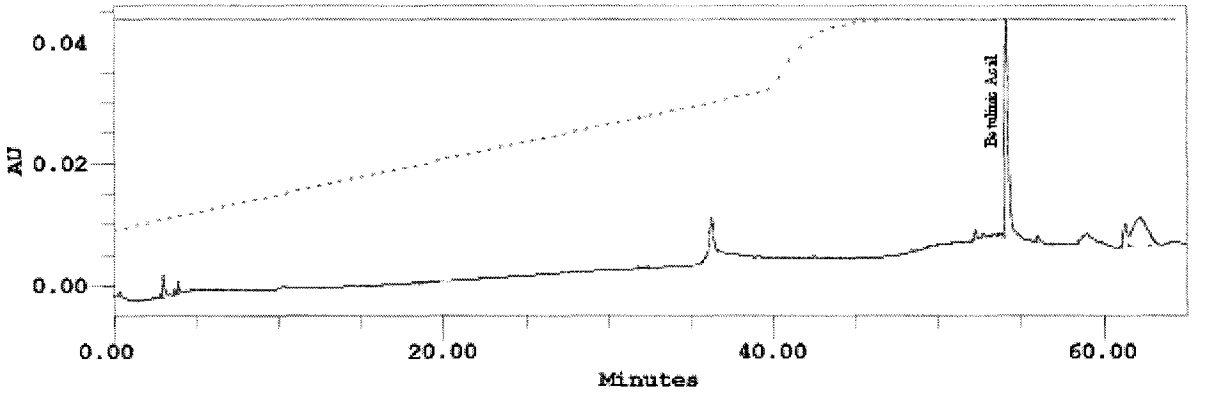

Figure 3. 3: Chromatograph of Betulinic Acid Standard.

3.5.2 Dry Weight Analysis

Roughly 2 g of sample was placed in an aluminum tin and its weight recorded. The samples were dried at 60 °C for 8 hours to remove moisture and weighed after 1 hour of desiccation.

m_t: mass of tin (g)

m_s: mass of sample and tin (g)

m_{ds}: mass of tin and dried sample (g)

% Solids: mass of dried extract per mass of extract (mg dried extract/g extract)

$$\% \, Solids = \frac{m_{ds} - m_t}{m_s - m_t} * \frac{1000 \, mg}{1 \, g} \qquad \text{[Eq. 1]}$$

3.5.3 Purity Calculations

Purity is defined as the mass percent of desired product within the dried extract.

m_b: Mass of biomass in extractor (g)

V_e: Total volume of extract (mL)

C_{pac}: Concentration of paclitaxel in extract (mg/mL extract)

C_{9dab}: Concentration of 9-Dihydro-13-Acetal-Baccatin III in extract (mg/mL extract)

C_{10dab}: Concentration of 10-Deacetyl Baccatin III in extract (mg/mL extract)

C_{bac}: Concentration of Baccatin III in extract (mg/mL extract)

m_e: Mass of dried extract per mass of extract, also referred to as % solids (mg dried extract/g extract)

The density of the extract is assumed to be that of the solvent due to the small percentage of dried extract to the total mass.

$$purity = \frac{C_{pac}}{m_e * \rho_{solvent}} \left[\frac{mg\ paclitaxel}{mg\ dried\ extract} \right] \quad \text{[Eq. 2]}$$

$$paclitaxel\ yield = \frac{V_e * C_{pac}}{m_b} \left[\frac{mg\ paclitaxel}{g\ biomass} \right] \quad \text{[Eq. 3]}$$

The other taxanes and betulinic acid would follow the same calculations as above by replacing the concentration of paclitaxel with the desired product.

4.0 Results and Discussions

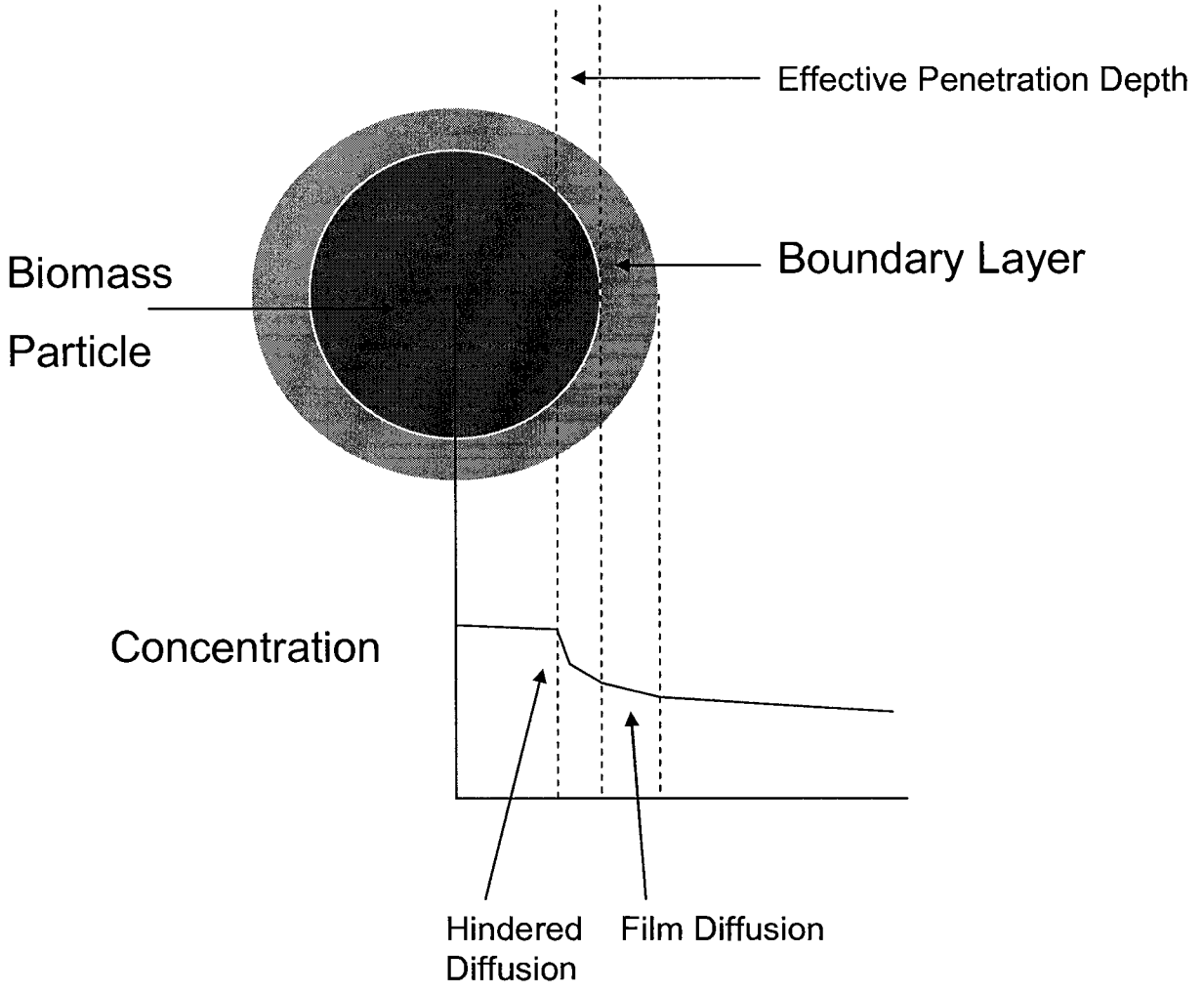

Figure 4. 1: Mass Transfer Diagram of Biomass Particle.

Ideal mass transfer of each biomass particle is assumed during the extraction process (Figure 4.1). Outside of the boundary layer, bulk mixing occurs, where a homogeneous mixture with a bulk concentration is assumed. Proceeding into the boundary layer, film diffusion occurs. Within the biomass particle, it is assumed that the pores of the particle allow limited penetration, thereby creating an effective penetration depth of the solvent. This case is especially true in Dynamic

Pressurized Liquid Extraction (DPLE) where the particles themselves are hydrophilic while the solvents, hexane and dichloromethane, are hydrophobic.

After this effective penetration depth, the solvent penetration into the particle is insufficient to break the solute-matrix interactions, the possible binding effects between the product and other impurities present within the particle. This effect results in a core where little to no diffusion occurs, leaving its solutes intact. Therefore, smaller particles allow for a more efficient extraction, since the effective penetration depth remains the same, but the diameter of the particle is reduced. The effective penetration depth would remain the same in the case of uniform pore distribution. Another possible advantage of the smaller particles is the increase in surface area allowing more surface contact with the solvent and resulting in increased mass transfer and therefore a quicker extraction.

4.1 Paclitaxel Extraction and Refinement

4.1.1 Reproducibility

To ensure that the results could be compared between runs, reproducibility testing was performed. The following figure (Figure 4.2) shows the results of the four runs under identical conditions:

The runs were performed using a 90 ^{o}C hexane pre-wash and a 90 ^{o}C dichloromethane extraction.

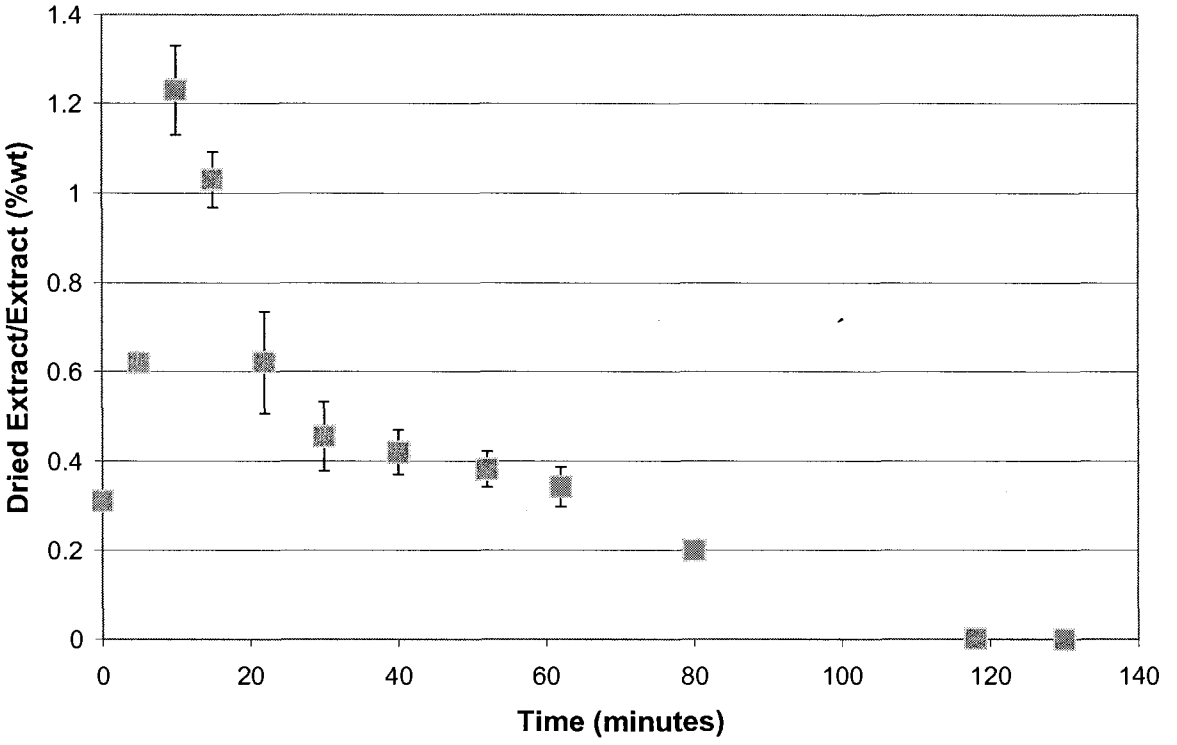

Figure 4. 2: Reproducibility Test Results Using 90 °C Hexane Pre-wash and 90 °C Dichloromethane Extraction.

4.1.2 Methanol Extraction

In order to compare DPLE from the standard extraction process, a baseline must be established. In the following sections, 100 % recovery refers to the total amount of paclitaxel found within the biomass as determined by HPLC analysis on soxhlet extractions.

Using a dynamic methanol extraction, tailing occurs (Figure 4.3); this tailing results in longer extraction times to achieve complete extraction. The extraction results in a high recovery of product; however, the methanol also extracts other compounds as seen in the amount of solids

extracted (Figure 4.4). The purity of the product had an average of 0.061% with respect to dried extract and a maximum purity of 0.080% with respect to dried extract. Therefore, the amount of dried extract greatly exceeds the amount of paclitaxel extracted.

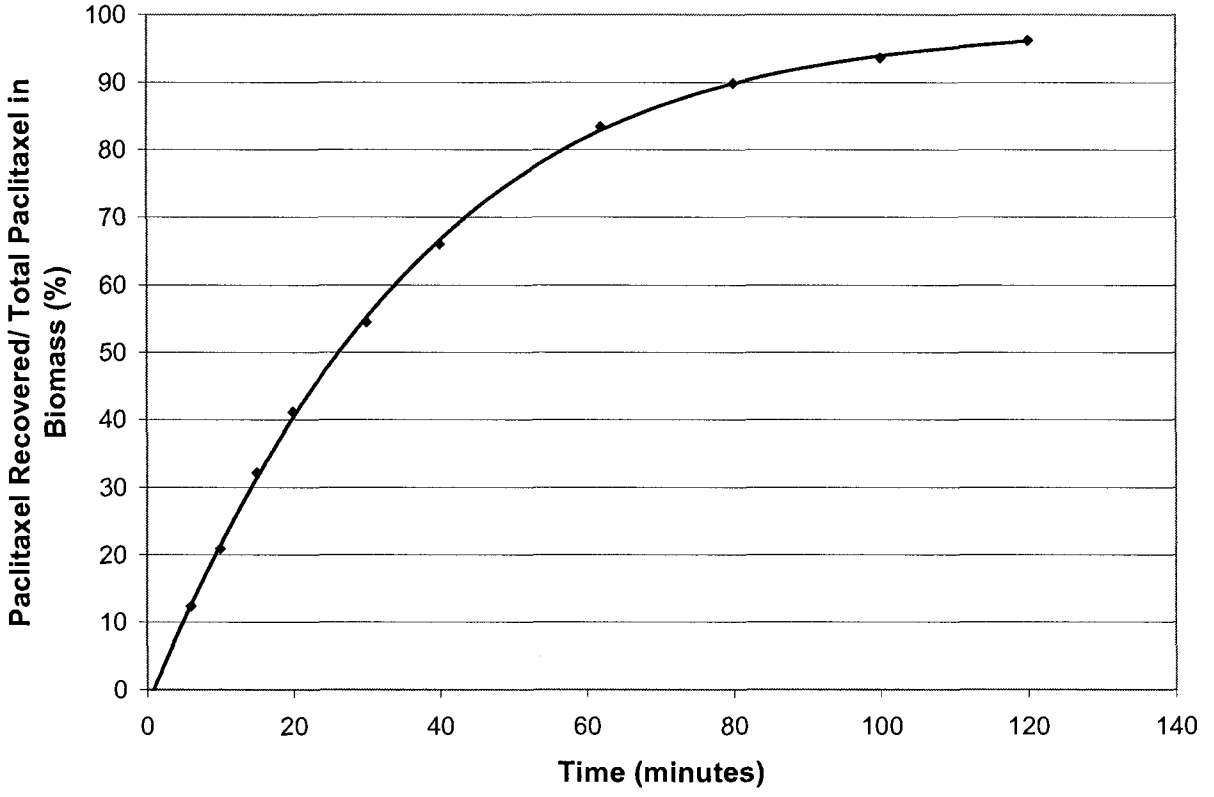

Figure 4. 3: Paclitaxel Concentrations from Methanol Extraction at 20 °C.

