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FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

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M. A. Sc. (Chemical Engineering)
GRADE - DEGREE

Department of Chemical Engineering
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TITRE DE LA THÈSE - TITLE OF THE THESIS

Study in Integrated Bioseparation Technologies for Mass Taxane Production

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DEAN OF THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

STUDY ON INTEGRATED BIOSEPARATION TECHNOLOGIES FOR MASS TAXANE PRODUCTION

Yuheng Wang

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of

Master of Applied Science in Chemical Engineering

Department of Chemical Engineering, University of Ottawa

December 2004

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Your file Votre référence
ISBN: 0-494-01635-3
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ISBN: 0-494-01635-3

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Abstract

In recent years, natural bioactive compounds have found ever increasing applications as pharmaceuticals, nutraceuticals and dermaceuticals. As these compounds are usually characterized with both complicated structures and delicate thermodynamic stability, it is usually not economical to obtain them through direct chemical synthesis in commercial production. Therefore, cost-effective separation and purification technologies under relatively mild conditions are crucial for the efficient utilization of the biomass resources in the mass production of affordable products.

In this work, the principles of chemical engineering, plant biology, and phytochemistry were successfully applied to the separation of bioactive compounds from plant materials, taking the taxane extraction and purification as an example. A new model, the Solute Distribution Model, was proposed and used to guide the experimental work. Based on the model, two approaches, the Dual-Solvent Extraction approach and the Extraction-Adsorption approach, were developed in order to enhance both selectivity and recovery rate in bioseparation. Separation from *Taxus Canadensis* of all valuable products, including paclitaxel, 9-DHB III, 10-DAB III, Baccatin III, and other possible by-products, was studied experimentally. Dynamic Pressurized Liquid Extraction, a new extraction technique in the area of natural product mass production, was applied, along with conventional solvent extraction means.

Based on the results of this work, the Solute Distribution Model, the Dual-Solvent Extraction approach and the Extraction-Adsorption approach were found to be effective

tools for the development of cost-efficient and environmentally friendly technologies in large-scale production of valuable products from plant materials. One novel taxane extraction and purification process, integrated as DPLE-SPE-Chromatography, was invented and proofed. Certain interesting experimental phenomena, particular to the system examined, were observed and explained with fundamental principles. Compared to the reported results of previous studies, the newly developed process could potentially offer higher product yields, milder operating conditions, and lower negative environmental impacts and, therefore, better economics.

Résumé

Ces dernières années, les composés bioactifs naturels ont trouvé des applications toujours croissantes comme pharmaceutiques, nutraceutiques et dermaceutiques. Étant donné que ces composés sont habituellement caractérisés par des structures compliquées et une stabilité thermodynamique fragile, il n'est habituellement pas économique de les obtenir par synthèse chimique directe pour la production commerciale. Par conséquent, la rentabilité des technologies de séparation et de purification dans des conditions relativement modérées est cruciale pour l'utilisation efficace des ressources de biomasse dans la production en série de produits abordables.

Dans ce travail, les principes du génie chimique, la biologie d'usine, et la phytochimie ont été appliqués avec succès à la séparation des composés bioactifs à partir de matières végétales tout en prenant l'extraction et la purification du taxane comme exemple. Un nouveau modèle, le modèle de distribution des corps dissous, a été proposé et employé pour guider le travail expérimental. Basé sur ce modèle, deux approches, l'approche d'extraction par duo de solvant et l'approche par extraction/adsorption, ont été développées afin d'augmenter la sélectivité et le taux de production dans le bioséparation. La séparation de tous les produits valables du *Taxus Canadensis*, y compris le paclitaxel, 9-DHB III, 10-DAB III, Baccatin III, et d'autres sous-produits possibles, a été étudiée expérimentalement. Une nouvelle technique d'extraction dans le secteur de la production en série de produit naturel, l'extraction liquide pressurisée dynamique, a été appliquée et comparée à des moyens conventionnels d'extraction par solvants.

Basé sur les résultats de ce travail, le modèle de distribution des corps dissous, l'approche d'extraction par duo de solvant et l'approche par extraction/adsorption ce sont avérés des outils efficaces pour le développement des technologies rentables et amicales pour l'environnement dans la production à grande échelle des produits valables provenant de matières végétales. Un nouveau processus d'extraction et de purification du taxane, caractérisé par la chromatographie DPLE-SPE, a été inventé et validé. Certains phénomènes expérimentaux, spécifiques au système examiné, ont été observés et expliqués à l'aide de principes fondamentaux. Comparé aux résultats rapportés par des études antérieures, le processus nouvellement développé pourrait potentiellement offrir un plus au taux de production, des conditions de fonctionnement plus douces, abaisser les incidences négatives sur l'environnement et, par conséquence, augmenter la rentabilité.

Acknowledgements

I would like to thank Dr. J. Zhang and Dr. Z. Duvnjak for giving me the opportunity to study in the exciting research area of bioseparation further after my over 10 years' professional practise. I would like thank them for their guidance, day to day supervision, motivation, encouragement, friendship and financial support.

My thanks go to our excellent technical support team members, especially Mr. L. Tremblay. I also would like to thank other faculty members and staffs in Department of Chemical Engineering, University of Ottawa. I would like to thank all the faculty members and staffs in Department of Chemical Engineering, University of New Brunswick as well.

I would like to express my deep love and appreciation to my parents, who brought me up, encouraged me to go to high school and financed my education while they lived in a poor life. This study, which is regarding anticancer drugs, is also inspired with the braveness of my father, who has been fighting with cancer for over 10 years and can not afford the expensive medication yet.

I also would like to express my deep love and appreciation to my wife, Feng Tian, and my son, Kevin Wang, for their support and apprehension while I worked in the lab after hours. My study is also inspired with their deep love to me and their dream of a better life that we are deserved.

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Abbreviation

Abbreviation	Meaning
API	Active Pharmaceutical Ingredient
ASE	Accelerated Solvent Extraction
cGMP	current Good Manufacturing Practice
CSE	Conventional Solvent Extraction
DPLE	Dynamic Pressurized Liquid Extraction
FDA	Food and Drug Administration
GMP	Good Manufacturing Practice
HPLC	High Performance Liquid Chromatography
LLE	Liquid-Liquid Extraction
NP-SPE	Normal Phase Solid Phase Extraction
PLE	Pressurized Liquid Extraction
RP-SPE	Reverse Phase Solid Phase Extraction
SDM	Solute Distribution Model
SFE	Supercritical Fluid Extraction
SPE	Solid Phase Extraction

Chapter I: Introduction

1.1. Pharmaceutical Aspect of Natural Products

Natural products are organisms or secondary metabolites generated by these organisms in response to external stimuli. These organisms range widely in size and diversity. Among natural products, animals and plants have been the main source of food, cloth and shelter for human beings. Even human bodies are natural products indeed. These natural products play an indispensable role in human health care as pharmaceuticals, nutraceuticals and dermaceuticals.

In addition to one of the main sources of food, cloth and shelter, plants have long been a source of medicines as well. Herbal medicine have been used for over thousands years in countries such as China and India (Cragg et al., 1998). Nowadays, eighty percent of the world's population relies on medicinal plants for their primary health care (Schuster, 2001). Approximately sixty percent of them rely entirely on plants for medication (Farnsworth, 1994).

Over the past two decades, interests in medicinal plants in the world have grown enormously and at all levels, from the use of herbal products as natural cosmetics and for self-medication by the general public to the scientific investigation of plants for their biological effects in human beings (Editorial of the Lancet, 1995). The increasing numbers of natural health products on the shelves of supermarkets provide a solid proof of increasing interest for using natural products in North America and elsewhere. In

Canada, new Natural Health Products Regulations were published and came into force on January 1st, 2004 (Canada Gazette, 2003).

Among these natural products, plant extracts and phytochemicals are far more important than others. Plant extracts show promising results to some chronic diseases which conventional medicine is perceived to offer little therapeutic success. Examples commonly seen are that the traditional Chinese medicines sometimes work better for some chronic diseases than western medicines. Most traditional Chinese medicines are usually prepared by extraction with boiling water or cooking. Chinese medicines now are expected to be better and more conveniently consumed through its modernizations. Another example is ginkgolides from *Ginkgo biloba* with specific platelet activating factor antagonist activity. Standardized extract of *ginkgo* leaves is one of the most frequently prescribed medicines in Germany and is taken to alleviate cerebral ischemia. Plant extracts have yielded active constituents with unique activity as well. Examples are vincristine and vinblastine derived from *Catharanthus roseus*, aryl-lignam etoposide from *Podophyllum* species, and the paclitaxel from *Taxus brevifolia* and *Taxus baccata* (Editorial of the Lancet, 1995).

Other natural products are also promising sources of medication for human beings. For example, edible and medicinal mushrooms not only can convert the huge lignocellulosic biomass waste into human food, but also can produce notable mycopharmaceuticals, myconutriceuticals and mycocosmeceuticals (Wasser, 2002). Its health potential is enormous but mostly untapped.

Although it has been reported that many species of organisms vanish every year, there are still more species that are unknown to us. For example, less than 10% of the world's biodiversity has been tested for biological activity (Harvey, 2000). Less than 10% of the estimated 250,000 flowering plant species in the world have been examined scientifically for their potential in medicine (Rouhi, 2003). An inventory of the known and estimated number of species in each group of organisms is shown in Table 1.1 (Pandey, 1998).

It should be noticed that there are still a lot of species that are not chemically examined for biological activities from Table 1.1. Therefore, there should be more exiting news ahead for natural product studies.

Table 1.1 Known and Estimated Numbers of Biological Species (Pandey, 1998)

Group	Known	Estimated	Known (%)
Viruses	5,000	130,000	4
Bacteria	4,760	40,000	12
Fungi	69,000	1,500,000	5
Algae	40,000	60,000	67
Bryophyta	17,000	25,000	68
Gymnosperms	750		
Angiosperms	250,000	270,000	93
Protozoa	30,800	100,000	31
Porifera	5,000		
Cnidaria	9,000		
Nematodes	15,000	500,000	3
Crustacea	38,000		
Insects	800,000	10,000,000	8-13
Other Vertebrates	132,460		
Mollusks	50,000		
Echinoderms	6,100		
Amphibians	4,184		
Reptiles	6,380		
Fishes	19,000	21,000	90
Birds	9,198		100
Mammals	4,178		100

1.2. Bioactive Components from Natural Products

The bioactive components are the compounds which show some specific bioactivities when they are introduced into the bodies of human or animals in certain ways. These bioactive components are the main active ingredients of pharmaceuticals, nutraceuticals and dermaceuticals named according to their functions and applications. Natural bioactive components can be produced from plants, fungi, bacteria, protozoans, insects, animals, etc.

Microorganisms, particularly bacteria and fungi produce a wide variety of secondary metabolites, bioactive components, which show actual or potential therapeutic application. Therefore, microorganisms have been an important source of bioactive components and the first group of candidates for natural drug discovery. Most of the antibiotics are secondary metabolites from microorganisms. The major families of antibiotics are (Walsh, 1998):

1. β-Lactams,
2. Tetracyclines,
3. Aminoglycoside antibiotics,
4. Macrolides,
5. Ansamycins,
6. Peptide/glycopeptide antibiotics,
7. Miscellaneous antibiotics.

The following well-known antibiotics, penicillin, chlortetracycline, chloramphenicol, streptomycin, erythromycin, rifamycin, lincomycin, cephalosporin C, vancomycin,

nalidixic acid and amphotericin B were obtained from microorganisms and currently commercially produced by large scale fermentation.

Microorganism derived bioactive components are large part of pharmaceuticals. Of the 20 best-selling non-protein drugs in 1999, nine were either derived from or developed as the result of leads generated by microorganisms. These 9 biopharmaceuticals, simvastatin, lovastatin, enalapril, pravastatin, atorvastatin, augmentin, ciprofloxacin, clarithromycin and cyclosporin A, offer combined annual sales of more than US$ 16 billion (Harvey, 2000).

Animals provide the vital source of modern biopharmaceuticals. Table 1.2 shows some of the bioactive components originally isolated from animal source (Walsh, 1998).

The majority of biopharmaceuticals currently found and used are from plants. With the discovery of new species, plants, especially flowering plants, will be the most important resource for new bio-pharmaceuticals. Table 1.3 shows partial pharmaceuticals derived from plants (Bidlack, 2000).

Table 1.2 Some Pharmaceutical Substances Originally Isolated from Animal Source (Walsh, 1998).