In regards to using methanol as an extraction solvent, there is also a problem with swelling. When using methanol, the biomass absorbs the solvent causing the biomass to swell. This swelling could aid in the diffusion of solutes from the core of the particles; however, it has operational drawbacks. This swelling may cause blockage of filters and screens used to hold the biomass in place within extraction equipment. Also, swelling causes an issue in terms of solvent recovery. Since the solvent is absorbed into the biomass particle, the task of recovering all of the

solvent becomes arduous. In this experiment, roughly 1 L out of the 6 L of methanol remained with the biomass and would require extra steps to recover the solvent. These steps tend to be energy intensive as well as time consuming.

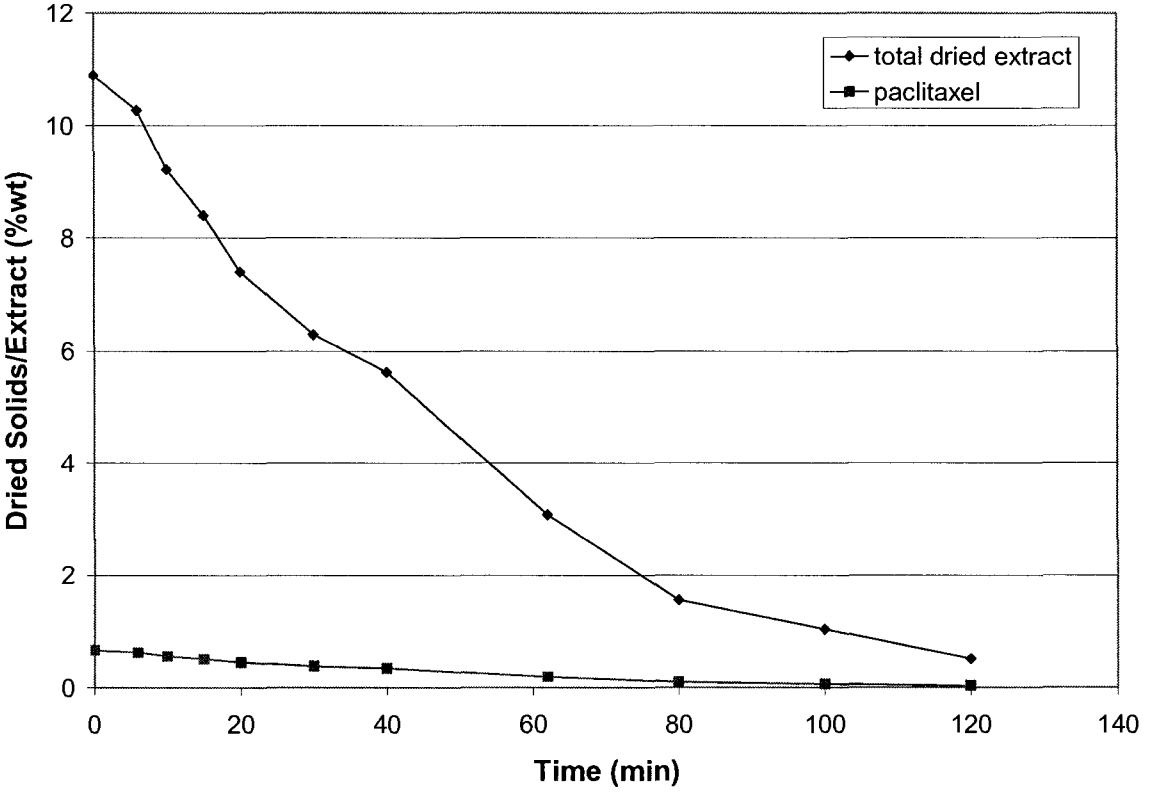

Figure 4. 4: Time Course of Solids Extracted per g Solvent Extract Using Methanol Extraction.

The solvents used in DPLE are preferable as the aforementioned problem is not an issue. Since the solvents are hydrophobic, they do not become trapped in the biomass, thereby offering enhanced recovery of the solvents as well as offering a possibly more efficient process.

4.1.3 Hexane Pre-wash

In order for a hexane pre-wash to be feasible, the pre-wash should be able to maximize the extraction of impurities while minimizing the amount of product to be removed, leaving the product for subsequent steps. The following figure shows the amount of solids removed using the hexane pre-wash (Figure 4.5).

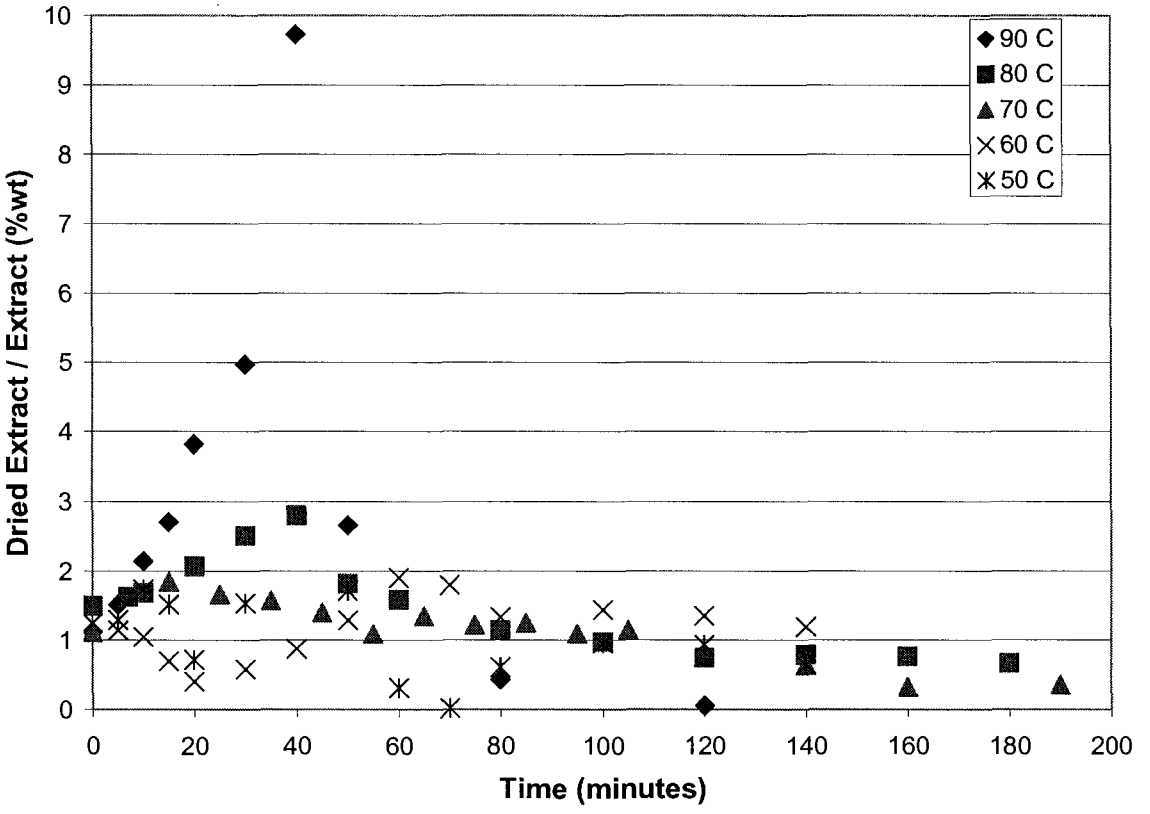

Figure 4. 5: Time Course of Solids Extracted per g Solvent Extract Using Hexane at Various Temperatures at 45 ml/min.

Under higher temperatures, it appears there could be an increase in the extraction of solids. At 90 °C, virtually no solutes are removed after 120 minutes. This effect could possibly be explained by the reduction of matrix binding effects. In order to study the possible trend, further test runs should be done between 80 and 90 °C. If the accumulated dried extract removed from the

biomass is calculated at each temperature to the same extraction time, the resulting figure can seen below (Figure 4.6).

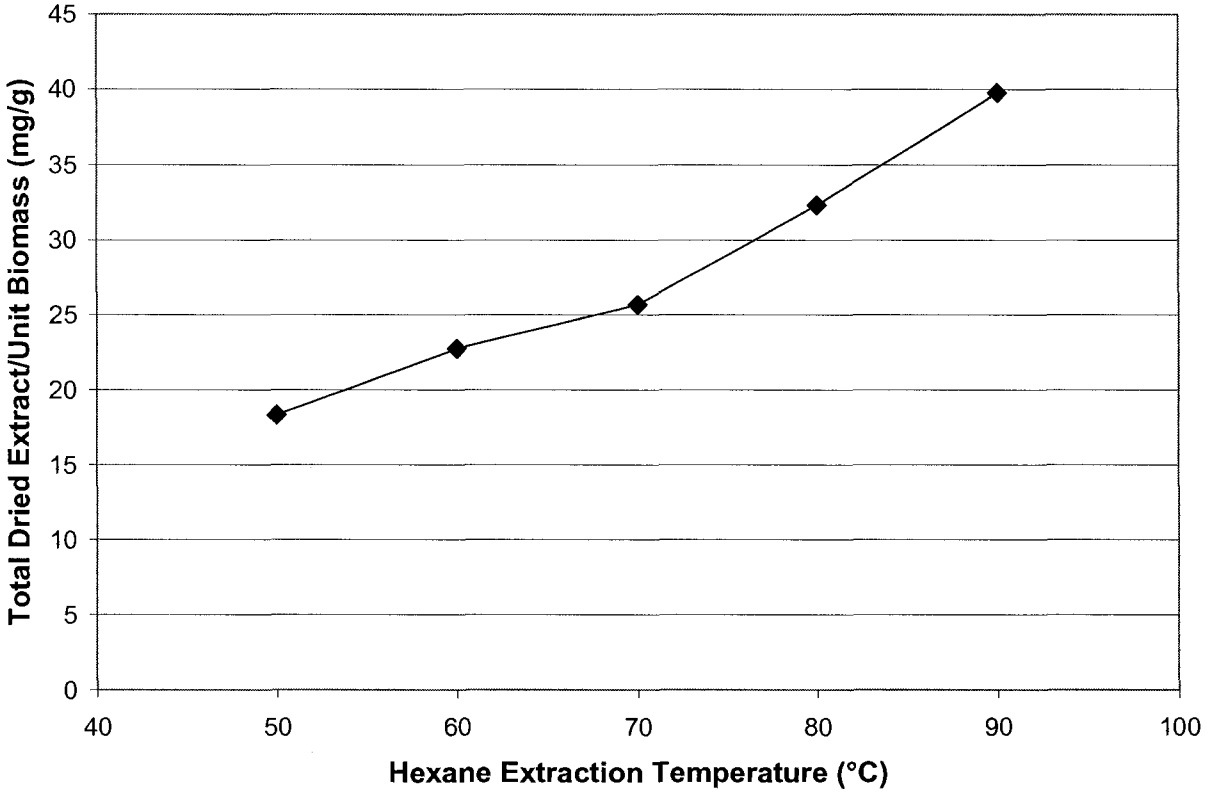

Figure 4. 6: mg Total Extracted Solids per g of Ground Hemlock Using Hexane at Various Temperatures at 45 mL/min.

It is observed that the total amount of extracted solutes increase with temperature. This could be the result of the higher temperature reducing the matrix binding effects, which would lead to an increase in dissolved solutes. In contrast to the dried extract, the paclitaxel extracted was not a significant amount (Figure 4.7). As can be seen, at 90 ^{o}C, the amount of paclitaxel extracted at 80 minutes is much less than 1% of the total paclitaxel content residing in the ground hemlock. If the pre-wash were to continue for 120 minutes, when the solids extraction becomes virtually zero, there would only be a paclitaxel loss of 1.1%.

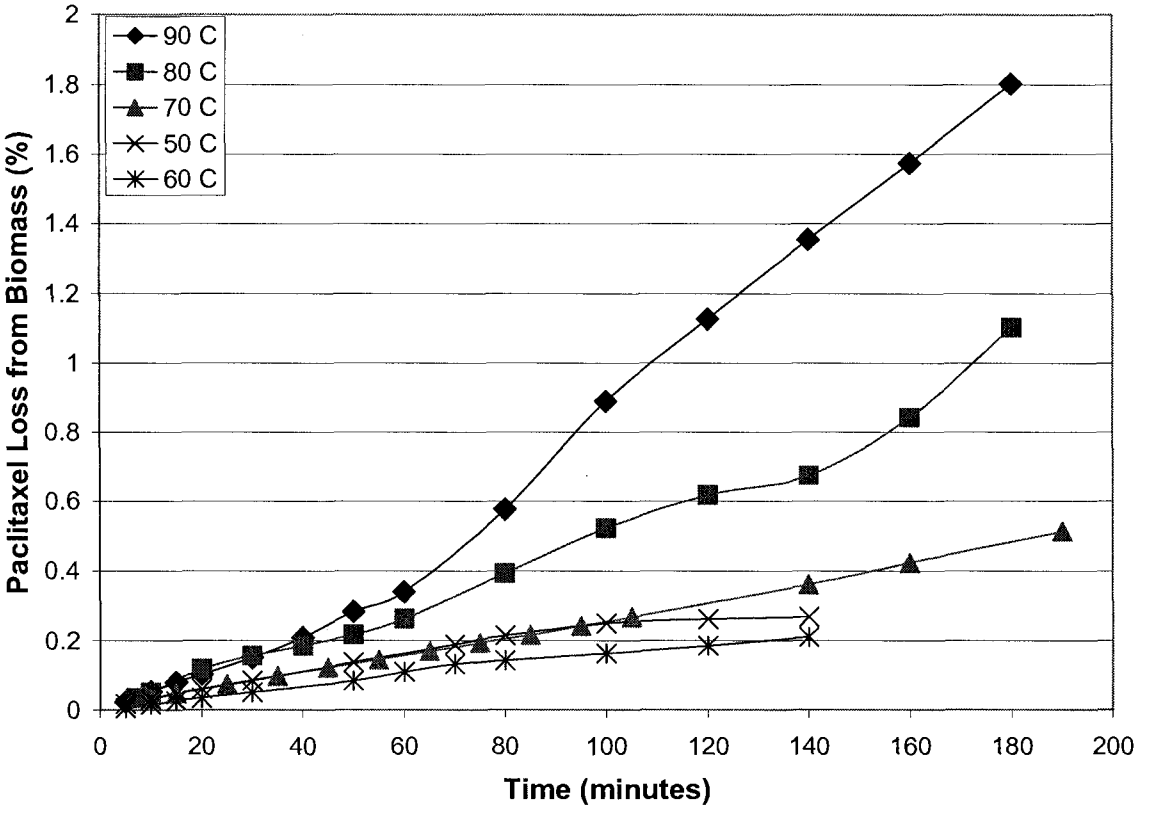

Figure 4. 7: Total Paclitaxel Extracted Using Hexane at Different Temperatures at 45 mL/min.