Product	Indication	Original Source
Insulin	Diabetes mellitus	Porcine/bovine pancreatic tissue
Glucagon	Used to reverse insulin-induced hypoglycaemia	Porcine/bovine pancreatic tissue
hGH	Treatment of short stature	Human pituitaries
FSH	Subfertility/infertility	Urine of post-menopausal women
hCG	Subfertility/infertility	Urine of pregnant women
Blood coagulation factors	Haemophilia and other related blood disorders	Human blood
HSA	Plasma volume expander	Human plasma/placentae
Polyclonal antibodies	Passive immunization	Serum of immunized animals/humans
HBsAg	Vaccination against hepatitis B	Plasma of hepatitis B carriers
Urokinase	Thrombolytic agent	Human urine
Peptide hormones	Various	Mostly from pituitary gland
Trypsin	Debriding agent	Pancreas
Pancrelipase	Digestive enzymes	Pancreas
Glucocerebrosidase	Gaucher's disease	Placenta
Steroid hormones	Various, including subfertility	Gonads
Corticosteroids	Adrenal insufficiency, anti-inflammatory agents, immunosuppressants	Adrenal cortex
Prostaglandins	Various, including uterine stimulants, vasodilators and inhibition of gastric acid secretion	Manufactured in most tissues
Adrenaline	Management of anaphylaxis	Adrenal gland

Abbreviations: hGH = human growth hormone; FSH = follicle stimulating hormone; hCG = human chorionic gonadotrophin; HAS = human serum albumin; HBsAg = hepatitis B surface antigen.

Table 1.3 Partial Lists of Pharmaceuticals Derived From Plants (Bidlack, 2000)

Pharmaceutical	Medicinal Use	Plant Source
Aspirin	Analgesic, anti-inflammatory	*Filipendula ulmaria*
Atropine	Pupil dilator	*Atropa belladonna*
Caffeine	Stimulant	*Camellia sinensis*
Camphor	Rheumatic pain	*Cinnamomum camphora*
Codeine	Analgesic, antitussive	*Papaver somniferum*
Dicoumerol	Antithrombotic	*Melilotus officinalis*
Digoxin	Atrial fibrillation	*Digitalis purpurea*
Ephedrine	Bronchodilator	*Ephedra sinica*
Eugenol	Toothache	*Syzygium aromaticum*
Pilocarpine	Glaucoma	*Pilocarpus jaborandi*
Quinine	Malaria propholaxis	*Cinchona pubescens*
Reserpine	Antihypertensive	*R. serpentia*
Sennoside	Laxative	*Cassia augustifolia*
Scopolamine	Motion sickness	*Datura stramonium*
Tubocurarine	Muscle relaxant	*Chondrodendron tomentosum*
Tetrahydrocanabinol	Antiemetic	*Cannabis sativa*
Theophylline	Diuretic, antiasthmatic	*Camellia sinensis*
Vinblastine	Hodgkin's disease	*Catharanthus roseus*

Plants are not only the main resource of pharmaceuticals, but also the main resource of nutraceuticals and dermaceuticals, the bioactive components that help people out of subhealth condition. With the increasing awareness of subhealth, life quality of people will likely be improved by nutraceuticals and dermaceuticals. Foods are important

resources of nutrition, but the concentrations of bioactive components in foods are usually very low. As a result, daily meals can not always provide enough bioactive components that are needed. Some bioactive components from food and their functions are listed in Table 1.4. Therefore, functional foods and food supplements are good addition to daily meals.

Table 1.4 Bioactive Food Constituents That May Prevent Disease (Adapted from Bidlack, 2000)

Active compounds	Food source	Potential health benefit
β-Carotene, lycopene, lutein, other carotenoids	Tomatoes, carrots, yams, cantaloupe, spinach, sweet potatoes, citrus fruits	Reduces coronary heart disease, reduces cancer
Epigallocatechin and epigallocatechin gallate	Green tea, grapes/wine	Reduces cancer, reduces heart disease
Daitzen, genestein, other isoflavones	Soybeans, soy foods	Prevents menopausal symptoms, prevents osteoporosis, and reduce cancer
Tocopherols, tocortrienols	Vegetable oils	Antioxidant, lowers serum cholesterol, inhibits cancer, decreases heart disease
ω-3 fatty acids	Fish oil, algae, flaxseed	Reduces serum cholesterol, reduces heart disease, reduces serum triacylglycerol, and immunosuppressant
Conjugated linoleic acid	Dairy products, processed vegetable oils	Anticancer, antiatherosclerosis
Diallyl disulfide and allicin	Garlic, onions	Anticancer, stimulates immune function, free radical scavenger, reduces serum cholesterol, and reduces serum TG
Sulforaphane and other organic isothiocyanates	Cruciferous, vegetables	Chemoprevention
Limonene	Citrus fruits	Anticancer
Coumarins	Vegetables, citrus fruits	Prevents blood clotting, anticarcinogenic activity
Nondigestible fermentable oligosaccharides, fructose oligosaccharides	Garlic asparagus, chicory	Intestinal fortification, stimulates immune function, inhibits tumorigenesis, and reduces serum cholesterol

Biotechnology industry has evolved significantly since the introduction of human insulin synthesized in Escherichia coli in 1982—the first Food and Drug Administration (FDA) approved recombinant therapeutic agent in the USA. Since then, over 75 other recombinant proteins have been introduced. The list is comprised of cytokines, hormones, monoclonal antibodies, and vaccines (Ahuja, 2000).

Recent research suggests that plants will be a facile and economic bioreactor for large scale production of pharmaceutical recombinant proteins. Plants have numerous advantages as production factories for proteins compared with human or animal fluids/tissues, recombinant microbes, transfected animal cell lines, or transgenic animals. Among these are (Hood et al., 2002):

1. Rapid scale-up production
2. Facile, genuine assembly of multimeric antibodies (unlike bacterial)
3. Increased safety, because plants do not serve as hosts for human pathogens, such as HIV, prions, hepatitis viruses, etc.
4. Low cost production of bulk crude material on an agricultural scale suitable for further GMP purification
5. Capitalization costs of manufacturing that are perhaps 10% relative to steel tank bioreactor methods

For that reason, more bioproducts are expected to be produced by the way of "molecular farming".

In recent years, marine natural product bioprospecting has yielded a considerable number of drug candidates, although most of these molecules are still in preclinical or early clinical development but some are already on the market, such as cytarabine, or are predicted to be approved soon, such as ET742(YondelisTM) (Haefner, 2003).

1.3. Bioseparation Engineering

Bioseparation engineering is the application of fundamental engineering and biological principles to the design of adsorbents, equipments, and processes for the separation of biological molecules (Ladisch, 2001). Bioseparations involves the separation and purification of compounds of biological origin, which are produced from cells grown in the bioreactors or by cells contained in animal or plant tissue (Harrison et al., 2003).

Biotechnology can only benefit human being by its industrialization. The success of biotechnology products is highly dependent on the successful development and application of reliable and sensitive bioseparation methods (Ahuja, 2000).

Most bioseparations in biotechnology industry have four similar steps, which occur sequentially (Belter et al., 1988):

1. *Removal of Insolubles.* Filtration and centrifugation is the principal unit operations used in this segment. Relatively little product concentration or improvement of product quality occurs in this stage.

2. *Isolation of Bioproducts.* These steps, which are relatively nonspecific, remove materials of widely divergent properties compared to the desired product. Appreciable concentration and product quality increases usually occur. Adsorption and solvent extraction are typical unit operations applied in the isolation of bioproducts.

3. *Purification.* These processing techniques are highly selective for the products and remove impurities of similar chemical functionality and physical properties.

Chromatography, electrophoresis, and precipitation are good examples in this stage.

4. *Polishing.* The end use of the products dictates the final sequence utilized. Crystallization is a typical unit operation here. In addition, most products must also be dried.

For the bioproducts that are derived from natural products, bioseparation processes usually begin with solvent extraction. Then solvent extraction is followed by the just mentioned four steps in bioseparation processes.

The importance of bioseparation has been recognized since the publication of the first book on the topic, *Bioseparations: Downstream Processing for Biotechnology* by Belter, Cussler and Hu in 1988. Despite the fact that numerous bioseparation technologies have been transferred from lab scale to industrial scale, bioseparation are still the bottle neck of the bioprocesses and are worth further studying.

1.4. Purpose of This Study

A bioseparation process comprises a sequent series of bioseparation techniques, namely unit operations. Details of numerous bioseparation techniques have been intensively studied, but the study on relations between two adjacent unit operations in bioseparation processes have not been reported to the author's knowledge. If the property of the products from the previous unit operation is exactly the same as that of the starting materials of following unit operation, then the two unit operations can be Integrated into

one. Therefore, one of purposes in this work is to study amalgamation or integration of various bioseparation techniques to save both natural resource and environment.

Considering isolation of bioproducts that are derived from natural products, bioseparation process usually begin with solvent extraction. Specifically, this study will focus on the extraction techniques suitable for amalgamation or incorporation with following isolation techniques in bioseparation processes.

More specifically, this study will take isolation of taxanes from twigs and needles of *Taxus canadensis*, the renewable resource of important anticancer pharmaceuticals, as an example, to discuss the incorporation of bioseparation techniques.

Pressurized liquid extraction (PLE) is an advanced sample preparation technique in the labs. Based on this new extraction technique, Dynamic Pressurized Liquid Extraction (DPLE) is proposed. The potential of DPLE for large scale extraction is also discussed in this work.

Chapter II: Background and Literature Review

2.1. Taxanes

To the general public with the exception of Viagra®, most well-known pharmaceuticals recently are two taxanes, paclitaxel (Taxol®) and docetaxel (Taxotere®) because these taxanes are the most significant anticancer drugs ever discovered. Other taxanes isolated from the biomass of *Taxus* species, such as 10-DAB III, baccatin III and 9-DHB III, are the intermediates for semisynthesis of paclitaxel and docetaxel.

2.1.1. Chemistry of Taxanes

The chemical structure of taxanes and related compounds is described in Suffness Ed., *Taxol®, Science and Applications*, 1995 and also in *The Merck Index*, 13th Edition, 2001. The term of "taxanes" refers to compounds comprising the tricyclic ring nucleus shown in Figure 2.1:

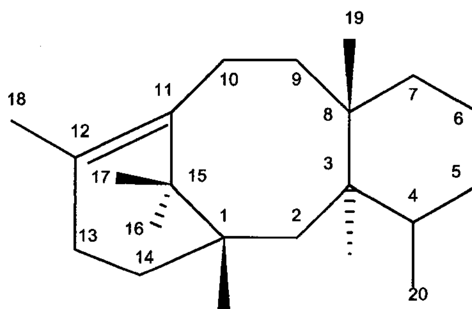


Figure 2.1 The Tricyclic Ring Nucleus of Taxanes

Paclitaxel (Taxol®), the most important taxane, was first isolated from the bark of *Taxus brevifolia* and characterized by Wani et al. (1971). Cephalomannine is known as the impurity that is chemically very similar to paclitaxel. The two chemicals only differ a little on their side chains. As a result, this compound is also the one that is most difficult to separate from paclitaxel by preparative chromatography. The two molecules' chemical structures are illustrated as Figure 2.2.

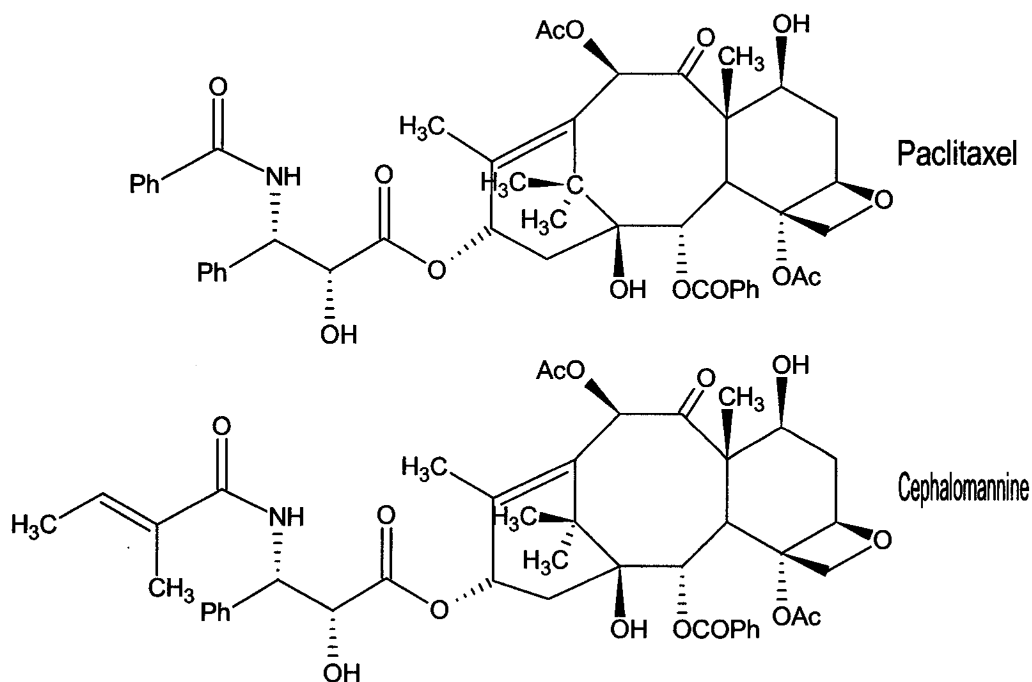


Figure 2.2 Chemical Structures of Paclitaxel and Cephalomannine

Other taxanes' structures are shown in Figure 2.3, Figure 2.4 and Figure 2.5. Although these molecules are chemically similar, their physical properties may differ a lot. Therefore, it is very difficult to recover all the valuable taxanes, such as paclitaxel, 10-DAB III, baccatin III and 9-DHB III synchronously.