The optimal temperature for the hexane pre-wash would be at 90 °C since it allows for the maximum removal of impurities while leaving 99% of the paclitaxel intact. Although higher efficiencies could occur at higher temperatures, we are limited by the stability of the taxanes. At temperatures above 90 °C, there is a risk of paclitaxel decomposition [Mattina et al, 1997].

The hexane pre-wash was also performed at a higher flowrate, 100 mL/min, to determine the effect of different flowrates (Figure 4.8).

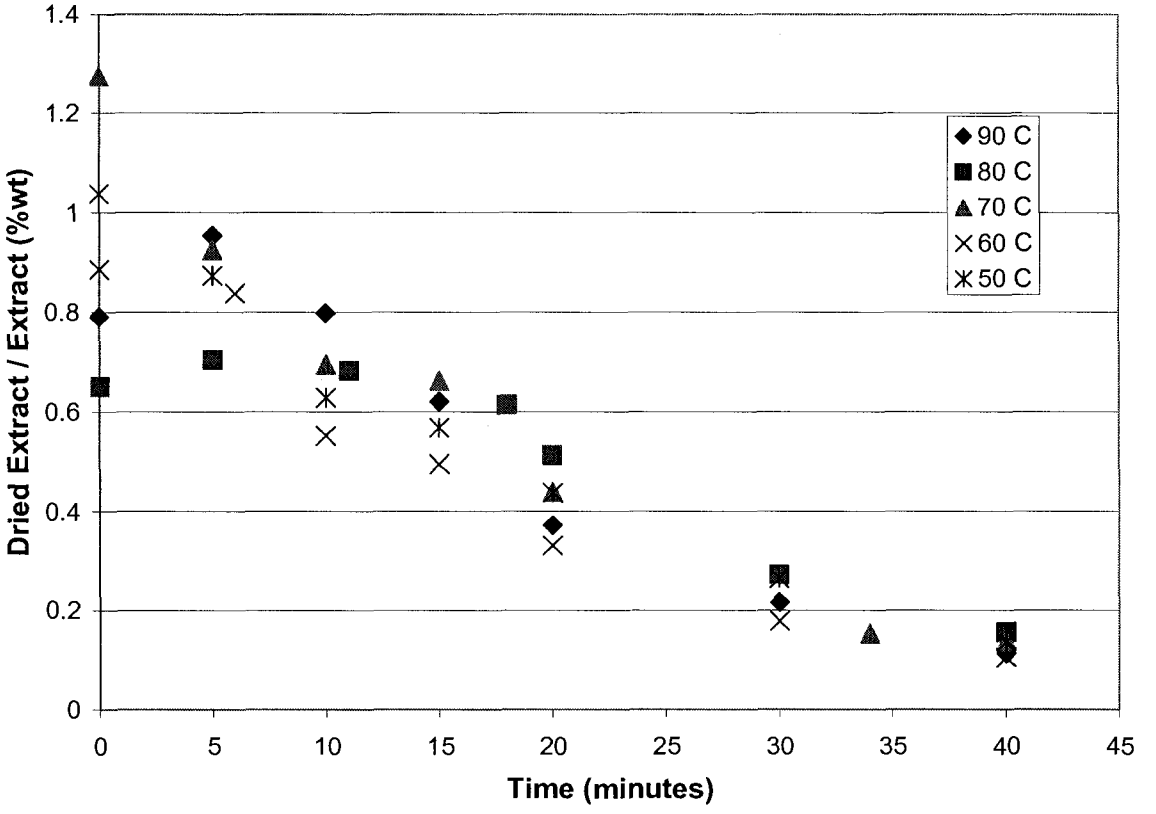

Figure 4. 8: Time Course of Solids Extracted per Unit Solvent Extract Using Hexane at 100 mL/min at Various Temperatures.

The extraction gives a similar trend regardless of extraction temperature. The total extractables in the extract amount is roughly 13 g which is significantly less than the amount extracted using a lower flowrate (32 g at 90 °C).

In the small scale work performed by Y. Wang, different flowrates during the hexane pre-wash were studied. Unfortunately, the work consisted of only flowrates up to an equivalent cross-section of 45 ml/min in our extractor. The small scale results indicated that the dried extract content gradually increased with increased flowrates until at the faster flowrates, the dried extract content reached a plateau.

Given sufficient time, it would be expected that the total dried extract removed from the biomass would be equivalent to the amounts achieved using a lower flowrate. Since the effective penetration depth is assumed to be the same, as pressure and temperature remain constant, the mass transfer to the core of the particle would remain unchanged, except for the bulk flow. The increased quantity of fresh solvent entering the system would offer a greater difference in concentration gradient. Since the mass transfer of solutes to the bulk solution would be driven by the concentration, an increase in the extraction time would be expected.

However, there is a significant difference, 32g versus 13g, in the extraction amounts calculated from the concentrations shown above (Figures 4.7 and 4.8). This may have been an effect of channelling, within the extractor. Channelling was concluded upon visual examinations of the biomass after the extraction process. Depressions were seen in the top of the biomass indicating possible channel areas. As there would be reduced contact with the biomass, the reduced mass transfer would account for the discrepancy. This effect could possibly be reduced with an introduction in the packing procedure. Within this work, the biomass was loosely packed. A biomass slurry, biomass and solvent, could be a possible solution to overcoming the effect.

Although the higher flowrate was less successful than a lower flowrate extraction, better flow distribution or a packing procedure may increase the effectiveness, which in turn could reduce processing time.

4.1.4 Dichloromethane Extraction

Next, the efficiency of the process using dichloromethane only without the pre-wash was investigated. It is imperative to determine the necessity of the hexane pre-wash to possibly streamline the process. As well, it would also allow for a direct comparison between methanol and dichloromethane in terms of paclitaxel extraction.

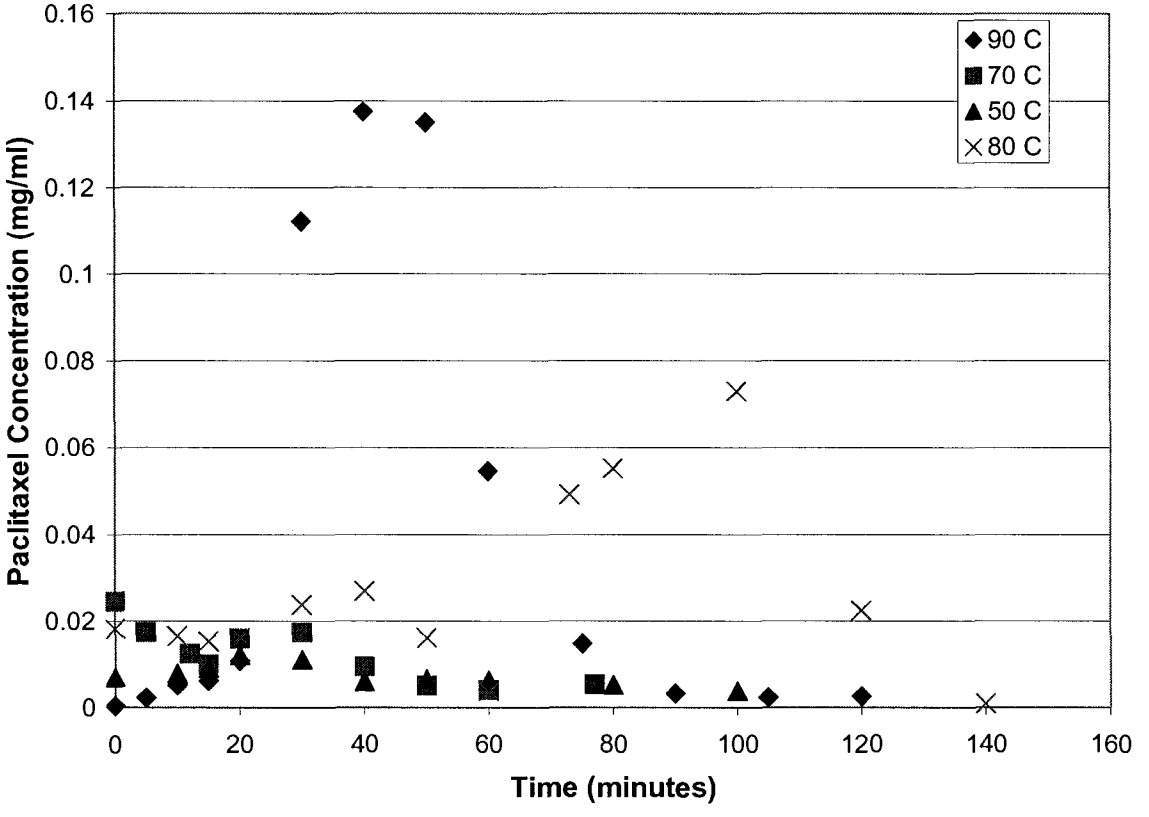

Figure 4. 9: Paclitaxel Concentration Using Dichloromethane in DPLE without Pre-wash at Various Temperatures and 45 mL/min.

As can be seen in Figure 4.9 above, at 90 °C the bulk of the paclitaxel is extracted from 20 to about 60 minutes. The peaks tend to start around the 20 min mark; however, there is a delay observed at 80 °C. Unfortunately, due to time constraints, the run could not be repeated in order

to observe the reproducibility of this peak. Should this peak be reproducible, it could be a result of increased mass transfer at higher temperatures

The values from Figure 4.9 translate into the following recoveries (Figure 4.10):

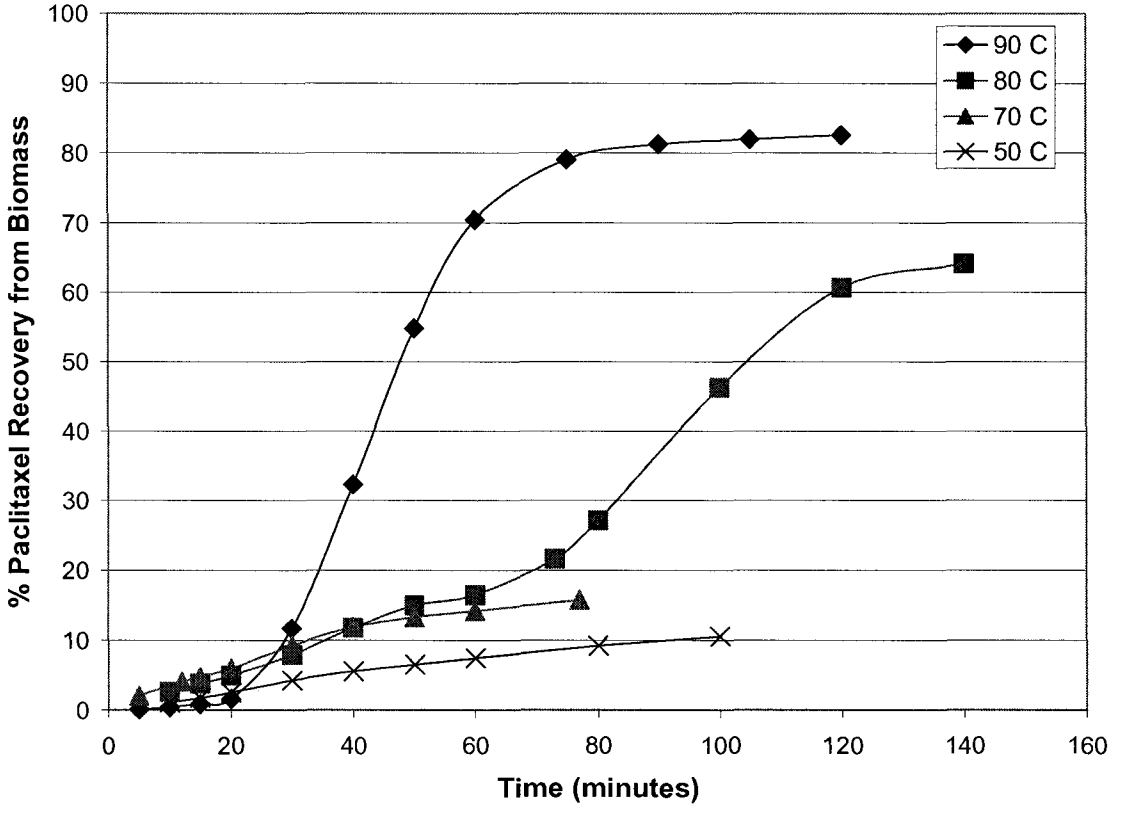

Figure 4. 10: Paclitaxel Recoveries from Total Paclitaxel Using Dichloromethane without Pre-wash at Various Temperatures and 45 mL/min.

At higher temperatures, higher recoveries are observed, but are still less than what is yielded through CSE. Although these recoveries are lower than the 100% from the small scale testing (Wang, 2004), there was a difference in operating conditions. The particle size of the biomass on the pilot scale was 20 mesh, while the material used in the small scale testing was less than 100 mesh. This could be a result of the smaller particle sizes reduciung mass diffusion hindrances unlike the longer diffusion path with larger particle sizes and limitation in penetration depth.

Further investigation would be required to determine if there is a point of exponential recovery at the lower temperatures. Longer extraction times would be necessary to determine whether this phenomenon was present.

The product purity of the recoveries from the above figures can be compared in the following, Figure 4.11.

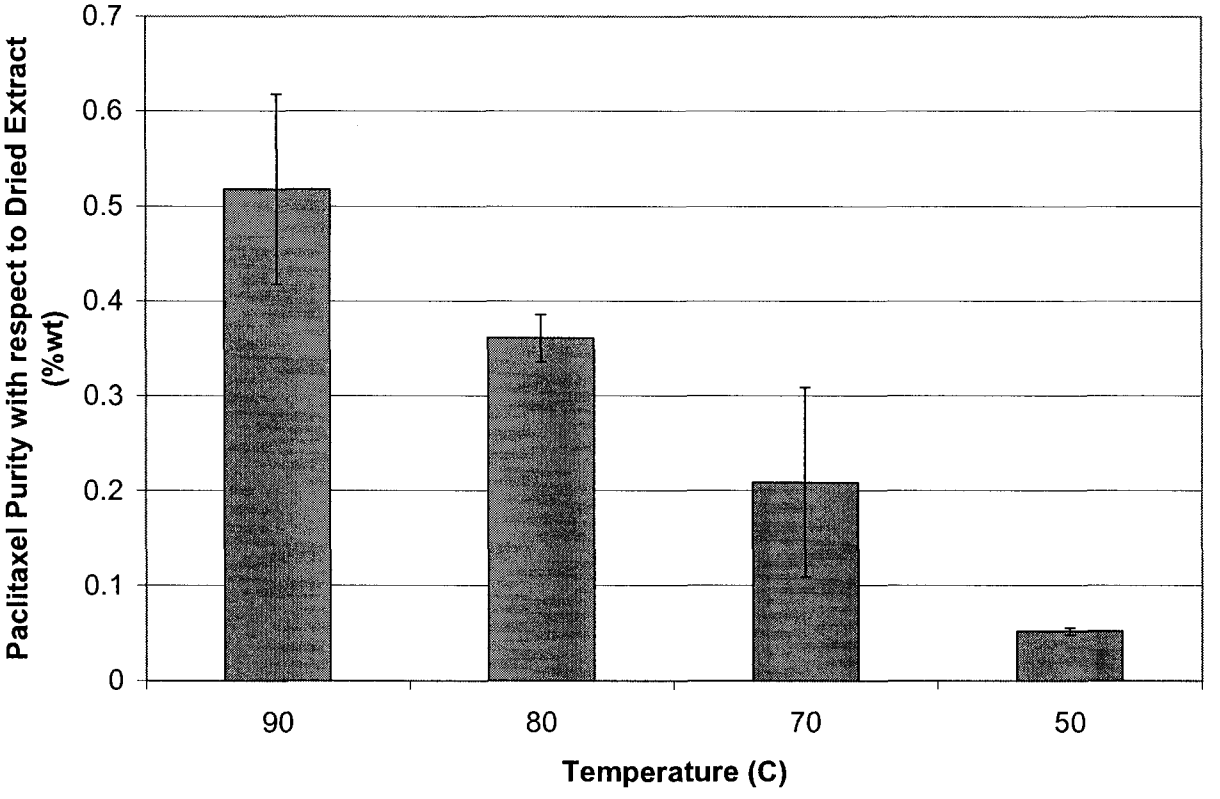

Figure 4. 11: Maximum Paclitaxel Purity Using Dichloromethane in DPLE without Pre-Wash at Different Temperatures at 45 mL/min.

At 50 °C, the purity is not higher than that of a methanol extract of 0.08 %; however, at higher temperatures, the separation factor increases dramatically. At 90 °C, the purity of the product is

almost ten times greater than that of a methanol extract. The increased selectivity results from the increase in mass transfer of paclitaxel while the bulk of impurities are still retained within the particle due to the polarity of the solvent.

Although the recovery rate obtained with dichloromethane are lower than those obtained with methanol, the purity of paclitaxel in the extract appears to be significantly greater.

4.1.5 Hexane and Dicholoromethane Extraction

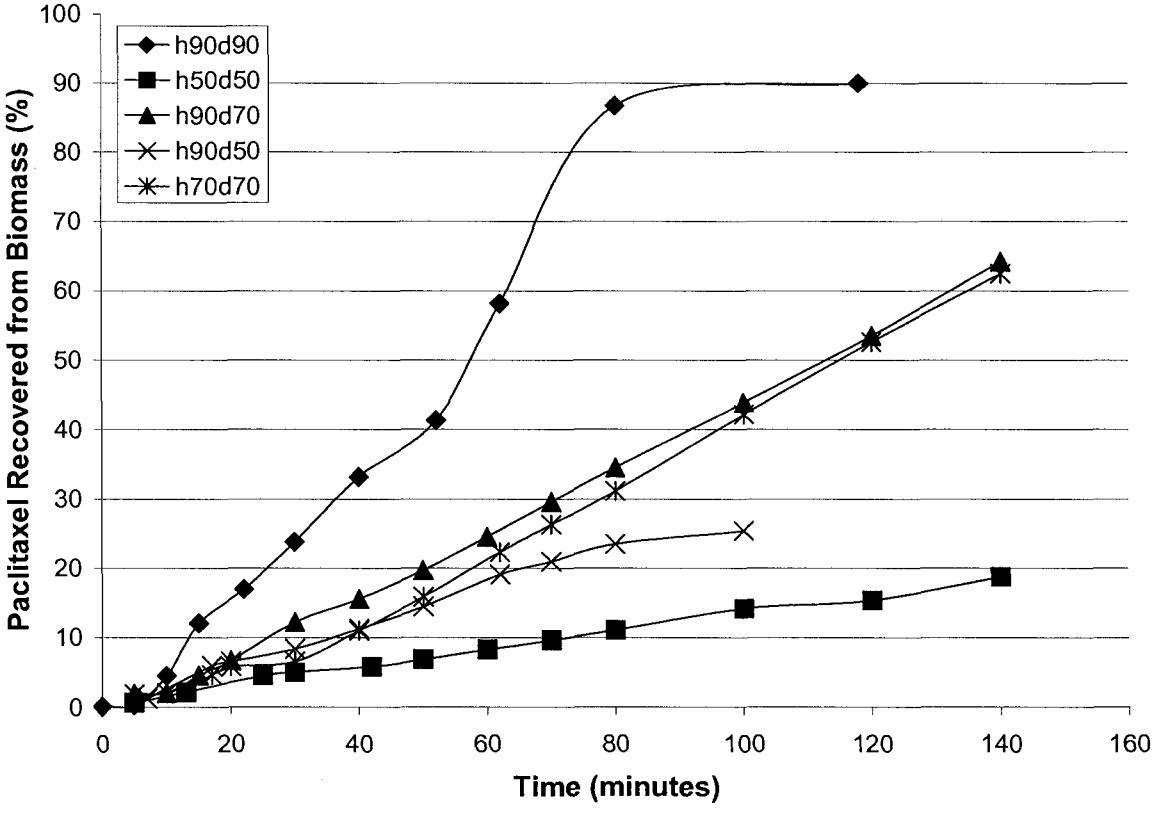

Figure 4. 12: Paclitaxel Recoveries at Different Temperatures Using a Hexane Pre-wash and Dichloromethane Extraction at 45 mL/min. (h: Hexane Pre-Wash Temp, d: Dichloromethane Extraction Temp)

Since dichloromethane has been shown to be a suitable solvent, it is necessary to see whether the hexane pre-wash has any effect on the extraction process.

Compared to the recoveries obtained from the use of dichloromethane only, using a hexane pre-wash resulted in higher recoveries (Figure 4.12). It is observed that a higher temperature hexane pre-wash also resulted in higher recoveries during the dichloromethane extraction step.
When comparing the dichloromethane extraction step with and without a hexane pre-wash, there is a noticeable difference in recovery rates. It can be seen that the use of a hexane pre-wash increases the amount of paclitaxel recovered. This could be a result of the removal of non-polar compounds, which reduces the matrix-binding effect. The effect reduction would then more easily allow the extraction of paclitaxel from the biomass particle. A more thorough investigation may reveal the reason or combination of reasons.

The product purity of the resulting extract can be seen in Figure 4.13 below:

At 90 °C, the purity has doubled, while at lower temperatures, the purity has increased by several times over their original purities. Using a hexane pre-wash at 90 °C and an extraction temperature at 90 °C, the product purity increased by almost twenty times when compared to methanol extracts.

The purities are significant, since that the higher purities can lead to fewer downstream processes. With fewer downstream processes, the overall yield could be improved if the remaining downstream process remained unchanged in terms of efficiency.

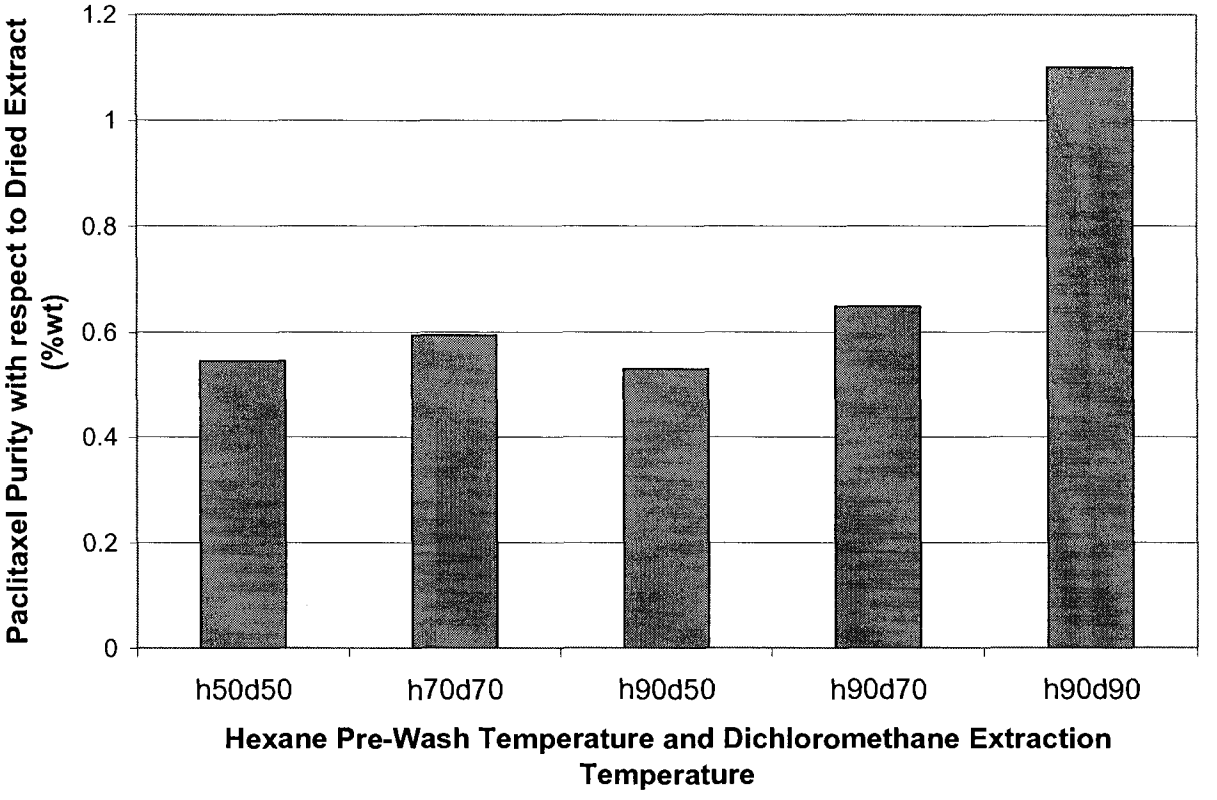

Figure 4. 13: Maximum Paclitaxel Purity Achieved with respect to Solids Extracted Using DPLE at Various Temperatures at 45 mL/min.

Not only is the purity of the product important, but the types of impurities found in the product can have an adverse impact. Since reversed phase chromatography is usually used in purifying paclitaxel in its downstream processes, reduction of non-polar impurities are paramount. The presence of non-polar impurities can impact downstream processes by reducing the life expectation and efficiency of resins when they become bonded.

If a methanol extract chromatograph was examined, the extract contains a large quantity of polar material and very little non-polar material was observed (Figure 4.14). In a dichloromethane extract without a hexane pre-wash, the amount of polar material is significantly reduced, but a

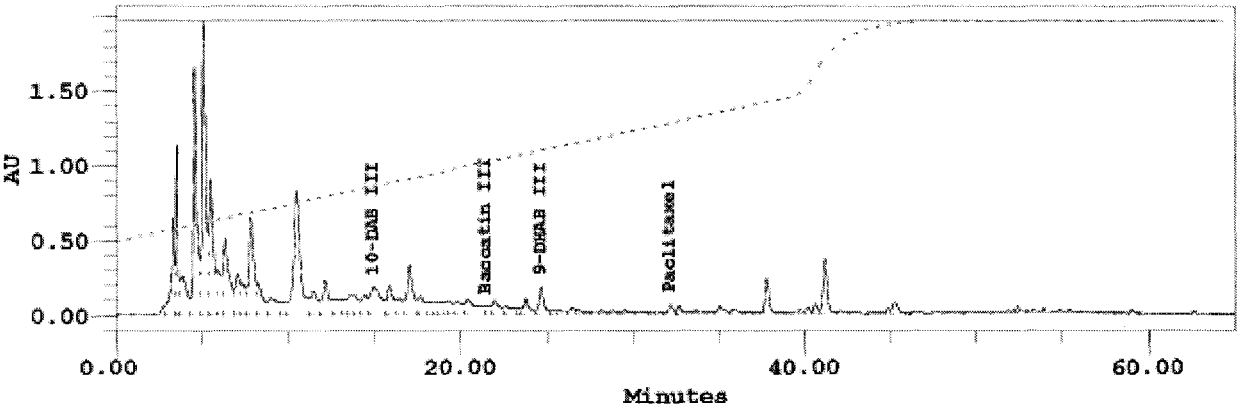

Figure 4. 14: HPLC Chromatograph of a Methanol Extraction of Ground Hemlock at 20 °C.

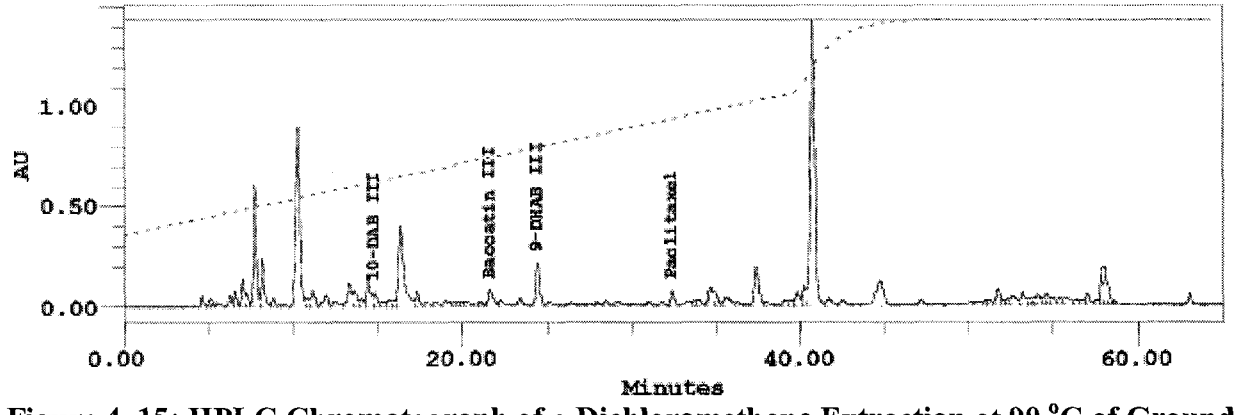

Figure 4. 15: HPLC Chromatograph of a Dichloromethane Extraction at 90 °C of Ground Hemlock.