Docetaxel, the chemical structure of which is shown in Figure 2.3, and the active pharmaceutical ingredient (API) of Taxotere® of Aventis Pharma Inc, is a successful analogue of paclitaxel.

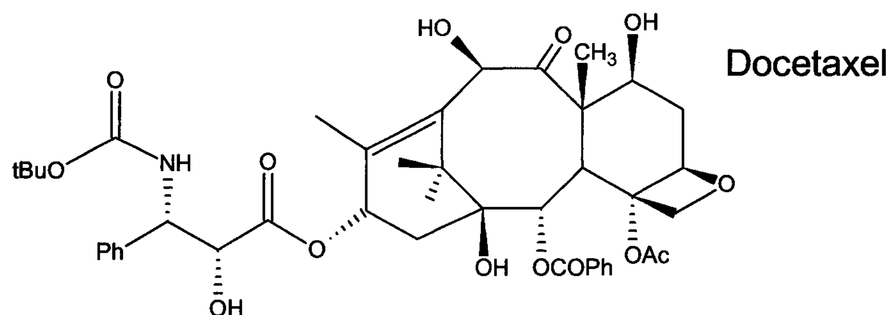


Figure 2.3 Chemical Structure of Docetaxel

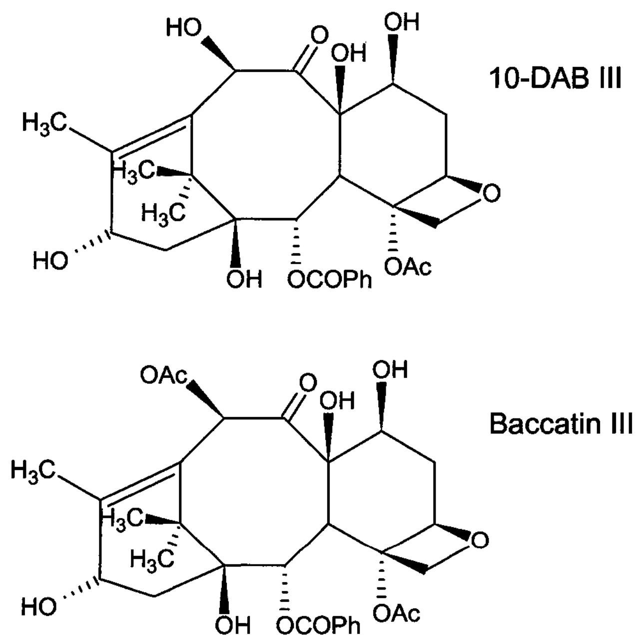


Figure 2.4 Chemical Structures of 10-DAB III and Baccatin III

10-DAB III and baccatin III are very important intermediates for semisynthesis of taxane anticancer drugs. Their chemical structures are shown in Figure 2.4.

9-DHB III, isolated from needles of *Taxus canadensis*, is a patented compound by Abbott Laboratories, Inc., USA and found as a useful intermediate for semisynthesis of taxane anticancer drug (Gunawardana et al., 1994). Its structure is shown in Figure 2.5.

Figure 2.5 Chemical Structure of 9-DHB III (Gunawardana et al., 1994)

2.1.2. Discovery of Paclitaxel

In the 1960s, the National Cancer Institute (NCI) initiated a program to screen large collections of compounds, natural or synthetic for anti-tumour activity. These activities lead to the discovery of the most significant compounds in the fight against cancer—taxanes. According to Goodman and Walsh (Goodman and Walsh, 2001), there were 3 major stages during the discovery and development of paclitaxel:

1. *The period from 1962 to 1975.* Over this period, *Taxus brevifolia* was made to reveal its molecular secret to the cancer research community and paclitaxel was shown to be a promising agent.

2. *The period from 1976 to 1983.* In this period, paclitaxel went through the developmental procedure established for all compounds at the NCI, from the screening against a panel of murine tumours to the filing of an application to begin human trials.

3. *The period from 1984 to 1989.* The Phase I clinical trials began in 1984 and the remarkable activity of paclitaxel were published at the end of this period. At the same time, supply issue of paclitaxel was serious concern because many of the approved trials were postponed because of lack of supplies of this compound.

Paclitaxel (Taxol®) is the most celebrated anticancer drug in the history. It has been hailed as the most promising anti-cancer drug by most oncologists (Stull et al., 1995). Its structure was determined using chemical methods and X-ray crystallographic analysis. Paclitaxel binds to microtubules and inhibits their depolymerization into tubulin; therefore, it blocks the cell's ability to break down the mitotic spindle during mitosis. With the spindle still in place the cell cannot divide into daughter cells. Table 2.1 shows the chronology of development of paclitaxel.

Paclitaxel has been approved by the Food and Drug Administration (FDA) for the treatment of breast, ovarian, non-small-cell lung cancer and AIDS related Kaposi's sarcoma. Adding paclitaxel may soon become a standard treatment option for some patients with head and neck cancer (Medical Post, 2003). Marketing of paclitaxel-coated coronary stents will also expand the market of taxanes (Jarvis, 2002).

Table 2.1 Chronology of Development of Paclitaxel (Adapted from Pandey, 1998 and Panchagnula, 1998)

1962 Collection of *T. brevifo*lia in Washington
1964 Cytotoxicity of bark extract
1966 Isolation of pure paclitaxel
1967 Antitumor activity detected
1971 Chemical structure elucidated
1979 Mechanism of action
1983 Taxol® IND filed
1984 Chemical trials begin
1986 Hypersensitive reactions observed
1988 NCI suggests premedication regimen
1989 Proved effective against ovarian cancer
1989 Selection of BMS as CRADA partner
1991 Proved effective against breast cancer
1992 Proved effective against non-small cell lung cancer
1992 NDA filed for Taxol®
1992 Approved by FDA for ovarian cancer
1994 Approved by FDA for breast cancer
1994 Total synthesis by Nicolaou and Holton, independently
1998 BMS exclusivity on Taxol® expires
2002 BMS exclusivity got extended

2.1.3. Production of Taxanes

The initial source of paclitaxel was the bark of the Pacific Yew, *Taxus brevifolia*, an understory tree growing in the forests of the Pacific Northwest from northern California into British Columbia (Goodman and Walsh, 2001).

More *Taxus* species were studied for taxanes content to meet the increasing demand of anticancer drugs and found that all the *Taxus* species have considerably high content of taxanes (Rozendaal, 2000). All the *Taxus* species can be sources for taxane production by large scale bioseparation. For the production of the natural bioactive components, isolation from the natural source often provides the most economically viable method of production (Cragg, 1998).

Because of the complex structure of taxane nucleus, total synthesis routes are very long and tedious and therefore appear uneconomical compared to semisynthesis. The biogenetic precursor of paclitaxel and docetaxel, 10-deacetyl baccatin III (10-DAB III), baccatin III and 9-DHB III are found in relatively high concentration in *Taxus* biomass. Since there is great interest in the semisynthesis of paclitaxel, docetaxel and new analogues to meet high demand of anticancer drugs, 10-DAB III and baccatin III have emerged as key precursors for taxane anticancer drug production. Table 2.2 indicates that the amount of semisynthetic paclitaxel using the precursors isolated from *Taxus* biomass is much more than that of natural paclitaxel.

Table 2.2 Theoretical Yield of Natural Plus Semisynthetic Paclitaxel per Metric Tonne of *Taxus brevifolia* Bark (Stull et al., 1995)

Recourse	Amount
Natural paclitaxel	300 g
Natural paclitaxel plus synthetic paclitaxel	1800 g

In summary, natural paclitaxel isolated from *Taxus* biomass plus synthetic paclitaxel and docetaxel with the precursors isolated from *Taxus* biomass will be the dominating source of API for taxane anticancer drugs. It is believed for the foreseeable future, taxanes will continue to be produced by biological means, mainly by plantation and thereafter separation and purification (Walke and Croteau, 2001).

2.1.4. Natural Resources of *Taxus* Species

Taxus species are distributed widely around the world. Table 2.3 shows the diversity of origin of various *Taxus* species (Pandey 1998).

Table 2.3 Distribution of *Taxus* (Pandey, 1998)

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

In Canada, there are two natural *Taxus* species available, *Taxus brevifolia* on the western coast and *Taxus canadensis* in Eastern Canada. The descriptions of *Taxus canadensis* in Table 2.4 is from Gymnosperm Database (2004). The natural resource of *Taxus canadensis* is abundant in Canada. Twigs and needles of *Taxus canadensis* are renewal.

Table 2.4 *Taxus canadensis* Marshall 1785 (Gymnosperm Database, 2004)

Common Names:	Canada yew, American yew, ground-hemlock, li du Canada, sapin trainard (Hils 1993).
Taxonomic notes:	Syn: *Taxus* baccata Linnaeus subsp. canadensis (Marshall) Pilger; T. baccata var. minor Michaux; T. minor (Michaux) Britton; T. procumbens Loddiges (Hartzell 1991).
Description:	"Shrubs to 2 m, usually monoecious, low, diffusely branched, straggling, spreading to prostrate. Bark reddish, very thin. Branches spreading and ascending. Leaves 1-2.5 cm ′ 1-2.4 mm, pale green abaxially, mostly without cuticular papillae along stomatal bands, dark green to yellow-green adaxially, epidermal cells as viewed in cross section of leaf wider than tall or ± isodiametric. Seed somewhat flattened, 4-5 mm. 2n = 24. Seeds maturing late summer - early fall" (Hils 1993).
Range:	Canada: extreme SE Manitoba, Ontario, Québec, Prince Edward Island, New Brunswick, Nova Scotia, Newfoundland; France: St. Pierre and Miquelon; USA: Connecticut, Illinois, Indiana, Iowa, Kentucky, Maine, Massachusetts, Michigan, Minnesota, New Hampshire, New York, Ohio, Pennsylvania, Rhode Island, Tennessee, Vermont, Virginia, Wisconsin, and West Virginia; at 0 - 1500 m elevation as an understory shrub in rich forests (deciduous, mixed, or coniferous), or in bogs, swamps, gorges, ravine slopes, and rocky banks (Hils 1993). See also Thompson et al. (1999).
Big Tree:	Some wild plants are said to reach 3.7 m tall and have an arboreal growth form (Hartzell 1991).

2.2. Current Taxane Bioseparation Technologies

Since taxanes are important anticancer drugs and isolation from biomass is a practical way for taxane production, there have been many researchers working in this interesting area. As a result, there are many publications about bioseparation of taxanes.

2.2.1. Extraction of Taxanes from *Taxus* Biomass

In screening for active pharmaceutical ingredients (API) from biomass of natural products, the first step is to separate bioactive ingredients from others, which involves both science and engineering. It is usually the beginning and bottleneck of the whole bioseparation process. Various extraction methods have been extensively studied, such as Soxhlet extraction, percolation, maceration, digestion, extraction under reflux, steam distillation, ultrasonication, turbo-extraction, pressurized liquid extraction (PLE) (Benthin et al., 1999), microwave-assisted extraction (Hao et al., 2002) and supercritical fluid extraction (SFE) (Kim et al., 2003). Although some of the methods have been proved effective for small scale extraction, none of them shows efficient in large scale extraction.

Taxanes are present at low concentrations; the volume of solvent used for their extraction is very large. Most of the published bioseparation processes are based on the conventional solvent extraction (CSE) (Table 2.5). The CSE based processes consume large amount of organic solvents and cause environmental problems. From Table 2.5 it can be noticed that the solvents used in CSE are methanol, 95% ethanol, or their mixture with dichloromethane. The extraction time is as long as 10 days for a single extraction operation.

Table 2.5 Some Conventional Solvent Extraction Methods for Recovery of Taxanes from *Taxus* Biomass

Starting Material	Solvent	Extraction Condition*	Reference
Ground twigs and needles	Methanol or ethanol/water (95%)	5:1 (v/w), 24 hours	US 2003/0032820
Ground twigs and needles	Methanol or ethanol/water (95%)	5:1 (v/w), 24 hours	US 2002/0151579
Ground twigs and needles	Methanol or ethanol/water (95%)	5:1 (v/w), 24 hours	US 6,469,186 B1
Ground yew biomass	Methanol	7:1 (v/w)	US 2003/0013899 A1
Water washed twigs and needles	CH_2Cl_2:CH_3OH=1:1 (v/v)	16 hours, 20°C	US 6,452,024 B1
Dry twig and needles	Methanol	6 hours+6 hours, 50-60°C	WO 02/38555 A1
Dry twigs and needles	Methanol	6:1 (v/w), 5 hours+5 hours, 65°C	US 6,229,027 B1
Dry twig and needles	Methanol	3:1 (v/w), 3 hours	US 6,066,748
Dry twigs and needles	Methanol	5:1 (v/w), 60°C	US 5,969,165
Ground plant material	CH_2Cl_2:CH_3OH=9:1 (v/v)	3:1 (v/w)	US 5,965,752
Ground twigs and needles	Methanol	10:1 (v/w), 12 hours, 15-45°C	US 6,124,482
Yew bark	Methanol	12:1 (v/w), 72 hours, 40°C	US 6,136,989
Yew biomass	Methanol or ethanol/water (95%)	16-21 hours	US 5,670,673
Yew bark, ground	Methanol	6-7:1 (v/w), 10 days, <40°C	US 5,654,448
Dry bark	Methanol	9:1 (v/w), 72 hours	US 5,856,532
Ground twigs and needles	Methanol	2:1 (v/w) + 3:2 (v/w)	CA 2,203,844
Ground needles	Methanol	10:1 (v/w), 12 hours	US 6,002,024

***In Extraction Condition, (v/w) is the ratio between solvents (liter) and biomass (kg) in extraction.**

In super-critical fluid extraction (SFE) as illustrated in Table 2.6, which is claimed to be environmentally friendly, organic solvents, have to be used as co-solvents because supercritical CO_2 alone as extraction solvent cannot yield satisfactory recovery in the extraction of taxanes. Some of the co-solvents such as methanol and acetonitrile are toxic. Therefore, SFE that uses toxic solvents causes the same environmental and health problems as CSE does.

Table 2.6 Extraction Conditions in Supercritical Fluid Extraction for Taxane Extraction from *Taxus* Biomass

Starting Material	Solvents	Extraction Condition	Reference
Ground bark, root, twig and needles	Supercritical CO_2 plus co-solvent (15-25%, v/v)	2-3 MPa, 60-100°C	US 6,503,396 B2
Yew biomass	Supercritical CO_2 plus co-solvent (methanol, ethanol or acetonitrile)	39-49 MPa, 34-45°C	US 2002/0051827 A1

In addition to CSE and SFE, some new extraction technologies are also studied at laboratory scale for taxane extraction from the biomass of *Taxus* species (Table 2.7). Unfortunately, the CSE is still the dominant extraction technique because there is no other extraction technique that can surpass and replace it in large scale extraction. Conventional solvent extraction is still the governing extraction technique at large scale taxane extraction because of its unique features such as low investment and easy handling.

Table 2.7 New Extraction Technologies for Taxanes Extraction from Biomass of *Taxus*

Technology	Starting Material	Extraction Condition	Conclusion	Reference
Pressurized Liquid Extraction	Dry bark, 40-60 mesh	Methanol, ethanol, dichloromethane or chloroform, 100°C, 10.13 MPa	Extraction recovery of paclitaxel with dichloromethane and chloroform is much less than that of conventional solvent extraction with methanol, ethanol or mixed solvents.	Kawamura et al., 1999
Microwave Assisted Extraction	*Taxus* biomass, 55% moisture	Methanol, ethanol (95%, ethanol/water) or chloroform	The taxanes recovery rate with chloroform is about half of that with methanol or ethanol.	Mattina et al., 1997

2.2.2. Preliminary Purification of *Taxus* Biomass Extract

Presently, there is no existing extraction technology that satisfies both high recovery rate and high selectivity. For example, the weight of extract of CSE with methanol, the dominant extraction organic solvent, can be 55% of that of dry *Taxus* biomass (Ketchum et al., 1999). Large amounts of hydrophilic and hydrophobic impurities are present in the extract of methanol or ethanol. These impurities can cause serious problems in purification of taxane using liquid preparative chromatography.

Preparative chromatography is the dominant purification technique in biopharmaceutical production. It is an essential bioseparation technique for production of bioproducts with high purity, but the purification cost with this technique is as high as 50% of the total

manufacturing cost (Ladisch, 2001). Therefore, the preliminary purification of extract before preparative chromatography, the most expensive separation technique, are required. The major objectives of preliminary separation are:

1. To reduce the mass loaded to the preparative chromatography column by removing most of the impurities.

2. To remove most very polar (hydrophilic) impurities that can damage the normal phase separation media by irreversible adsorption of hydrophilic impurities to it in a normal phase chromatography based process.

3. To remove most very non-polar (hydrophobic) impurities that can damage the reverse phase separation media by irreversible adsorption of the hydrophobic impurities to it in a reverse phase chromatography based process.

The preliminary purification processes differ widely (Table 2.8); any change in the preliminary separation processes could lead to valuable progress.

Table 2.8 Preliminary Separations in Some CSE Based Bioseparation Processes

Organic Solvents Used	**Preliminary Separation Before Preparative Chromatography***	**Number of Unit Operations**	**Reference**
Methanol or ethanol, Hexane, Dichloromethane	Filtration, 1st Concentration, Partition, 2nd Concentration, Absorb on solid, Elution and 3rd Concentration, Sample preparation, loading	8	US 2003/0032820 A1, US 2002/0151579 A1, US 6,469,186 B1
Methanol, Hexane, Acetone	Soak and wash with water, Filtration, 1st Concentration, Absorb on solid, Elution and 2nd Concentration, Sample preparation, loading	8	US 2003/0013899 A1
Methanol, Dichloromethane, Hexane, Acetone	Filtration, Concentration, 1st Dissolution in methanol, 1st Precipitation, 1st Centrifugation, Dissolution in Acetone, Percoloration by hexane, Evaporation, 2nd Dissolution in methanol, 2nd Precipitation, Centrifugation, Sample preparation and loading	12	US 6,452,024 B1
Methanol, Hexane, Ethyl acetate	Filtration, Concentration, Blow to dry, Dissolution in mixed solvents, Sample Preparation and loading	6	WO 02/38555 A1
Methanol, Dichloromethane, Hexane, Ethyl acetate	Filtration, 1st Concentration, Partition, 2nd Concentration, Sample preparation and loading	6	US 6,229,027 B1
Methanol, Chloroform, Benzene, Acetone	Filtration, 1st Concentration, Partition, 2nd Concentration, Pass through resin, 3rd Concentration, Pass through silica gel	7	US 6,066,748; US 5,969,165
Methanol, Dichloromethane, Chloroform, Toluene, Petroleum Ether	1st Filtration, 1st Concentration, Partition, Filter over charcoal, 2nd Concentration, Dissolved in toluene, 2nd Filtration, Rinse, Air dry, Dissolved in methanol, 3rd Filtration, Sample preparation	13	US 5,965,752
Methanol, Acetone, Toluene, Dichloromethane	Filtration, 1st Concentration, dissolved in acetone, filtration, dry, precipitate with water, extraction, 2nd concentration, drying, 10-DAB III only, without chromatography	10	US 6,124,482
Methanol, Dichloromethane, Acetone	Filtration, 1st Concentration, Extraction, Partition, 2nd Concentration, Sample preparation and loading	7	US 6,136,989
Methanol or 95% ethanol, Chloroform, Petroleum Ether	Filtration, 1st Concentration, Partition, 2nd Concentration, Drying, Sample preparation and loading	7	US 5,670,673
Methanol, Acetone, Dichloromethane, Petroleum Ether	Filtration, 1st Concentration, Partition, 2nd Concentration, Precipitation, Drying, Sample preparation and loading	8	US 5,654,448
Methanol, Ethyl Acetate	Filtration, Concentration, Precipitation in water, Drying, Sample preparation and loading	6	US 5,856,532
Methanol, Hexane, Chloroform, Acetone	Filtration, Concentration, 1st Precipitation, 2nd Precipitation, Dissolution, Sample preparation and loading	7	CA 2,203,844
Methanol, Acetone, Toluene, Dichloromethane	Filtration, 1st Concentration, Precipitation, Filtration, 2nd Concentration, Dry, 1st Partition, 2nd Partition, Sample preparation and loading	10	US 6,002,024

***Based on the examples in the patents**

2.2.3. Purification

Liquid preparative chromatography, either normal phase or reverse phase, is the dominant and irreplaceable industrial technique for taxanes purification.

Reverse phase liquid chromatography prevails recently as a result of rising environmental concerns because its gradient eluant are mixture of water and an organic solvent. The organic solvents used in reverse phase chromatography for taxanes purification are usually nontoxic or less toxic, such as ethanol and acetone that are miscible with water.

Crystallization and drying usually are the final unit operations for biopharmaceutical production. These unit operations are not problematic because the amounts of final products are usually very small. For example, 50 kilogram cyclosporin A, which is an antibiotic, is the typical productivity monthly for a large biopharmaceutical company.

2.2.4. Summary of Bioseparation Process for Taxanes Production

From Table 2.5 and Table 2.8, it may be noticed that all the CSE based bioseparation processes developed and currently used have two things in common:

1. A large amount of toxic organic solvents is used in these processes. This causes not only severe environmental problems but also problems for the processes themselves, because extra fine distillation units are required to recycle these organic solvent mixtures.
2. There are at least 6 unit operations in preliminary separation because of the complexity of the extracts and low concentration of taxanes in the extracts.

Although there are many publications available and each of them claims quite a few disadvantages of previous ones, there is not a revolutionary breakthrough in the bioseparation process research and development even over 10 years after paclitaxel discovery.

Figure 2.6 shows a typical CSE based bioseparation process for taxane production from the twig and needles of renewable *Taxus* biomass. This typical process originates from the large number of publications in which Liquid-Liquid Extraction (LLE) is used to remove lipids and to recover taxanes from extract in preliminary separation processes.

It should be noticed that Liquid-Liquid Extraction in preliminary separation of this typical taxane bioseparation process deals with a large amount of mixed solvents. Most of the solvents are toxic and expensive, such as methanol and dichloromethane. In this typical process, the large amount of discarded toxic solvents results a solvent loss and causes environmental problems.