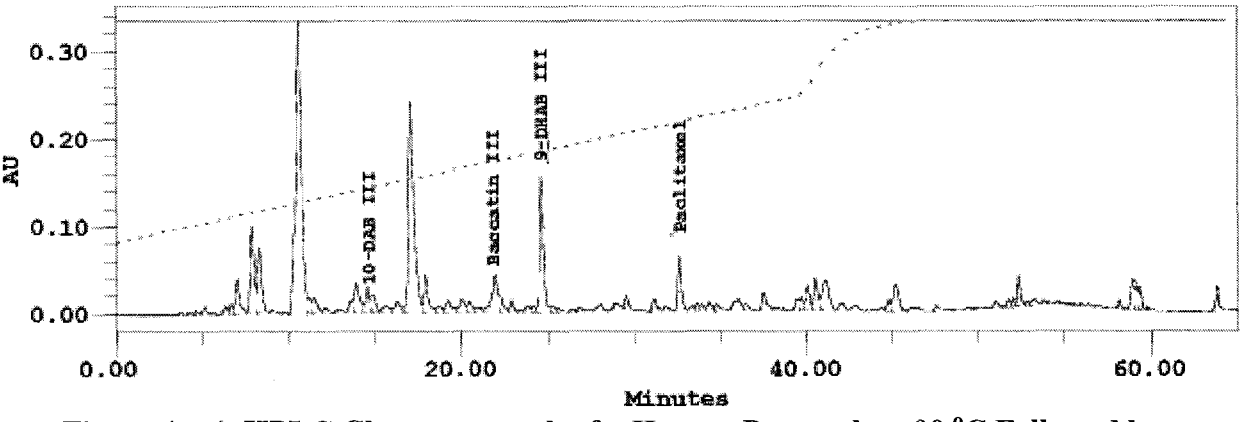

Figure 4. 16: HPLC Chromatograph of a Hexane Pre-wash at 90 °C Followed by a Dichloromethane Extraction at 90 °C.

significant amount of non-polar compounds was also detected (Figure 4.15). However, if a hexane pre-wash was used then followed by a dichloromethane extraction; a product free of polar and non-polar compounds was obtained (Figure 4.16).

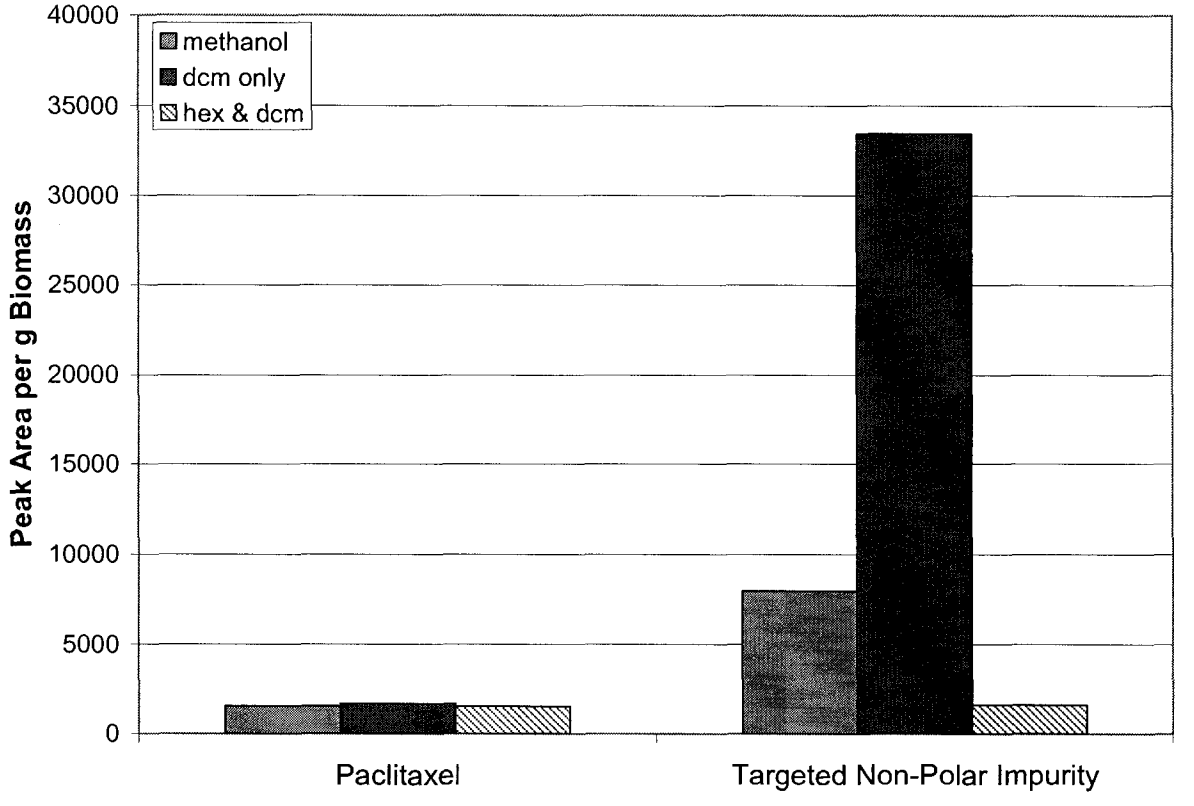

Figure 4. 17: Paclitaxel and Non-Polar Impurity Content in Extracts with Different Solvents.
Methanol was done at 20 °C, the other solvents were used at 90 °C.

When comparing the paclitaxel concentrations and the concentration of a non-polar impurity found at the 41.133 minute elution mark (Figure 4.17), it is observed that the paclitaxel concentrations are similar, but the levels of impurity vary as a direct result of the polarity of the solvent. Although a methanol extraction performed at 90 °C would have been preferable for comparison, the current methanol extract allows comparison with current industry practices. As

well, an extraction at 90 °C without pressurization would not have been feasible since the boiling point of methanol at atmospheric pressure is roughly 60 °C.

The different processes are distinguished by their separation factor. Although the use of a dichloromethane extraction has the disadvantage of the highest amount of the non-polar impurity, it has the benefit of reduced polar impurities that are achieved in methanol extracts. Therefore, the hexane pre-wash should remove the non-polar impurities first. It is observed that the hexane pre-wash has a noticeable effect on the dichloromethane extract. The targeted non-polar impurity present in the extract is lower than the amount present in a methanol extract.

4.1.6 Inline Adsorption

Process integration can be crucial for a variety of reasons. Most importantly, it reduces the number of process steps, which can reduce areas of product loss. As a result of reducing the process steps, it is also possible to reduce the overall processing time. One such integration for DPLE is the addition of an inline adsorption column directly to the extraction column.

In order to benchmark various resins, a standard elution solvent must be used. From previous work (Wang, 2004), the optimum ethanol concentration for extracting paclitaxel was determined to be 60%. Therefore, 60% ethanol/water was used as the elution solvent. Various resins were loaded with paclitaxel extract and eluted (Figure 4.18).

It is observed that silica gel retained the most paclitaxel, followed by HP2MG. Although silica gel appears to retain the most paclitaxel, it also has the least selectivity as shown below (Figure 4.19). The peaks represent the elution times

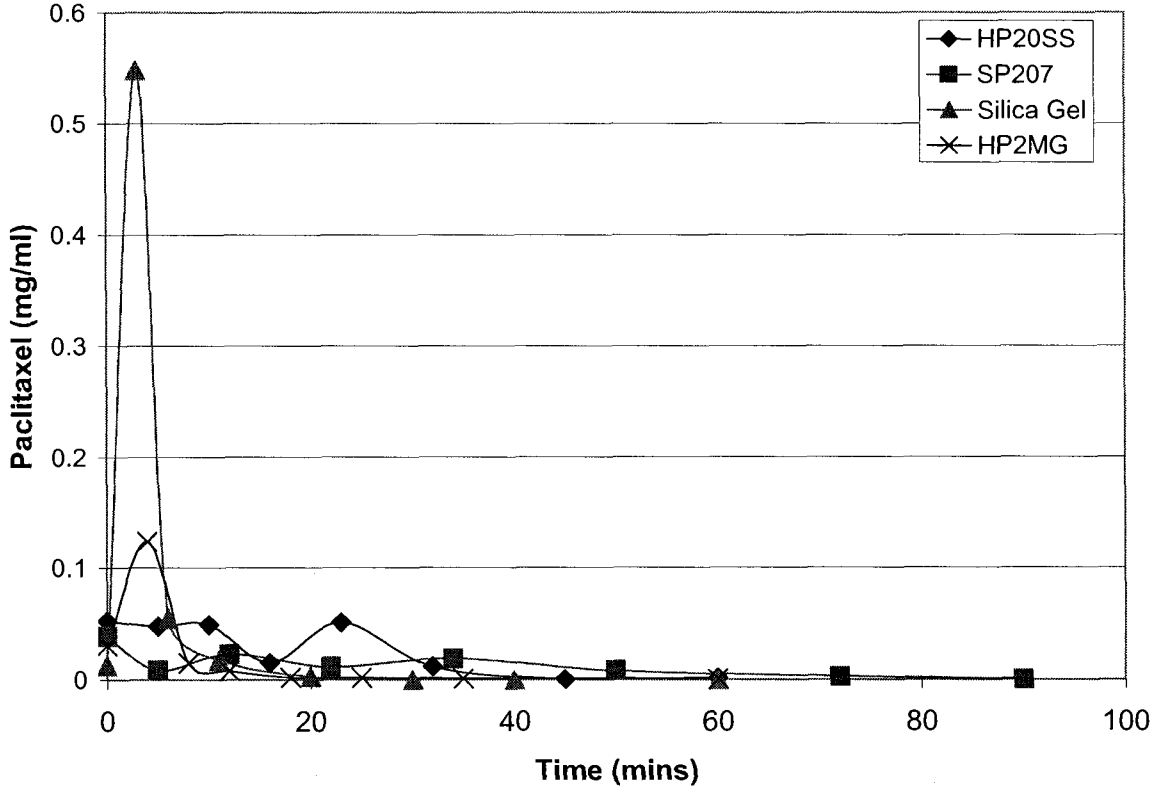

Figure 4. 18: Paclitaxel Concentrations from Different Adsorbents Using 90 °C Hexane Pre-wash and 90 °C Dichloromethane Extraction in DPLE, Followed by 60% Ethanol/Water Elution at 20 °C.

It is observed that the purity of the product while using silica gel was lower than the extract feed. Therefore silica gel is more selective towards the impurities than paclitaxel.

As shown, HP2MG was the best adsorbent when using 60% ethanol/water as eluent, based on product purity. Proceeding on this data, HP2MG was chosen as the adsorbent for use in further

testing. If a wash step was used, it may be possible to further purify the product by selectively removing impurities while leaving the product attached to the adsorbent. After the wash step, the product would then be removed with the normal elution process resulting in an improved

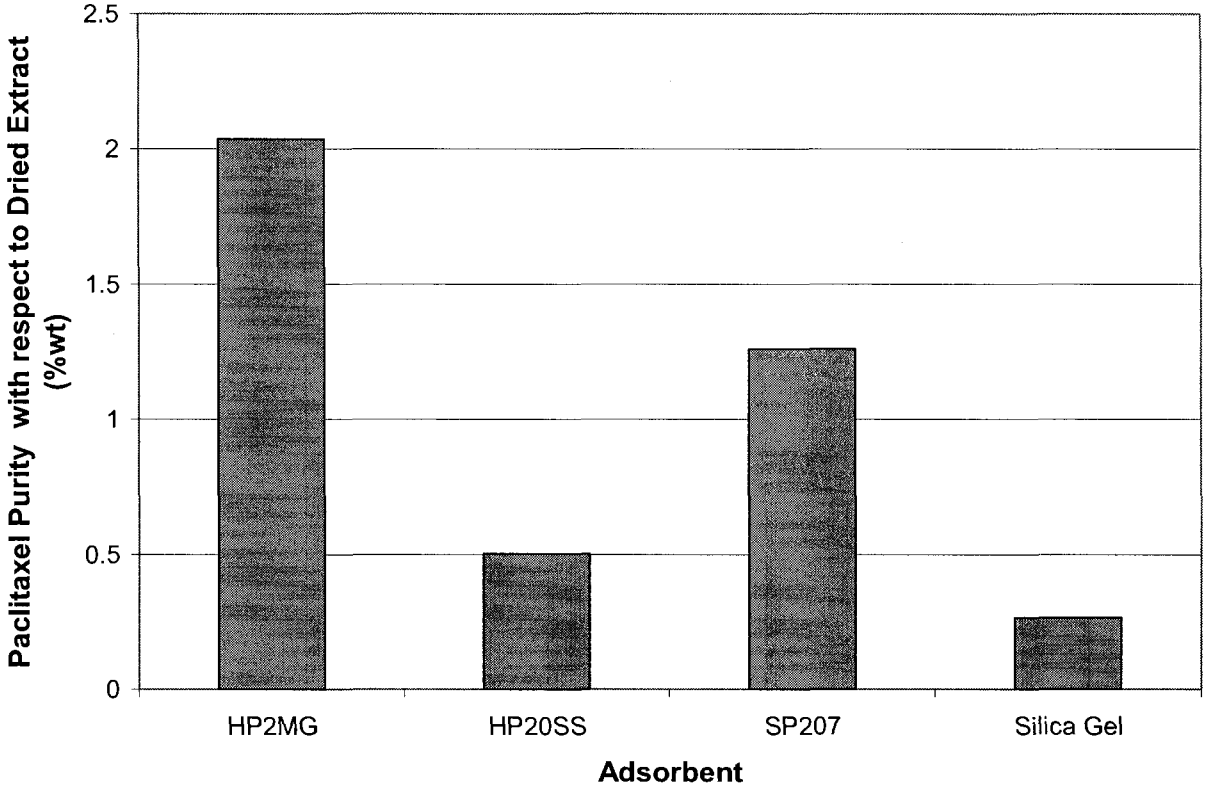

Figure 4. 19: Maximum Paclitaxel Purity with respect to Solids Extracted from Different Adsorbents Using 90 °C Hexane Pre-wash and 90 °C Dichloromethane Extraction Using DPLE and 60 % (v/v) Ethanol/Water Concentrations Used for Elution.

product. Therefore several different elution concentrations were tested with HP2MG resin (Figure 4.20).

It was found that 30% ethanol/water would prove to be the most promising as a wash, since it elutes the least amount of paclitaxel from the concentrations studied. It was also found that 90%

ethanol/water eluted the most paclitaxel from the resin. In order to determine the effectiveness of the elution concentrations, the purities have to be compared to determine that the product is being isolated (Figure 4.21).

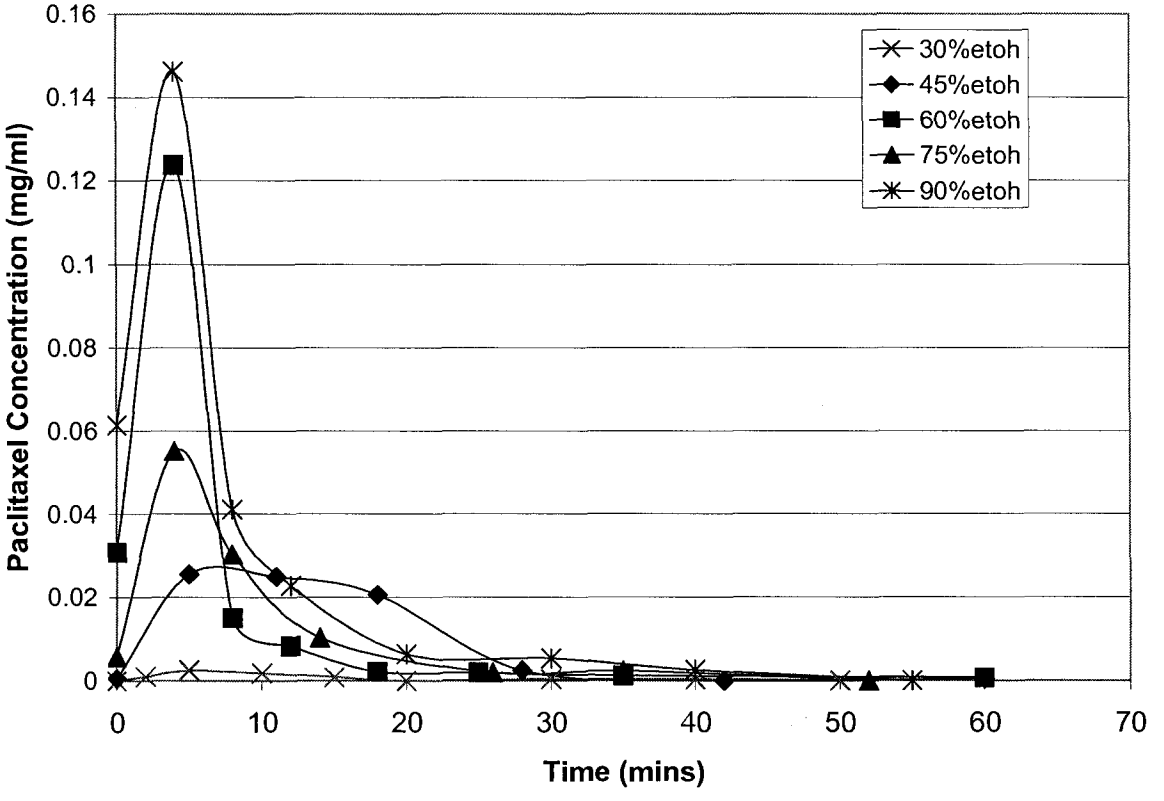

Figure 4. 20: Paclitaxel Concentrations from Eluting HP2MG with Different Concentrations of Ethanol/Water Mixtures.

Since the purity at 30% ethanol/water (v/v) is the lowest, it could be used as a wash concentration as more polar undesired compounds are being removed than at other concentrations. However, there is some paclitaxel being lost to this wash step. This step will have to be closely monitored to ensure that paclitaxel losses are kept to a minimum.

Another result was that the 90% ethanol seems to be able to elute more of the adsorbates from the adsorbent, however, more of the undesirable impurities are also eluted. Therefore, 90% ethanol may be a superior elution solvent, if a wash was used to remove impurities. From the above results, the use of 90% could result in higher purity and higher product recovery.

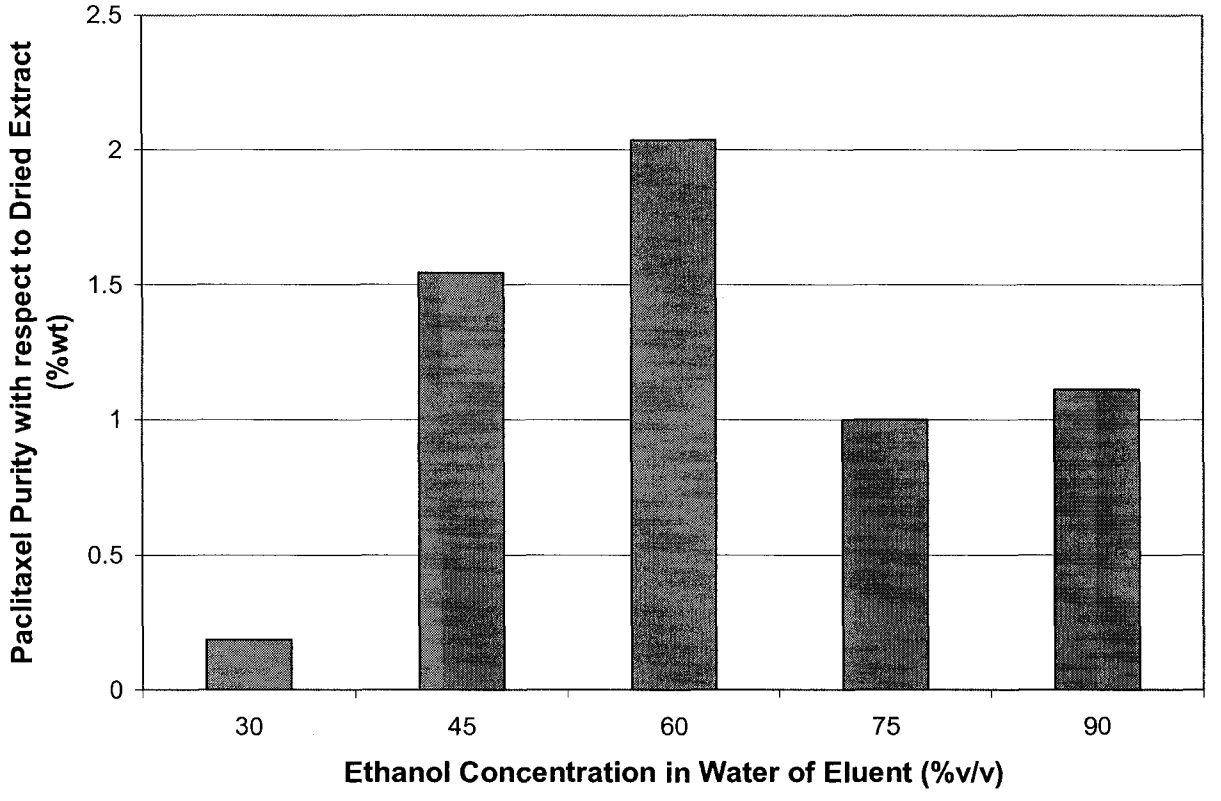

Figure 4. 21: Maximum Paclitaxel Purity Using HP2MG as the adsorbent at Different Ethanol/Water Concentration Elutions.

Although high purity extracts were obtained, the adsorption step resulted in low recoveries (Table 4.1).

It is observed that the recoveries increase as the elution concentration of ethanol increases. This is expected as the phytochemicals have a higher affinity for ethanol than water. However, as

mentioned earlier, the higher concentration was also less selective towards paclitaxel and removed impurities from the resin as well. It is also noted that the silica gel has a higher recovery than the adsorbents at similar elution concentrations as expected from Figure 4.18. Although the recoveries are similar between HP2MG, SP207, and HP20SS, Figure 4.18 shows that the retention on the resin vary greatly, since HP20SS elutes at a low constant rate, while HP2MG elutes very quickly, in comparison. From a production standpoint, quick elutions would be preferable to the constant elution since normally higher concentrations are achieved and shorter process times, as long as the selectivities were comparable.

Table 4. 1: Paclitaxel Recoveries from Adsorption Step

Type of resin and Ethanol/Water Elution Concentration	% Recovery
HP2MG – 30	1.35
HP2MG – 45	21.66
HP2MG – 60	22.86
HP2MG – 75	20.14
HP2MG – 90	33.01
HP20SS – 60	17.32
SP207 - 60	21.71
Silica Gel - 60	65.16

During the adsorption testing, there were indications that the size of the adsorption column was insufficient to handle the flow directly from the extractor. There were significant amounts of products being detected during the loading; it would indicate that breakthrough was occurring. As industry standards are in the 90-95% recovery range [Ladisch, 2001], therefore with a

properly sized column, the purity of the extract could be higher than our results, as well as improved recoveries.

To avoid this problem in the future, especially during scale-up, one of two following options will have to be taken. Either the column will have to be significantly larger or the extract will have to go into a holding tank so that the adsorption column can be fed at a different rate than the extraction column. Both options have their advantages and disadvantages.

Enlarging the column would allow a suitable flow rate and loading through the column. Unfortunately, this would also require a larger capital investment with more resin and a larger column. This method would also have the added advantage of possibly reducing processing time as the column would become an integrated process in addition to the extraction.

Using a holding tank, would allow for adjustments for smaller flow rates, which in turn would allow for a smaller adsorption column. However, this in turn would most likely require an additional pump to feed the adsorption column as well as increase processing time for the same volume of extract. This option does not offer any advantages over what is currently done.

4.1.7 Comparison from bench scale to pilot scale

Bench scale testing (Wang, 2004) shows that the efficiency of the extraction process can achieve close to 100% recovery. However, to achieve that yield, the particle size of the biomass was

smaller than 100 mesh in diameter. The particle size is very important as it directly affects the distance of the diffusion path.

In all the testing in this work biomass of 20 mesh was used for extraction since it is the standard size that is produced by Chatham Biotec Ltd and the standard material currently used in industry. This allowed for a reasonable comparison of the DPLE technology compared to the standard methanol extraction process used in industry. Smaller biomass particle sizes were examined to study the effect of biomass particle size on recoveries (Figure 4.22).

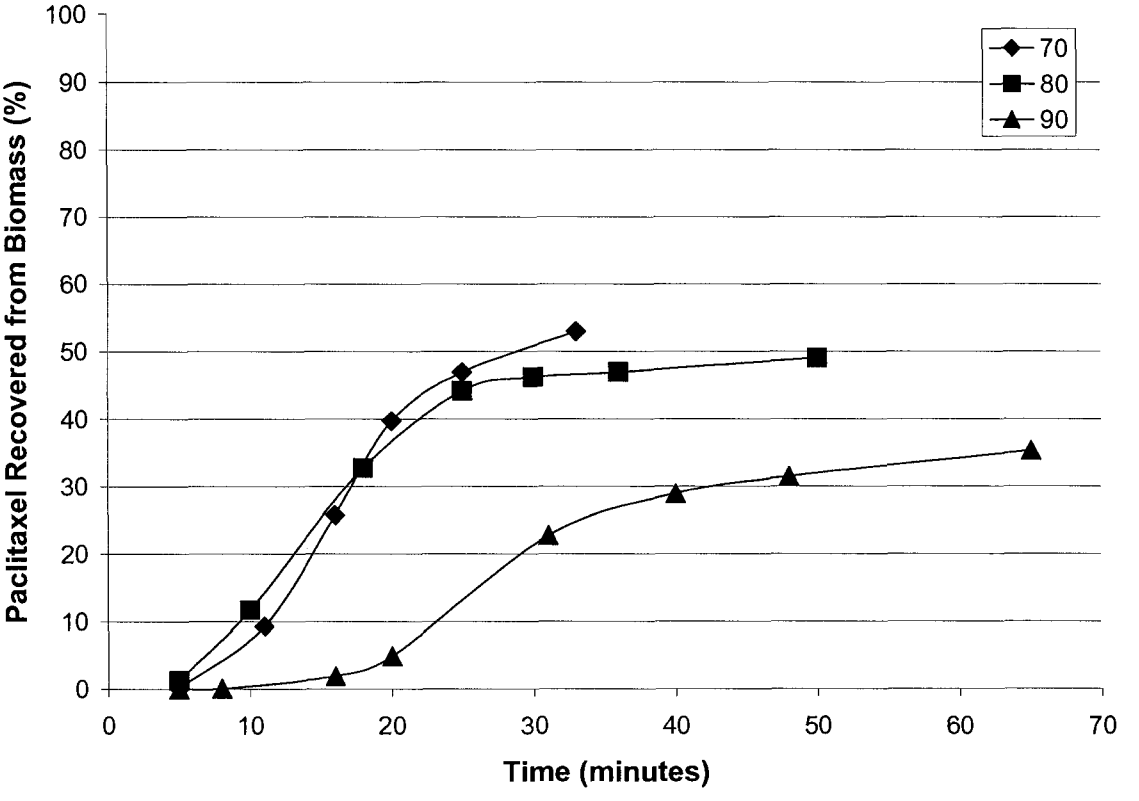

Figure 4. 22: Paclitaxel Recovery Using Small Particle Biomass (< 100 mesh) in DPLE Procedure with Hexane Pre-wash and Dichloromethane Extraction.

Higher yields were expected with the use of smaller particle sizes, since the penetration depth of the solvent should have allowed more of the material to have been contacted as opposed to the larger particle size. However, visual observations of the biomass after the extraction process would suggest that channelling occurred. The top of the biomass showed depressions where the solvent could have passed, which reduces solvent contact with the biomass. As higher temperatures were used, the mass transfer of the products should be more easily facilitated and higher yields would be expected.

Unfortunately, the use of the smaller particle sizes will require further design. However, the results obtained, prove that DPLE can be a competitive technology in industry even using the larger particle sizes. Using standard methanol extraction technology produces a high efficiency extract that has a very low level purity of less than 0.1% of all the solids extracted. In industry, a separate unit process would then be used to purify the extract to around 2% with efficiencies around 90-95%. Using the DPLE procedure we are able to obtain greater than 1% purity with a recovery of 90%, using the same sized particles. This is an improvement over the standard method as it reduces the number of steps involved. The recovery of the product will become more efficient would be expected from the use of smaller particle sizes since the diffusion path will have been reduced.

4.2 Betulinic Acid Extraction

The following section examines the results obtained pertaining to the extraction of betulinic acid from birch bark under batch experiments under CSE with agitation.

4.2.1 HPLC Analysis

The isolation of betulinic acid poses a specific problem due to betulin being similarly structured. The betulin will normally co-elute with betulinic acid resulting in overlapping peaks. In order to determine a proper analytical methodology, a methanol extract of birch bark was analyzed at UV wavelengths of 210, 230 and 559 nm. Both isocratic and gradient methods were studied. Separations were also tested on different types of columns. Although several solvent systems are mentioned in literature, acetonitrile and water were chosen since they were readily available from analyses on paclitaxel extracts. The best results, without any overlap of the product peaks, were obtained using the gradient in Table 3.1 at a UV wavelength of 210 nm and a C18 column.