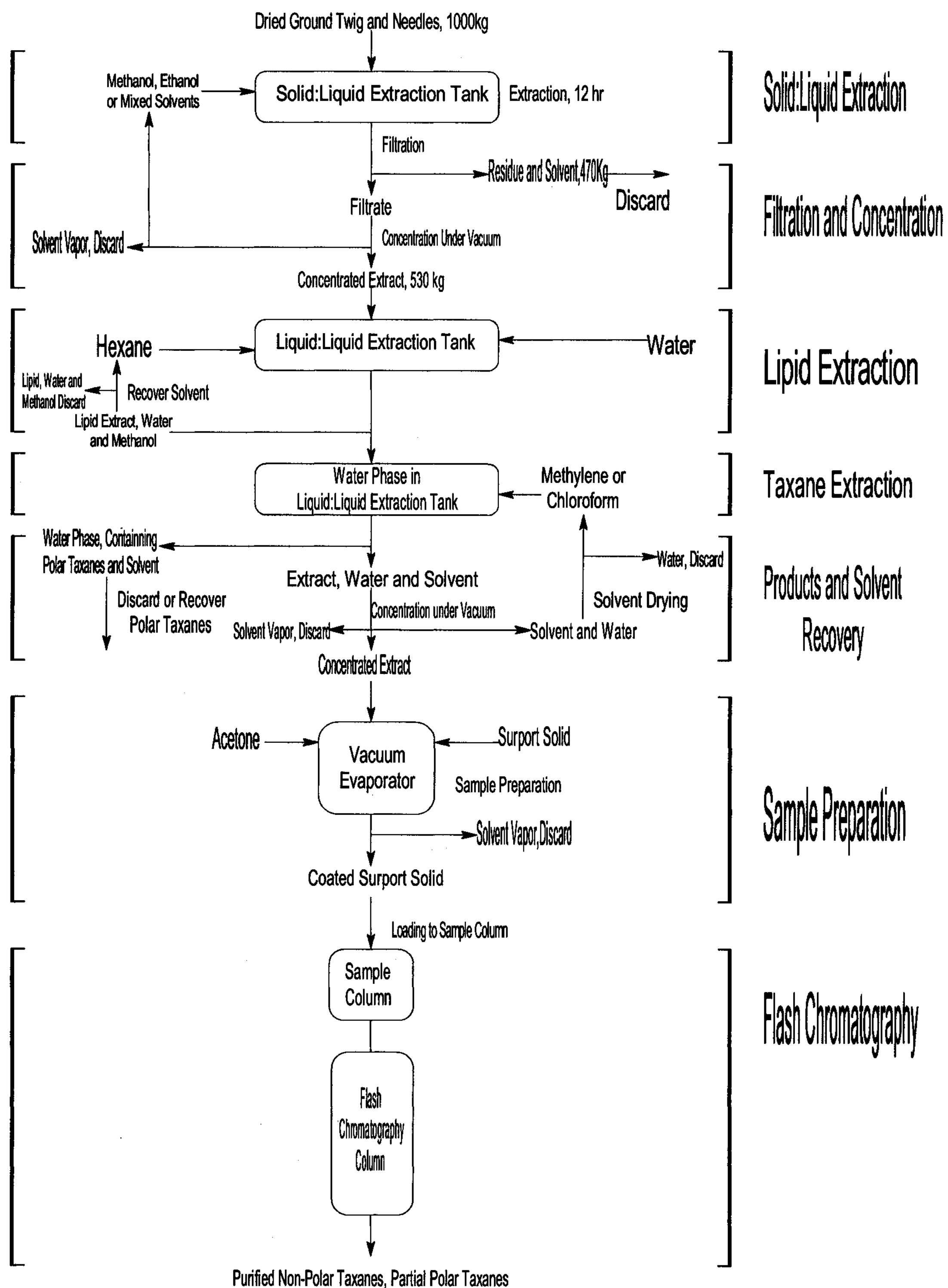


Figure 2.6 A Typical CSE Based Bioseparation Process for Taxanes Production

2.3. Pressurized Liquid Extraction

A new extraction method, which was introduced to the public in 1996 and is known as Pressurized Liquid Extraction (PLE; ASE® by Dionex Inc.), has shown to be promising on overcoming some of the major drawbacks encountered in CSE in very small scale extraction (Choi et al., 2003).

Pressurized Liquid Extraction (PLE) or Accelerated Solvent Extraction (ASE®) uses organic solvents at high pressures and temperatures above the solvent boiling point (Richter et al., 1996). Since then, PLE or ASE has been intensively studied as a new sample preparation technique using special instrument at laboratory scale (Benthin et al., 1999, Alonso-Salces et al., 2001, Lee et al., 2002 and Choi et al., 2003). This technique provides many advantages compared with other extraction methods in sample preparation shown in Table 2.9. According to the data in that table, PLE (ASE®) is the most economic extraction technique in small scale sample preparation. However, there is no publication available on applying PLE at large scale to recover bioactive components from biomass since it was invented.

Since PLE involves high temperature, the degradation of some heat sensitive bioactive ingredients likely occurs. Therefore, PLE is still mainly used as sample preparation method in environmental studies and is approved by Environmental Protection Agency (EPA) of USA as Standard Method 3545A.

The major hurdle for large scale PLE is the required high pressure, normally 8.27 MPa. This pressure is just a little bit lower than that of SFE. If the efficiency of PLE at relative low pressure is satisfied, it will be a promising extraction method comparable to SFE.

Table 2.9 Evaluation of Currently Available Extraction Technologies for Laboratory Scale Sample Preparation (Adapted from Dionex Website, 2004)

Name of Extraction Technology	**Average Solvent Used per Sample**	**Average Extraction Time per Sample**	**Average Cost per Sample***	**Instrument and Method**
Conventional	200-500 ml	4-48 hr	US $27	Sample and Solvent in Beaker, Dynamic or Static
Soxhlet	200-500 ml	4-48 hr	US $27	Glass Soxhlet Extractor, Static
Automated Soxhlet	50-100 ml	1-4 hr	US $16	Soxhlet Extractor with Automation, Static
Sonication	100-300 ml	0.5-1 hr	US $24	Beaker in Ultrasonic Bath, Static
Super-Critical Extraction	8-50 ml	0.5-2 hr	US $23	Super-Critical Extractor, Static
Microwave Extraction	100-300 ml	10-20 min	NA	Microwave Extractor, Static
Pressurized Liquid Extraction	15-40 ml	12-18 min	US $14	PLE or ASE® Extractor, Static

***Based on 2000 samples per year.**

2.4. Solid Phase Extraction

As an important sample preparation method for modern instrument analysis, Solid Phase Extraction (SPE) is known as a method in which a solid stationary phase is typically packed into a syringe barrel and used to selectively extract, concentrate, and purify target analytes prior to analysis by High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC).

During the last two decades, SPE has steadily gained acceptance within the analytical community and is now rapidly replacing traditional LLE as the sample preparation technique of choice around the world (Liska, 2000). In the early 1970's, the commercialization of SPE device provides an excellent alternative choice to the troublesome liquid: liquid extraction with better results and fewer complications in sample preparation (Liska, 2000).

According to Thurman (2001), SPE provides many advantages over LLE:

1. Improved selectivity and specificity.
2. Higher recoveries.
3. Enhanced removal of interferences and particulates.
4. More "environmentally friendly" solvents and reduced exposure of personnel to toxic and flammable solvents.
5. Enormous decreases in solvent consumption and a concomitant reduction in hazardous waste.
6. Greater flexibility in terms of solvents miscibility.
7. Elimination of emulsions in LLE.
8. Greater reproducibility.

It is obvious that SPE offers a variety of technical advantages and economic benefits that classic LLE cannot match. As SPE prevails in sample preparation in the laboratory and development of SPE sorbents suitable for industrial application, more and more researchers who are involved in R&D of bioseparation processes are willing to take the advantages that SPE provides.

2.5. Preparative Liquid Chromatography

A chromatographic method can be considered simply a physical method of separation in which components to be separated is distributed between two phases (Ahuja, 2003). According to general classification, chromatographic methods can be classified as (Ahuja, 2003):

1. Column chromatography
2. Paper chromatography
3. Thin layer chromatography
4. Gas chromatography
5. High pressure liquid chromatography
6. Ion exchange chromatography
7. Gel filtration chromatography
8. Supercritical fluid chromatography

Based on retention modes in the stationary phase, chromatographic methods can be classified as (Ahuja, 2003):

1. Sorption Chromatography
2. Exclusion Chromatography
3. Ion exchange Chromatography

Based on the manner in which the sample is introduced into the bed and migrates through it, chromatographic methods can be classified as (Ahuja, 2003):

1. Frontal chromatography
2. Displacement chromatography
3. Elution chromatography

In pharmaceutical and biotechnological industries for industrial bioseparation, the liquid phase, column, sorption and elution chromatography is the most commonly used chromatographic method. Based on the mechanism of sorption between solutes and stationary phase, preparative chromatography can be further classified as normal phase chromatography and reverse phase chromatography.

Preparative chromatography is the most important and continues to be the dominant purification technique in the production of bioactive components.

Chapter III: Theoretical Aspects of Bioseparation

3.1. Comments on Current Taxane Bioseparation Processes

New bioseparation technologies come into sight quickly. Therefore, bioseparation processes have been improved by these new technologies. No matter what fancy technologies involved in bioseparation processes, state-of-the-art or not, it is believed that there are only 3 factors that can be used to evaluate a bioseparation process:

1. *The manufacturing cost of unit bioproduct.* This is a criterion from the economic aspect of the process evaluation. Some other commonly used criteria such as recovery rate and number of unit operations are included in this criterion.
2. *The quality of the finished bioproduct.* This is an integrated criterion that stands for all the quality criteria such as current Good Manufacturing Practice (cGMP) and Good Laboratory Practice (GLP) in bioseparation processes.
3. *Environmental impact of the bioseparation process.* This is a criterion from the social aspect of the process evaluation. For most bioproduct manufacturer, low pollution generally means high manufacturing cost. Therefore, a bioprocess which can provide bioproducts at low manufacturing cost with low pollution is desired by both the manufacturers and the community to obtain economic as well as social benefits.

Research and development of bioseparation processes involves and depends on information and knowledge from many aspects. The required information and knowledge should include:

1. Both aspects of pure science and engineering.
2. Practice of research and development of successful bioseparation process at various scales, from laboratory, pilot plant to commercial scale.
3. Economic aspects such as process engineering economics.
4. Regulations such as cGMP and GLP.

If any aspect of information and knowledge is insufficient, the developed process will fail by evaluation from this aspect. Therefore, both research scientists and engineers who work in research and development of bioseparation process area are facing new challenges and should work together. As the designed processes are scaled up, the scientists who designed the processes will face more engineering problems that usually should be solved by research engineers. Some of the processes which are designed by scientists in the lab could face fatal engineering problems when these processes are scaled up and therefore, have to be given up. While the innovative division of biochemical engineering is booming, chemical engineers seem not familiar with the extremely complex biological system, which comprised thousands of thermodynamically instable components, such as biochemicals and phytochemicals, with unusual complex chemical structures and physical properties.

All current bioseparation processes for taxane mass production from *Taxus* biomass begin with solvent extraction. As a result, the processes fall into 3 categories based on the extraction techniques involved:

1. Conventional Solvent Extraction based processes.
2. Supercritical Fluid Extraction based processes.
3. New extraction technologies based processes.

Extraction is the first and the most important unit operation in a bioseparation process, although solvent extraction has been overlooked by many researchers. This is not only because the unit operation of extraction deals with the largest amount of starting material compared with any others in a bioseparation process, but also it determines the following unit operation. As a result, the largest amount of solvents is needed in extraction comparing with other unit operations in a bioseparation process because of the largest amount of biomass. Taxanes in the biomass of *Taxus* species present in very low concentrations, e.g., the concentration of paclitaxel is only 285 ppm in dry needles of *Taxus canadensis* (van Rozendaal et al., 2000). As a result, the following unit operation that recovers taxanes from extract is also important because the concentration of target molecules is extremely low.

The more unit operations the process has, the lower overall recovery rates of target molecules is. The environmental problems and manufacturing cost of unit bioproducts will increase as the number of unit operations increases as well. As previously discussed in Chapter II, CSE is the dominant extraction technique. The CSE based bioseparation processes comprise 6-13 unit operations before purification with preparative chromatography. Many unit operations in preliminary separation are required to remove most impurities that can damage separation media in preparative chromatography. As shown in Figure 3.1, the overall recovery rate drops dramatically as the numbers of unit operation increases. For example, if the recovery rate in each unit operation is 80%, after 6 unit operations, the overall recovery rate drops to less than 30%.

SFE is commonly considered as an impractical technique for taxane extraction because of the extremely low concentration of taxanes in *Taxus* biomass. The manufacturing cost of

taxanes will be considerably high if supercritical liquid extraction is involved in the bioseparation process.

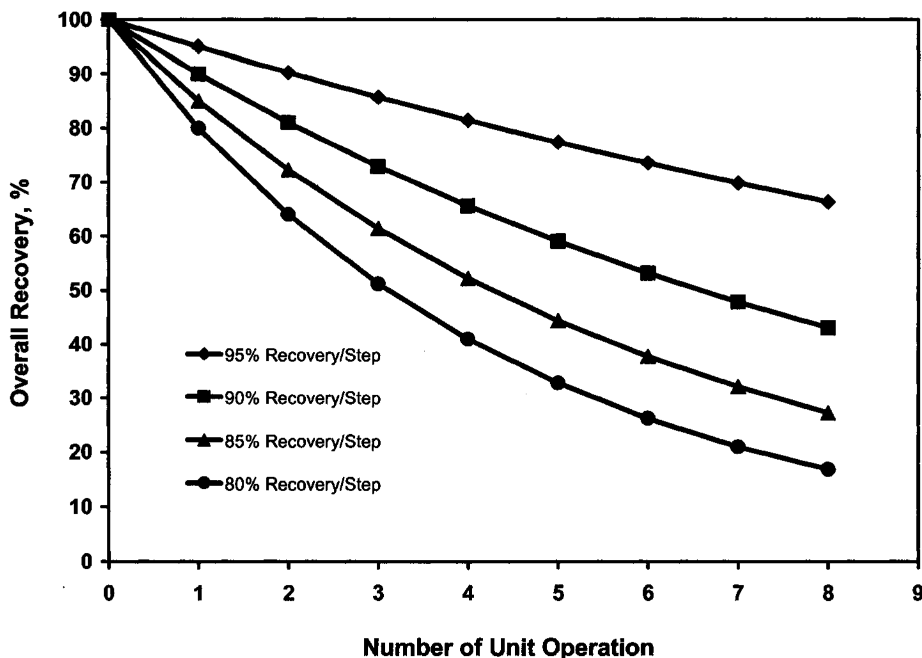


Figure 3.1 Relationships Between Overall Recovery Rates and The Numbers of Unit Operation.