4.2.2 Batch Extraction

To determine the most suitable solvent, several different solvents were used under batch extraction (Figure 4.23 and Figure 4.24). The amount of betulin was determined based on the HPLC chromatograms from peak concentration, as betulin should have a concentration greater than 10 times that of betulinic acid (Zhao et al., 2007).

Methanol and ethanol were the most proficient at extracting betulinic acid. Although chloroform was able to extract betulin well, it was less efficient with betulinic acid. Chloroform is also a little more difficult to handle due to its high volatility. Therefore methanol and ethanol were selected as solvents to study the effect of solvent concentration on extraction (Figure 4.25 and Figure 4.26).

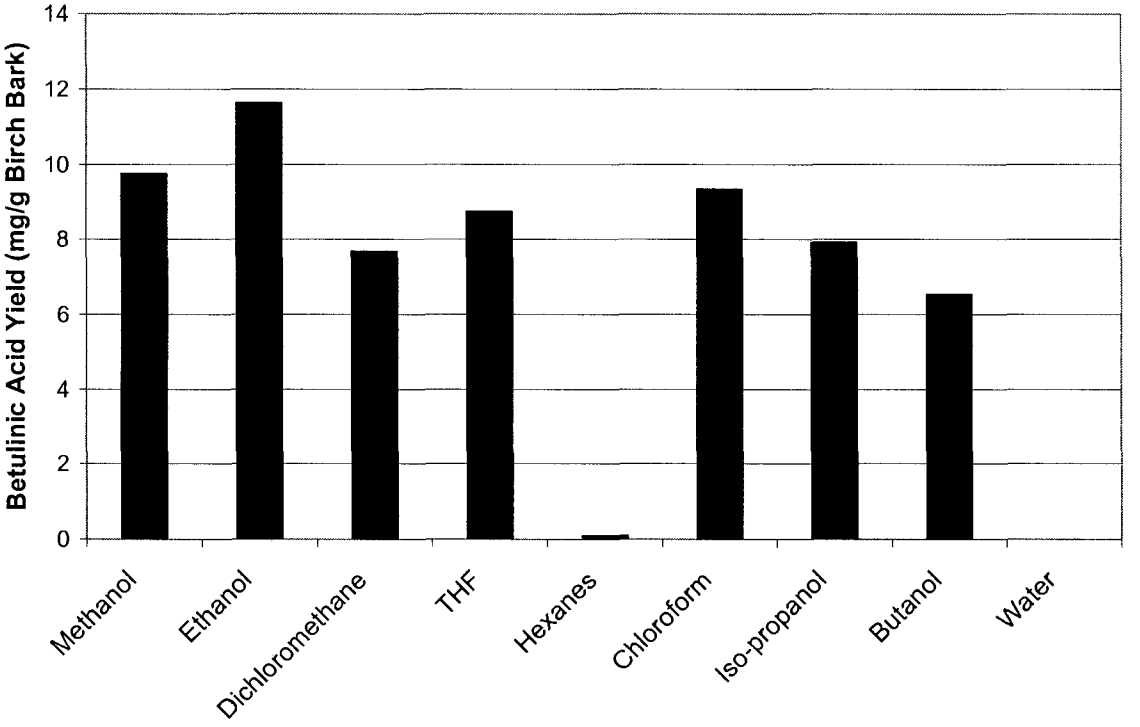

Figure 4. 23: Betulinic Acid Extracted Using Variouis Solvents at 20 °C.

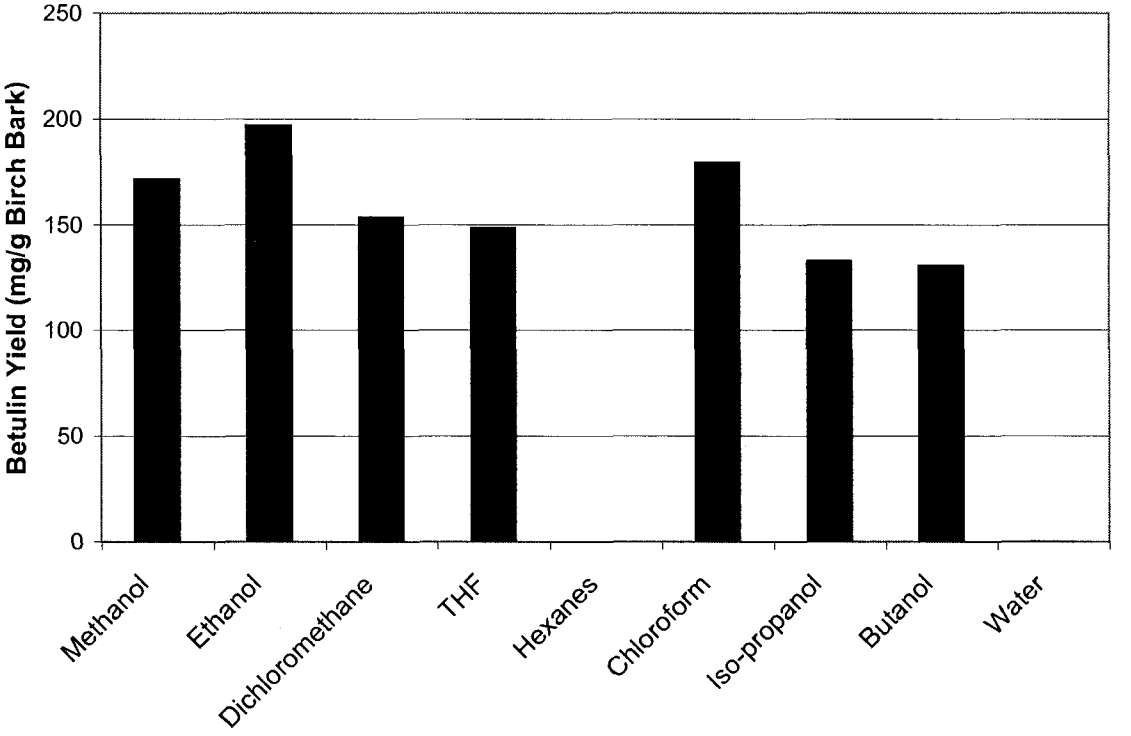

Figure 4. 24: Betulin Extracted Using Various Solvents at 20 °C.

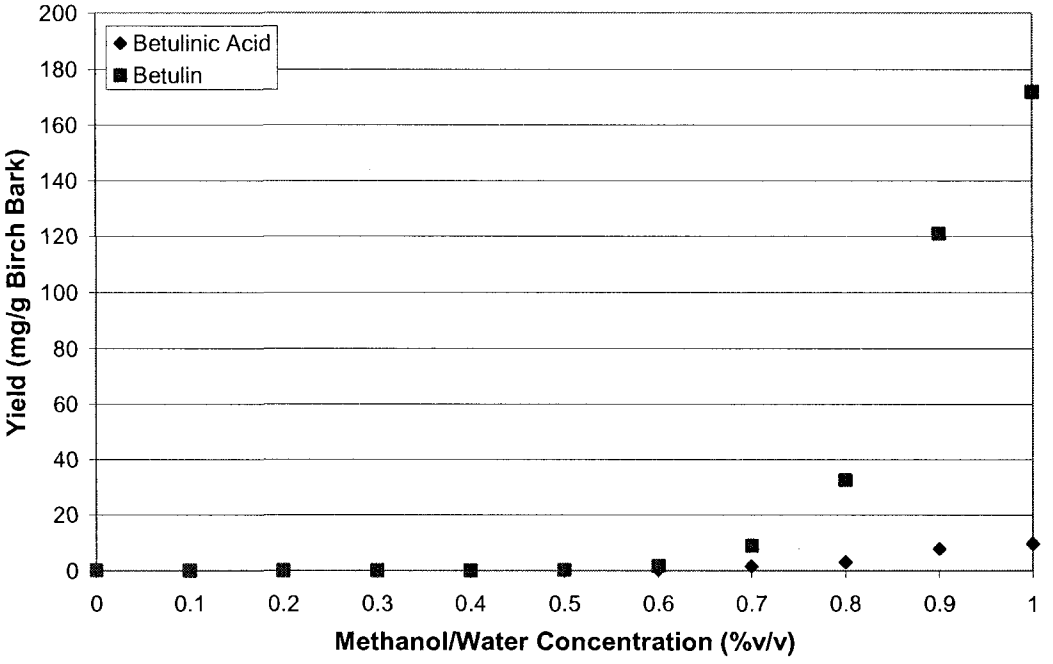

Figure 4. 25: Betulin and Betulinic Acid Extracted at Different Methanol/Water Concentrations at 20 °C.

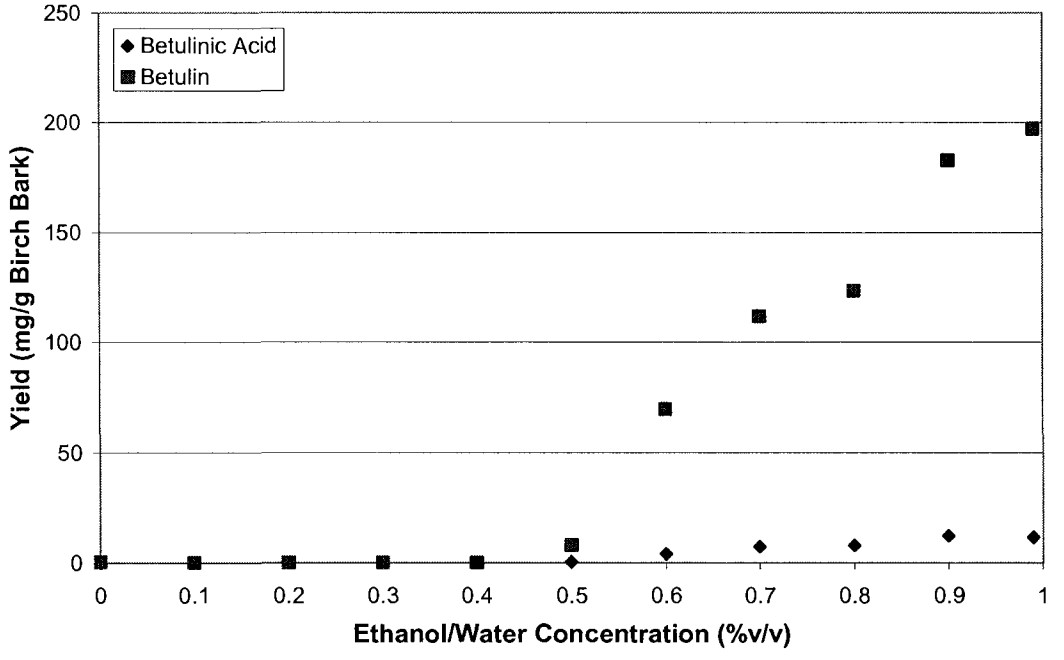

Figure 4. 26: Betulin and Betulinic Acid Extracted at Different Ethanol/Water Concentrations at 20 °C.

It was observed that the higher the concentration of ethanol or methanol resulted in a higher yield of betulin and betulinic acid. Therefore, in future experiments, only pure solvents will be used.

The extraction efficiency was studied in order to determine the total amount of betulinic acid present in the biomass (Figure 4.27).

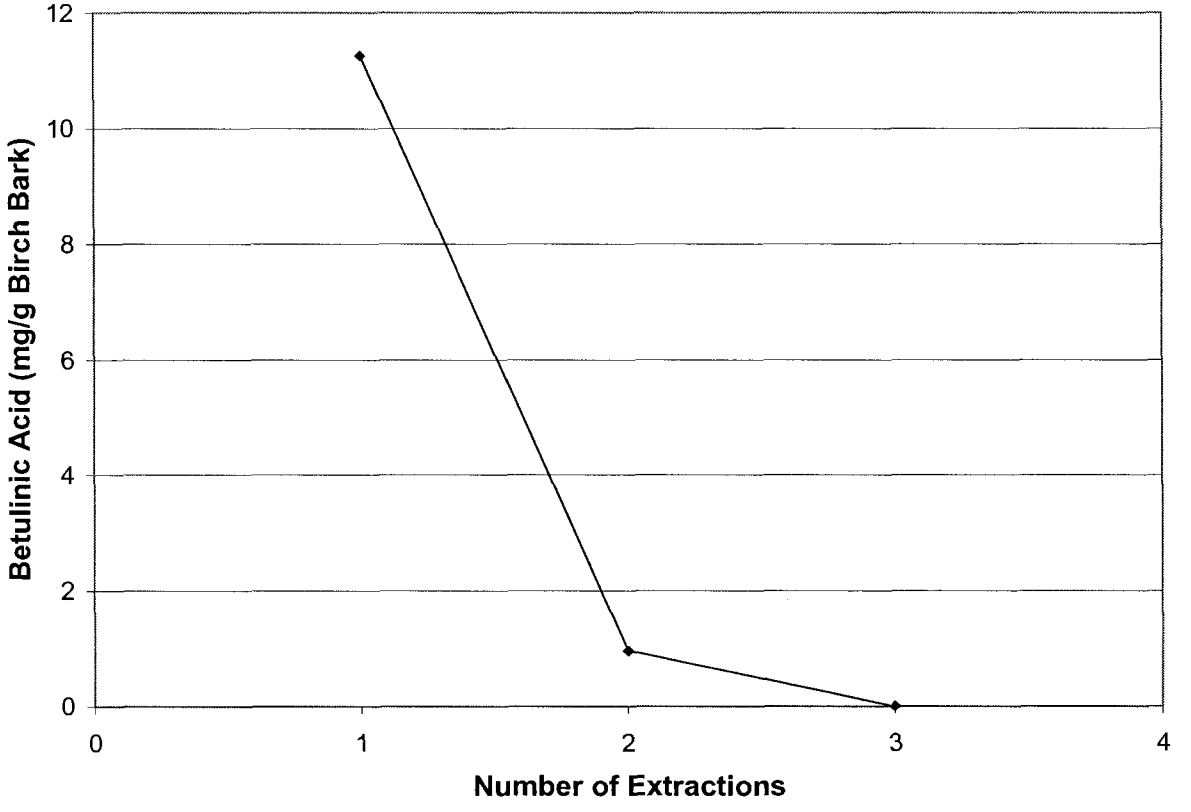

Figure 4. 27: Methanol Soxhlet Extractions of White Birch Bark for 4 hours per Extraction.

The initial extraction was able to remove 92.2 % of the betulinic acid. Subsequent extractions of the same biomass resulted in very low quantities of residual products, with no product extracted on the third extraction.