Pressurized liquid extraction (PLE) is a promising extraction technique, although it is still studied only at laboratory scale and in static mode currently. The only hurdle for large scale PLE to overcome is the pressure issue. A typical PLE at laboratory scale is conducted under 3.4-20.7 MPa and holds up for 15 minutes. As PLE is conducted at static mode rather than dynamic, high pressure is not only required to maintain the solvent in liquid form at high temperature, but also required to force the solvents into pores of biomass occupied by water or air and dissolve the solutes that are trapped in them (Richter et al., 1996).

3.2. Rationale of Dynamic Pressurized Liquid Extraction

A new extraction technique, Dynamic Pressurized Liquid Extraction (DPLE) is proposed based on the rationale of PLE and is studied in this work.

In DPLE, solvents in liquid form are continuously forced thought the matrices of the biomass in extraction column under a pressure that is just above the boiling point of the extraction solvent. Water or air that block the way of solutes in the pores from solvents are gradually dissolved and removed by solvents. Therefore, compared to PLE, no high pressure is required to force the solvents into pores of matrices occupied by water or air in DPLE.

In DPLE, the pressure required to maintain the solvents from boiling is relatively low comparing with that of PLE currently used. For example, a pressure of 490 Pa is enough to keep acetone in liquid form at 100°C (Richter et al., 1996). If solvent in PLE is forced continuously through the biomass in the extraction column at a certain temperature and under a pressure that is just above the boiling point of the solvents used at the extraction temperature, namely in dynamic manner, then the extraction technique is dynamic pressurized liquid extraction (DPLE). As a result, the main hurdle for large scale PLE will be solved by DPLE.

Elevated extraction temperature is necessary because it facilitates extraction in either PLE or in DPLE, which is studied in this work, because of the following mechanisms (Richter et al., 1996):

1. Increased solubility and mass transfer rate.
    a. The use of higher temperature increases the capacity of solvents to solubilize lipids such as waxes and tar in the needle powder.
    b. Faster transfer rate is achieved as a result of elevated extraction temperature.
2. Disrupted surface equilibriums.
    a. Increased temperature can disrupt the strong solute—matrix interactions caused by van der Vaals forces, hydrogen bonding, and dipole attractions of the solute molecules and active sites on the matrix.
    b. Higher temperature can also decrease the viscosity of solvent and allow the solvent to penetrate the matrix much better.

Extraction solvents are important for DPLE as well. In addition to extraction efficiency and selectivity, low viscosity is another important criterion for solvent selection in DPLE because a pressure drop will be caused by the dynamic solvents which are forced through the biomass bed in extraction column.

3.3. Rationale of Integrated Bioseparation Process

There is a starting material and a product for each unit operation in bioseparation process. The subsequent unit operation is determined by the property of product from the preceding unit operation. For any two unit operations in a bioseparation process, if the product from a unit operation is exactly the same as starting material of another unit operation, then the two unit operations can be conducted together as one unit operation. Therefore, 2 unit operations are integrated as one.

If the property of product from the preceding unit operation is different from that of the starting material of subsequent one, one more unit operation is required between the two. The property of product from a unit operation can be adjusted easily to match the requirement of a starting material for another unit operation to reduce the number of unit operations in a bioseparation process. This is the rationale on which integrated bioseparation process is based.

One example of dynamic liquid extraction process, namely leaching, is shown in Figure 3.2 where 3 unit operations, leaching, concentration and filtration, are Integrated together. When solvent with low viscosity is forced through the extraction column, which is packed with ground twig and needle powder of *Taxus canadensis*, the extract gets thicker and thicker as it flows from the top to the bottom. The concentrated extract is filtrated at the bottom of the extraction column and separated from the solid residue in the column.

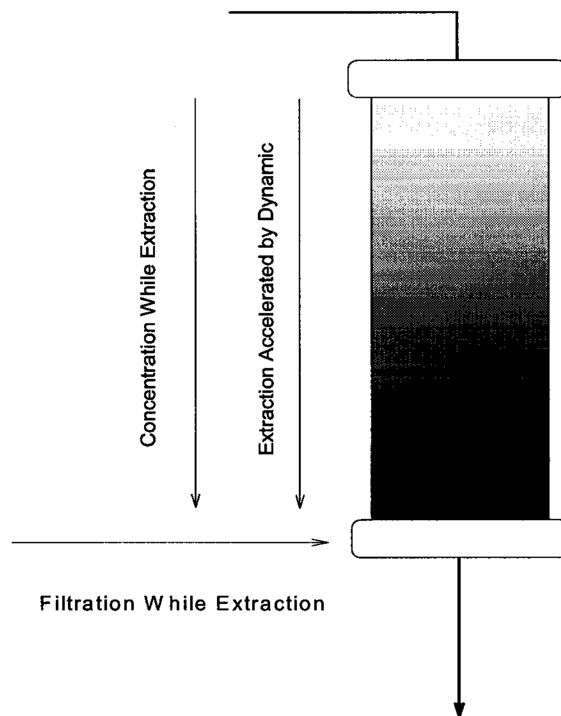


Figure 3.2 Dynamic Extraction Is Integrated With Concentration and Filtration

Another example is given in Figure 3.3 where 4 unit operations are integrated as one. All the current bioseparation processes for taxanes production from *Taxus* biomass use solid phase sample loading technique in preparative chromatography. The sample to be purified by preparative chromatography is coated on the inner surface of pores of porous material such as diatomite by drying the mixture of sample, solvent and porous material. The porous material coated with sample is packed into a sample loading column. Chromatography purification is conducted by forcing eluant to flow through the sample loading column and consequently through the preparative chromatography column. All the products are washed out from the sample loading column and thereafter into the

preparative chromatography column. Sample loading is also an extraction process in which some solutes in the matrices of porous material are dissolved by eluant.

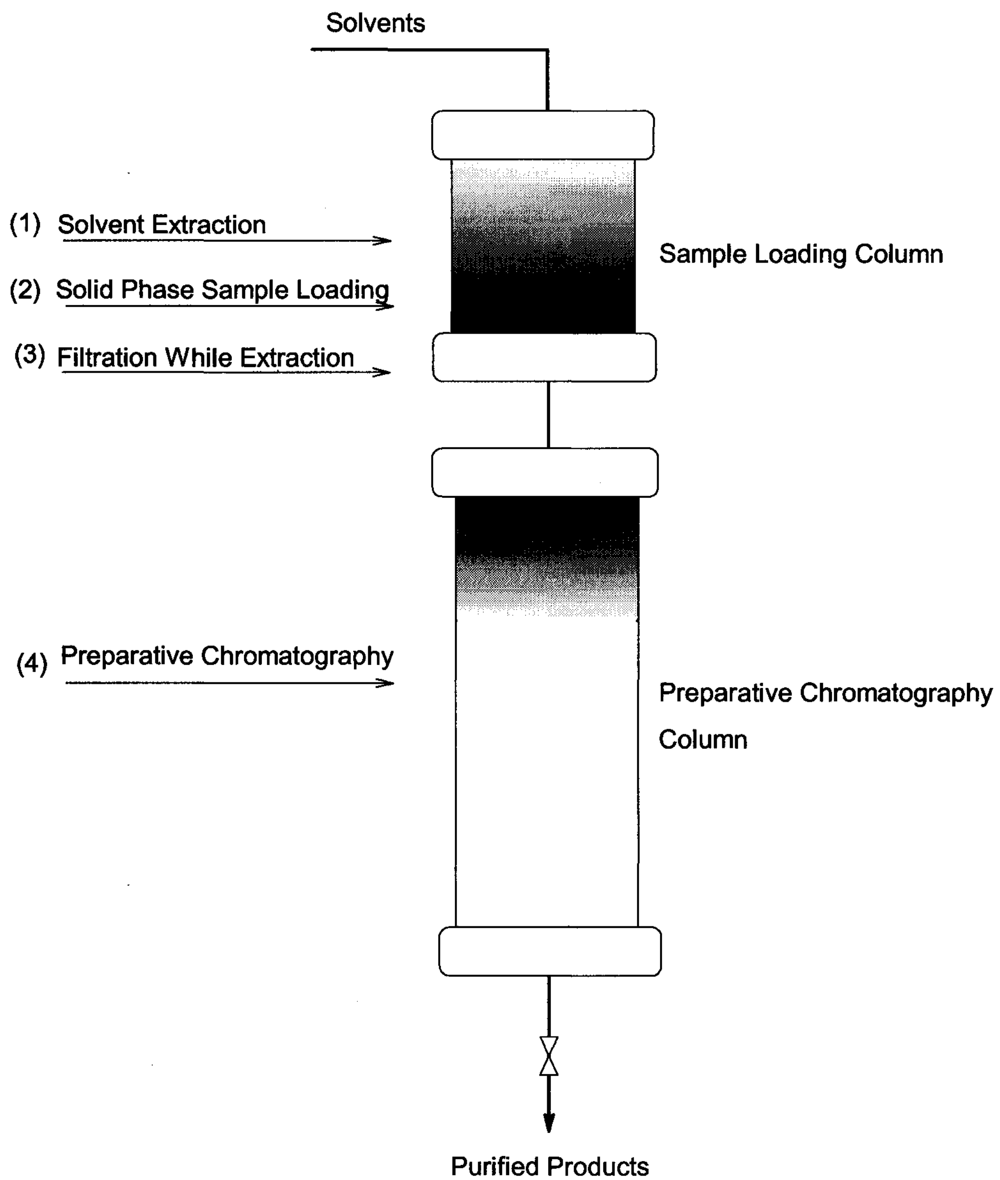


Figure 3.3 Four Unit Operations Are Integrated As One

3.4. Solute Distribution Model for Extraction and Recovery

In isolation of bioproducts from biomass, solvent extraction is usually the first and most important step. It determines the following unit operations. The extraction process must be rethought and explored in a new approach so that a true breakthrough can take place.

Figure 3.4 is typical HPLC chromatogram of a 70% acetone/water extract of ground needle powder of *Taxus canadensis*. Figure 3.5 is part of the HPLC chromatogram in Figure 3.4. From these chromatograms it can be noticed that there are few impurities with retention time close to those of taxanes. Therefore, there are baselines adjacent to the peaks of paclitaxel which the retention time is 36 minutes and 9-DHB III, which the retention time is 26 minutes. But there are large amount of hydrophilic impurities at the beginning of the chromatogram with retention time less than 10 minutes. There are also hydrophobic impurities near the end of the chromatogram. This means that most of the impurities are extremely hydrophilic or extremely hydrophobic. The properties of most of the impurities in terms of hydrophilicity or hydrophobicity are far from those of taxanes.

The solubility of hydrophilic compounds in polar solvents, such as water and methanol, is very high. However, their solubility in nonpolar solvents, such as hexane and dichloromethane, is very low. On contrary, the solubility of hydrophobic compounds is high in nonpolar solvent and low in polar solvents. This is also a criterion in solvent selection in the study of CSE in this work.

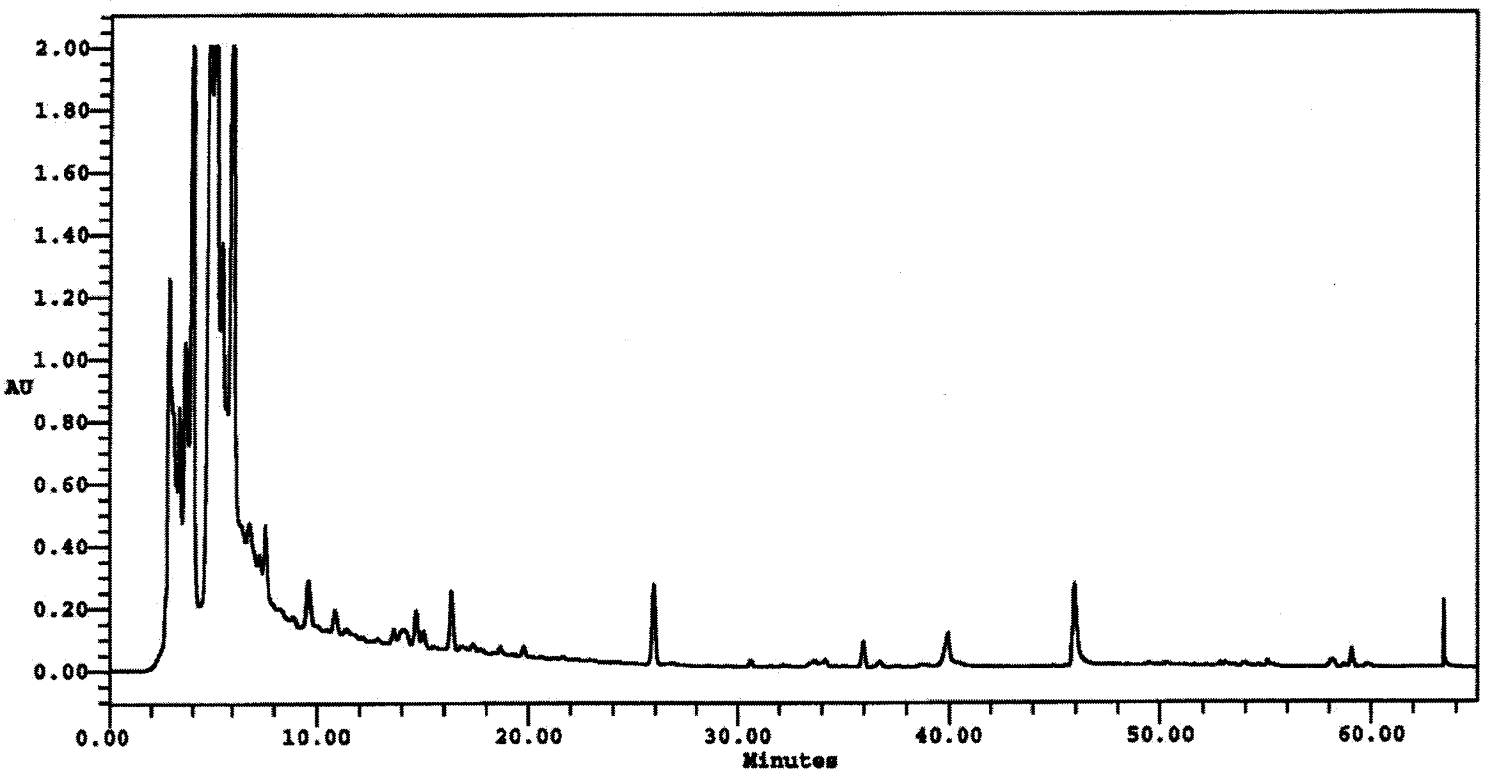


Figure 3.4 HPLC Chromatogram of 70% Acetone/Water Extract of *Taxus canadensis*. Extraction Condition: <100 Mesh Needle Powder with Celite 545; Extraction Temperature is 25°C; Time is 10 Minutes; Retention Time (min): Paclitaxel, 36.0; 9-DHB III , 26.0

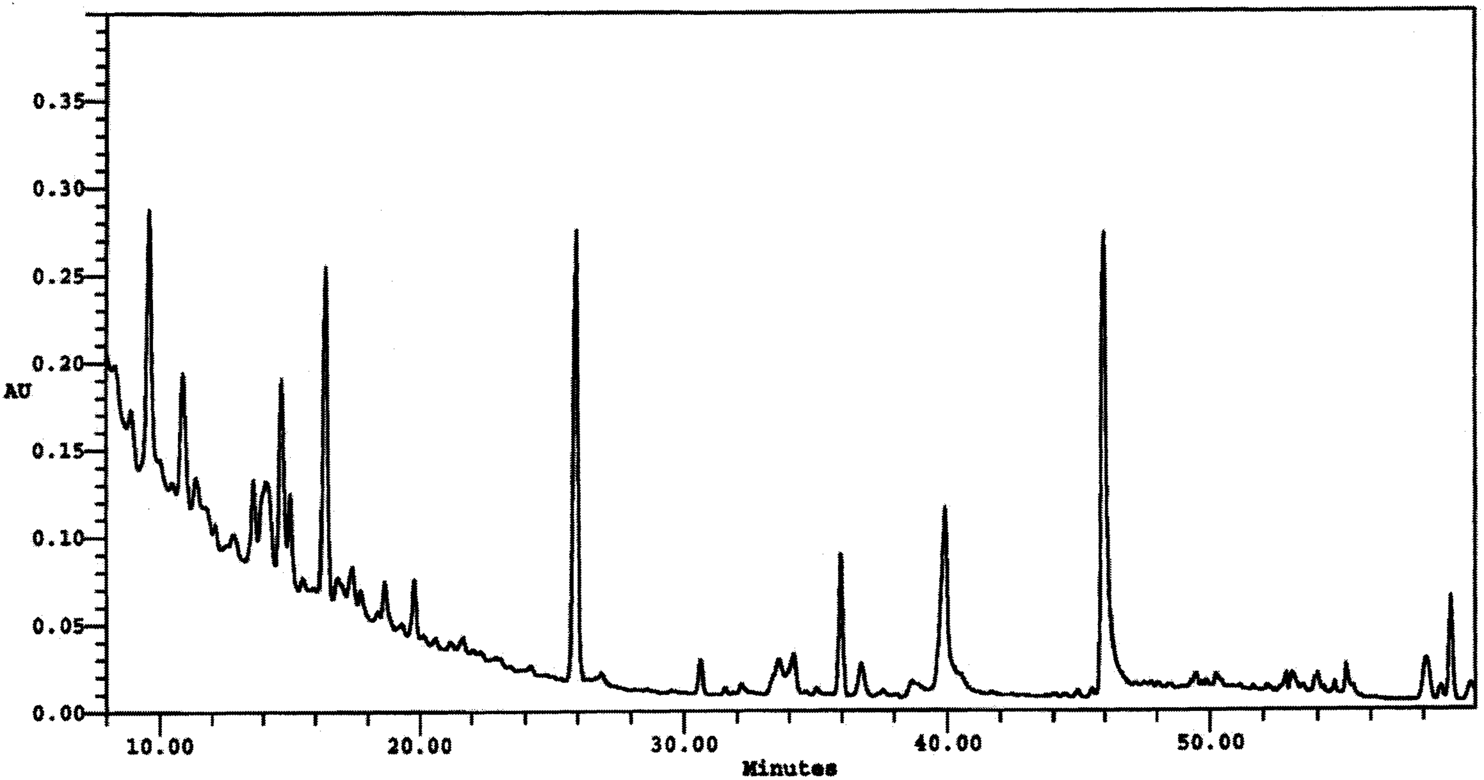


Figure 3.5 Detail Show of HPLC Chromatogram of 70% Acetone/Water Extract. Extraction Condition: <100 Mesh Needle Powder with Celite 545; Extraction Temperature is 25oC; Extraction Time is 10 Minutes; Retention Time (min): Paclitaxel, 36.0; 9-DHB III, 26.0

Based on the HPLC chromatograms of extract in Figure 3.4 and Figure 3.5, in term of hydrophilicity or polarity, the relative amount distribution of all the solutes in the biomass of the *Taxus canadensis* is illustrated approximately as Figure 3.6. Among the four taxanes, 10-DAB III is the most hydrophilic one and therefore appears far left in this figure. Paclitaxel is the most hydrophobic one and appears far right.

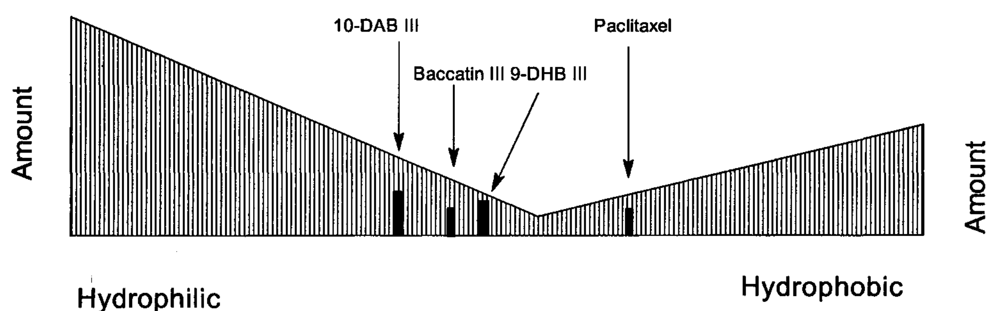


Figure 3.6 Schematic Sketch of Solute Amount Distribution in *Taxus* Biomass in Terms of Hydrophilicity or Hrdrophobicity Based On the HPLC Chromatogram of Extract

For the solubility of solutes in a solvent, "like dissolves like" means that only the solute with specific property in terms of polarity and chemical structure similar to the solvent has high solubility in the solvent. If the property of a solute varies from that of the solvent, its solubility decreases.

The solubility of solutes in a specific solvent is symbolized by a triangle as shown in Figure 3.7. Most of the solutes from *Taxus* biomass can be dissolved in a chosen solvent. As a result, there is not a solvent that has been found with satisfactory selectivity for taxane extraction so far. All the currently used solvents can extract, besides taxanes, a vast amount of impurities which widely differ in polarity.

Although methanol is considered as a good solvent for dissolving taxanes, it can also dissolve large amount of impurities as shown in Figure 3.7 (1). A solvent with increased polarity, as shown in Figure 3.7 (2), can dissolve more hydrophilic impurities than methanol does. But it dissolves much less hydrophobic impurities compared with methanol. Although this solvent has lower solubility for taxanes than methanol, it can also completely dissolve all the taxanes because they are present in a very low concentration.

Similarly, as shown in Figure 3.7 (3), a solvent with decreased polarity dissolves more hydrophobic impurities and less hydrophilic ones than methanol does.

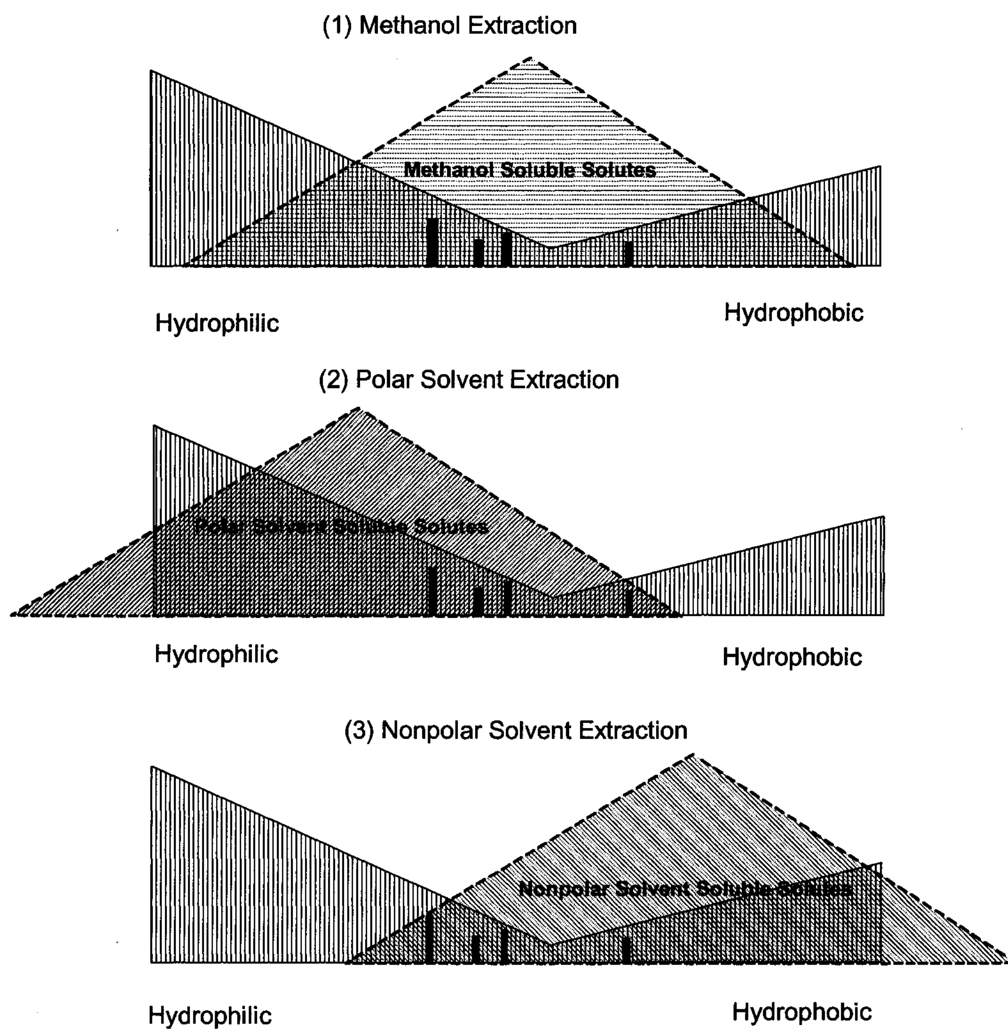


Figure 3.7 Schematic Sketches of Extractions with Solvents with Various Polarities

3.5. Approaches to Increase Selectivity in Bioseparation

Increased selectivity in solvent extraction is desired. Unfortunately, there is not any single solvent available with fulfilled selectivity in extraction of taxanes from biomass. Some means to increase extraction selectivity, which are based on the proposed solute distribution model, are invented and studied in this work.