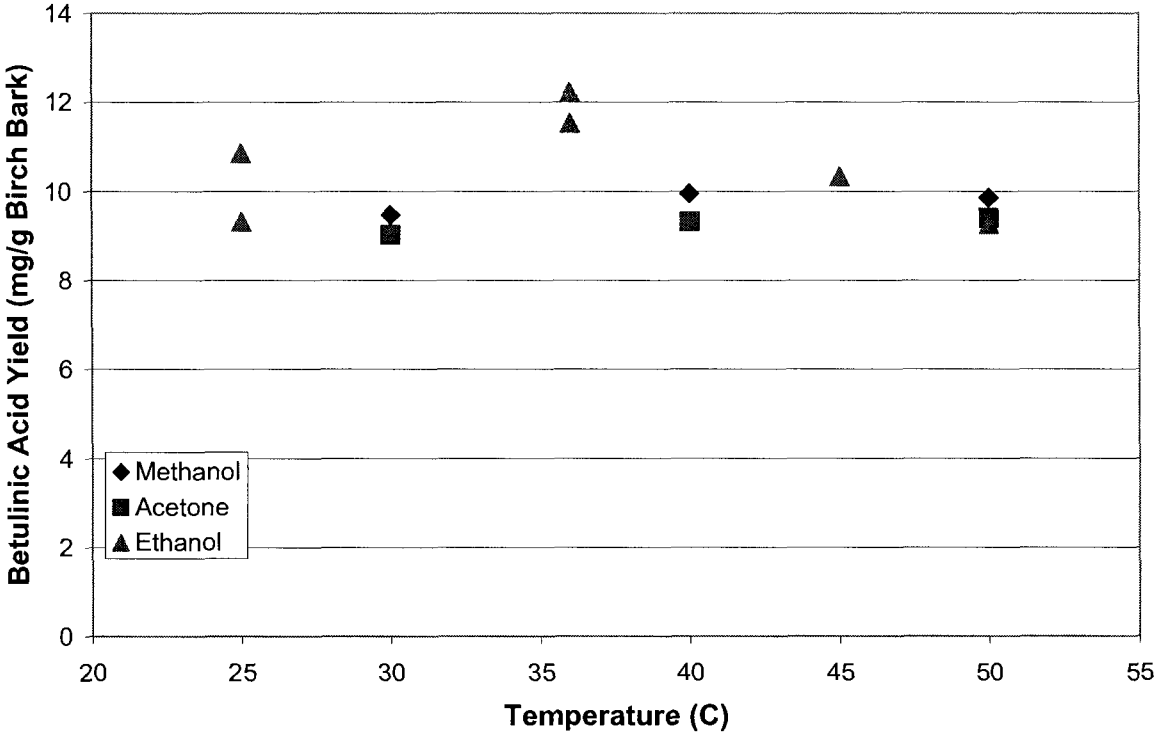

Figure 4. 28: Betulinic Acid Yield at Various Temperatures Using a Batch Extraction.

The temperature effect on extraction efficiency was then studied (Figure 4.28 and Figure 4.29). Temperature does not seem to affect the extraction capability of the selected solvents for either betulin or betulinic acid (Figure 4.28 and Figure 4.29). Furthermore, there is no apparent trend of the extraction efficiency, indicating that higher temperatures may not increase yields.

This effect is expected since the alcohols do not have the same mass transfer hindrances found when using dichloromethane for extraction purposes. Since methanol and ethanol are both polar solvents, they would not have difficulty penetrating the polar biomass particle, facilitating mass transfer of the solutes.

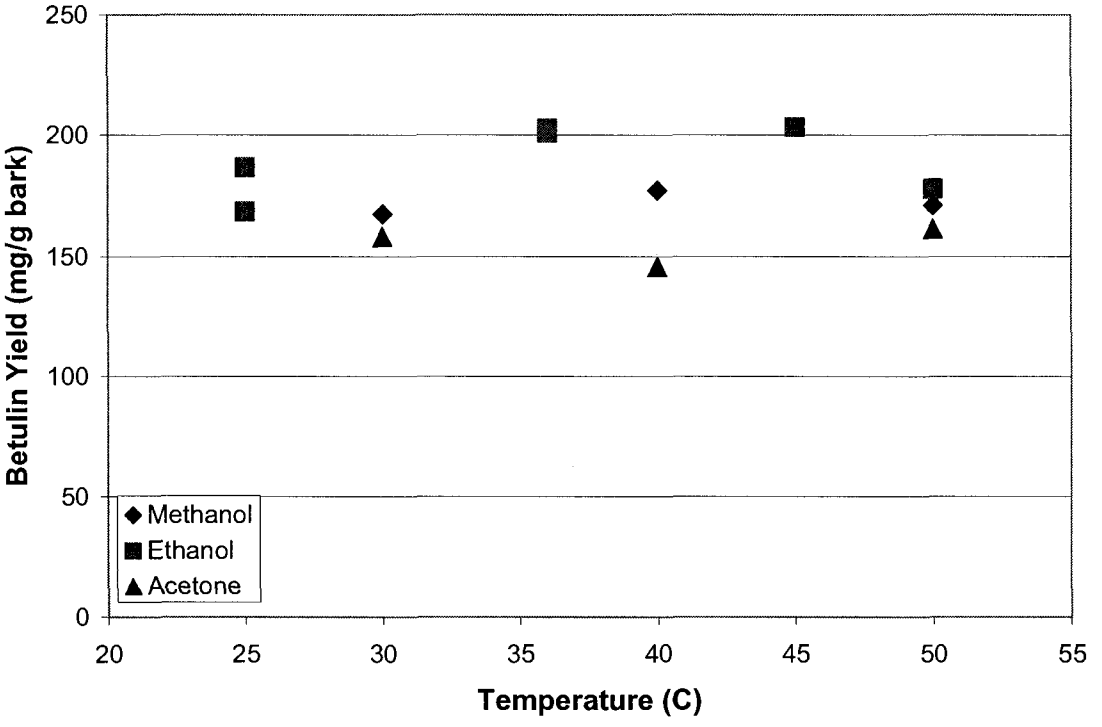

Figure 4. 29: Betulin Yield at Various Temperatures Using a Batch Extraction.

5.0 Conclusions and Recommendations

Dynamic Pressurized Liquid Extraction (DPLE) using dichloromethane with and without a hexane pre-wash was compared with methanol extraction for the recovery of taxanes from *Taxus Canadensis*. The effect of extraction temperatures was studied. Integrated adsorption, with different adsorbents and elutions, was studied for selectivity and purity improvement.

Extractions for betulinic acid were performed on birch bark under batch conditions to determine the optimum solvent and temperature conditions.

5.1 Conclusions

1. Chlorophyll and lipids can be isolated during hexane pre-wash step for use as a by-product.
2. A hexane pre-wash appeared to have improved the paclitaxel recovery with dichloromethane.
3. DPLE using a 90 °C hexane pre-wash and 90 °C dichloromethane extraction was able to recover 90% of the total paclitaxel found in the biomass with 1.1% wt purity.
4. A target non-polar impurity found in methanol extraction of taxanes is reduced to 20% of original amount when using DPLE.
5. An inline adsorption column was able to double product purity with breakthrough. Eliminating breakthrough would significantly increase product recovery.

6. Ethanol was determined to be the best solvent for extracting betulinic acid and betulin from birch bark at room temperature.
7. Pure ethanol and pure methanol were better at extracting betulin and betulinic acid than their aqueous solutions.
8. Temperature does not seem to affect the extraction efficiency of betulin and betulinic acid in either methanol, ethanol or acetone.
9. An analytical HPLC method for quantifying betulin and betulinic acid was found that could isolate the betulin and betulinic acid peaks.

5.2 Recommendations

1. The use of a better distribution header should help alleviate channelling problems in the future, such as a spray nozzle head. A change in packing procedure, such as pre-soaking the biomass into slurry for loading into the extractor, may alleviate the channelling issue as well.
2. An extended extraction of using a dichloromethane extraction in DPLE mode without a hexane pre-wash could be carried out at the lower temperatures to investigate the mass transfer effects of the exponential product recovery.
3. A repeat of the 80 °C dichloromethane only extraction should be duplicated to verify reproducibility of the result.
4. Temperatures between 80 and 90 °C should be investigated for the hexane pre-wash step to investigate a possible take-off point and possible trends in solute removal.
5. An investigation of the inline adsorption column should be studied further. The use of a wider adsorption column should alleviate the breakthrough problems encountered in this work. Although a variety of resins (hydrophilic, hydrophobic, ion exchange) were used, there could be more specialized resins more suitable in this operation.
6. After the distribution problems have been resolved, the use of smaller mesh biomass should be investigated further, in order to see if there is a significant difference in recoveries depending on particle size.
7. Quicker analytical techniques could greatly aid in the verification of reproducibility, since there is a large number of samples per run and analytical times of over an hour per sample in the work performed.

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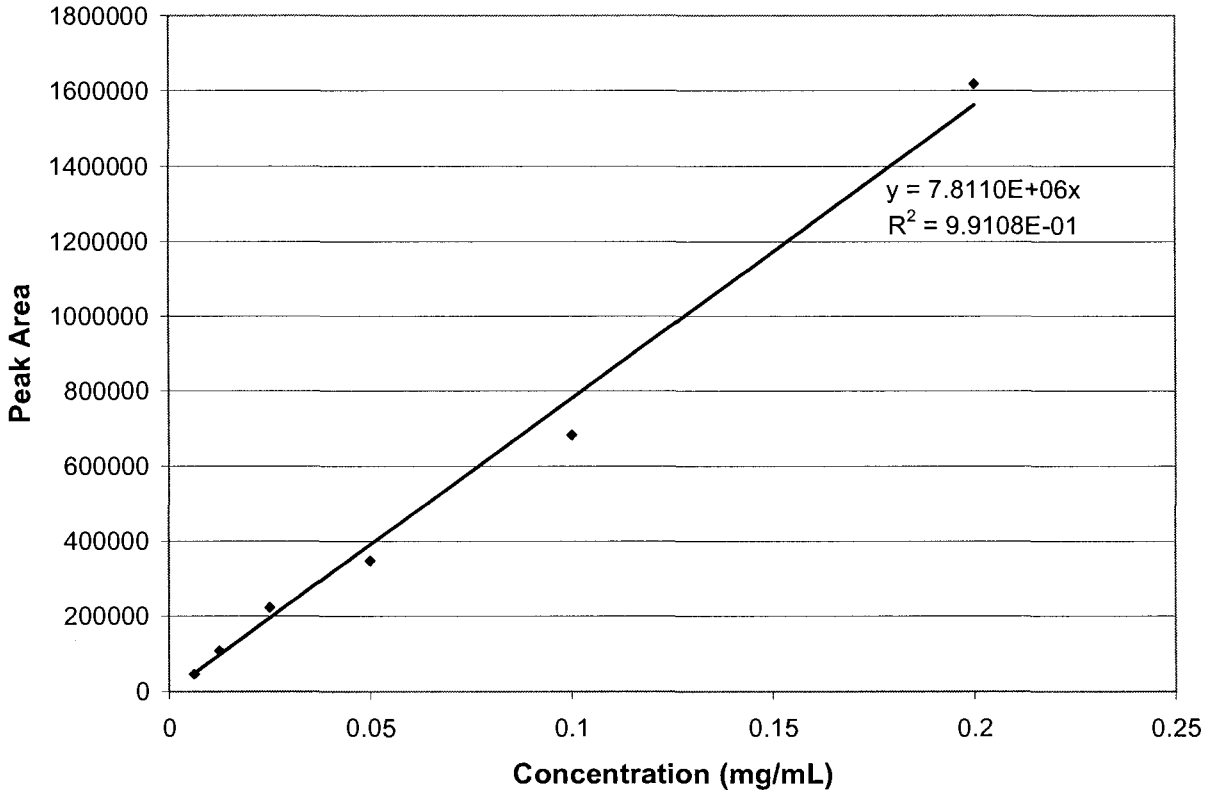
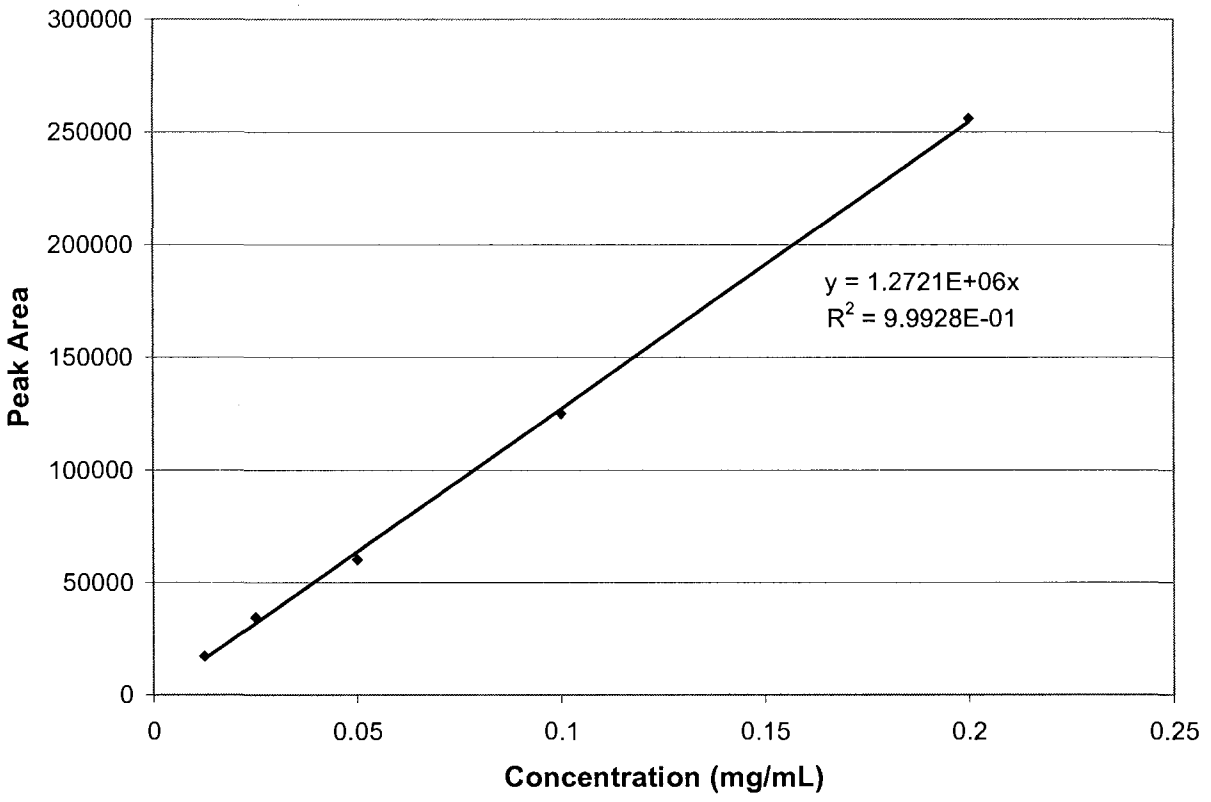
Appendix A – Standard Calibration Curves



Paclitaxel Calibration Curve for HPLC.



Betulinic Acid Calibration Curve for HPLC.