3.5.1. Dual-Solvent Extraction Approaches

As shown in Figure 3.8, extraction with two solvents with different properties can achieve high selectivity with a high product recovery rate. The two solvents may be different solvents; they also may be the same solvent under different temperature. In the dual-solvent extraction process, the extraction matrices may be either the same matrices or different ones.

In Figure 3.8 (1), most hydrophilic impurities can be removed with water first. Then the taxanes left in the biomass are extracted with a suitable organic solvent. Alternative way is that most hydrophilic impurities and taxanes are extracted with a suitable solvent, and then the hydrophilic impurities are removed with water by means of precipitation or extraction from the second dry matrix containing all the solvent dissolved solutes.

Similarly in Figure 3.8 (2), two nonpolar solvent are used consequently. Nonpolar solvents usually possess low viscosity and are suitable for leaching. Dynamic Pressurized Liquid Extraction (DPLE) studied in this work utilizes this approach to increase extraction selectivity.

Both polar solvent and nonpolar solvent are used in the approach shown in Figure 3.8 (3) to increase extraction selectivity. This approach is also used in DPLE based process to increase extraction selectivity.

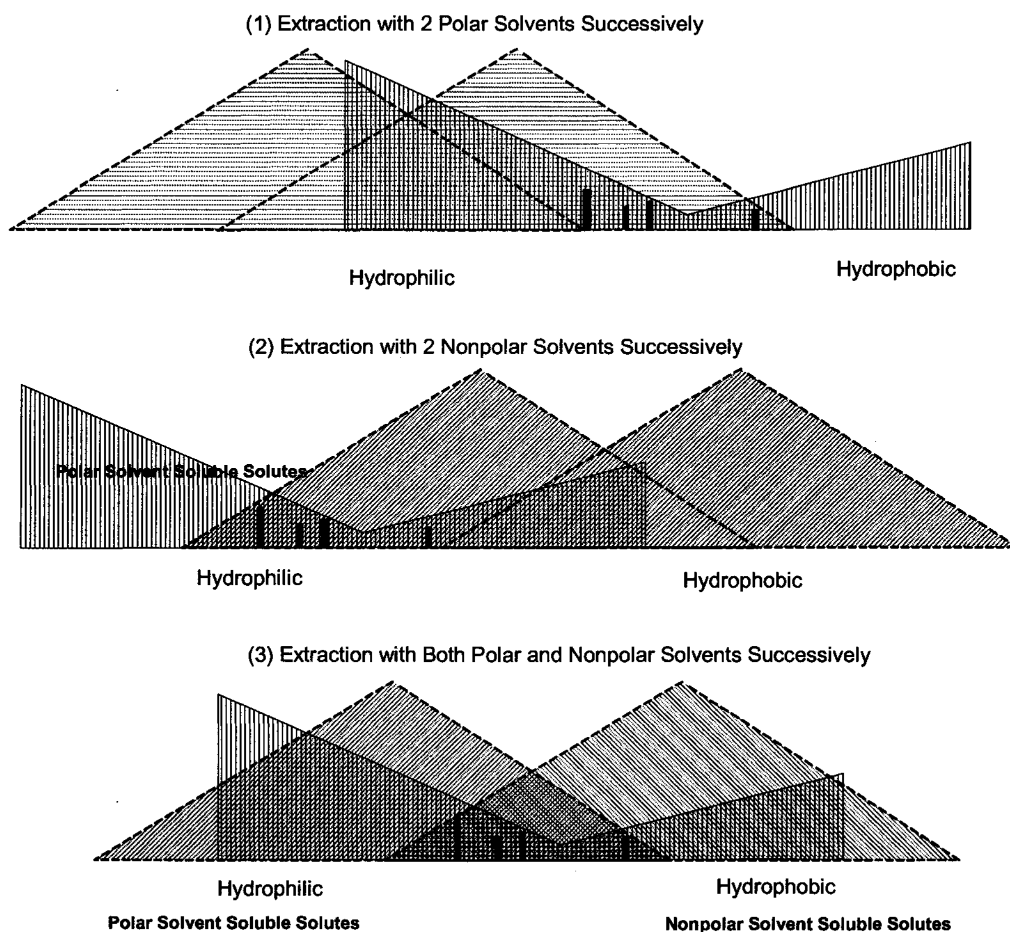


Figure 3.8 Schematic Sketches of Strategies to Increase Extraction Selectivity By Means Of Dual-Solvent Extraction

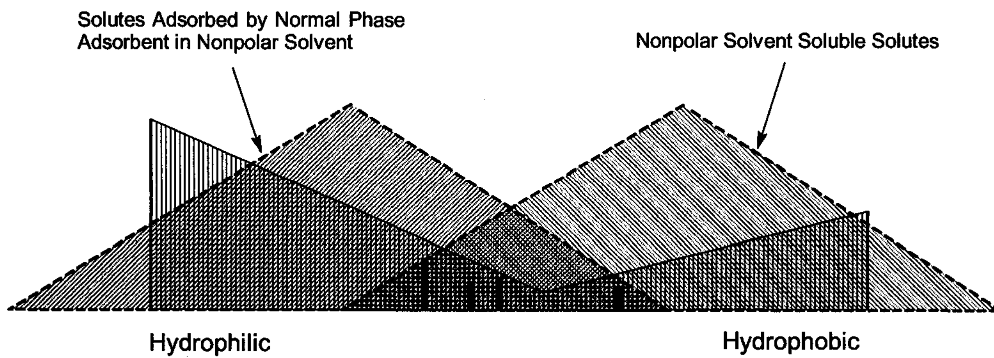
3.5.2. Extraction-Adsorption Approaches

In addition to dual-solvent extraction approaches, there are other approaches to increase selectivity in the taxane recovery: Solvent Extraction followed by Solid Phase Extraction as shown in Figure 3.9. In the strategy shown in Figure 3.9 (1), taxanes extracted by non-polar solvents, such as dichloromethane can be selectively adsorbed with normal phase adsorbent, such as silica gel, by normal phase solid phase extraction (NP-SPE) while the most of hydrophobic impurities are eluted and separated from taxanes in this process. The NP-SPE may be followed by normal phase preparative chromatography to yield products with high purity. Therefore, the two unit operations are Integrated as one.

In strategy (2), taxanes extracted by polar solvents in CSE can be selectively adsorbed on the surface of reverse phase adsorbent, such as resin or activated charcoal, in reverse phase solid phase extraction (RP-SPE) while most of the hydrophilic impurities can not effectively absorbed and are separated from taxanes. Similarly, the RP-SPE can be followed by reverse phase preparative chromatography to obtain high pure taxanes.

Both dual-solvent extraction approaches and extraction-adsorption approaches are ideal cases. In fact, biological systems are extremely complex. The solutes are not simply distributed homogenously in the biomass. Extraction is not merely just dissolution. The appropriate approaches can be selected based on specific solute distribution model and the experimental results. For example, according to the result of this work, most of impurities are hydrophilic. The weight of the hydrophilic impurities is up to 50% of the dry needles. Therefore, extraction using polar solvents should be avoided because it produces a large amount of toxic waste water with low extraction selectivity.

(1) Nonpolar Solvent Extraction followed by Normal Phase Solid Phase Extraction



(2) Polar Solvent Extraction followed by Reverse Phase Solid Phase Extraction

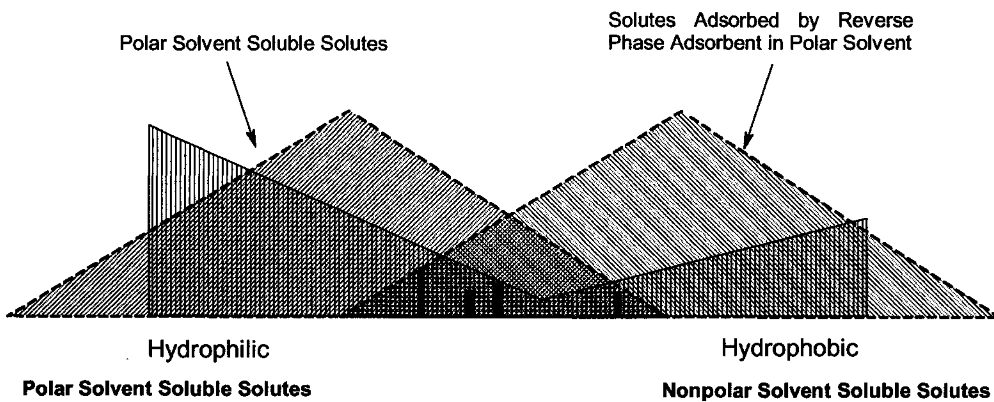


Figure 3.9 Schematic Sketches of Strategies to Increase Selectivity By Means of Extraction-SPE

Chapter IV: Experimental Methodology and Analysis

4.1. Material

4.1.1. *Taxus Canadensis* Needle Powder

Fresh twigs and needles of *Taxus canadensis* were picked at Hartland and Rexton, New Brunswick, Canada in May, 2003. After drying for 7 days in darkness at ambient temperature and humidity, the needles were stripped manually from stems and ground to powder with particle sizes that are finer than 20 mesh. The ground needle powder was stored in fridge at a temperature below -10°C. Before each experiment, the material of ground needle powder was freshly prepared by grinding again with household coffee mill (Braun, Type 4041, and Model KSM2), sieved and dried at 60°C for 4 hours in air ventilation dryer with digital temperature controller (Fisher Scientific, Model 737F). The sieved needle powder was mixed thoroughly to obtain homogenous needle powder for experiment.

4.1.2. Solvents and Water

The purity of ethanol was 99% ethanol/water. All other solvents were HPLC grade (EM Science, Gibbstown, NJ).

Water used in this study was HPLC grade prepared by a Zenopure® four-cartridge system (Type QUATRA 90LC, Zenon Environmental Inc., Burlington, ON, Canada).

4.1.3. Solid Phase Extraction Absorbents

Silica gel was purchased from Fisher Scientific (Selecto Scientific, Georgia, USA). Its particle size ranges from 32 μm to 63 μm. It is used without any further treatment in this study.

Macro-porous resins (Type: SP207 and HP2MG) were purchased from Mitsubishi Chemical Corporation, Tokyo, Japan.

Charcoal (20-40 mesh and <100 mesh, activated) was from EM Science, Gibbstown, NJ. They were used without any further treatment in the laboratory.

Celite® 545, which was used as filter aid in CSE experiments, was purchased from EMD Chemicals Inc., Gibbstown, NJ, USA.

4.1.4. Taxanes External Standards

Paclitaxel (99%), 10-deacetyl-baccatin III (10-DAB III, 95%), baccatin III (90%) were purchased from Fisher Scientific (by Acros® Organics) and used as external standards.

9-dihydro-13-acetyl-baccatin III (9-DHB III, 95%) was from NaPro BioTherapeuitcs, Inc., Boulder, CO, USA and was also used as external standard.

4.2. Dynamic Pressurized Liquid Extraction

4.2.1. Equipment

Dynamic pressurized liquid extraction was carried out using the experimental setup shown in Figure 4.1.

Waters® 501 HPLC pump was used to force the solvents through the system in this setup. The flow rate ranged between 0.0 ml/min and 9.9 ml/min.

The extraction cell and solid phase extraction cell were Omnifit® medium pressure preparative chromatography columns (15 mm inner diameter, 100 mm in length, pressure rate 2.1 MPa) made of borosilicate glass. Each of them is with a fixed endpiece and an adjustable endpiece.

The heat exchangers were made of copper tubing with 150mm in length, 2mm in inner diameter.

The relief valve was Swagelock®, Type RL3; relief pressure is adjustable, and purchased from Ottawa Fluid System Technologies Inc., Ottawa.

The valves were Omnifit® three-way valves made of polytetrafluoroethylene (PTFE). The Swagelock® pressure gauges with a range of between 0 and 690 Pa were purchased from Ottawa Fluid System Technologies Inc., Ottawa.

The hot water bath was Ultra-Thermostat®, Model NB-35 703.

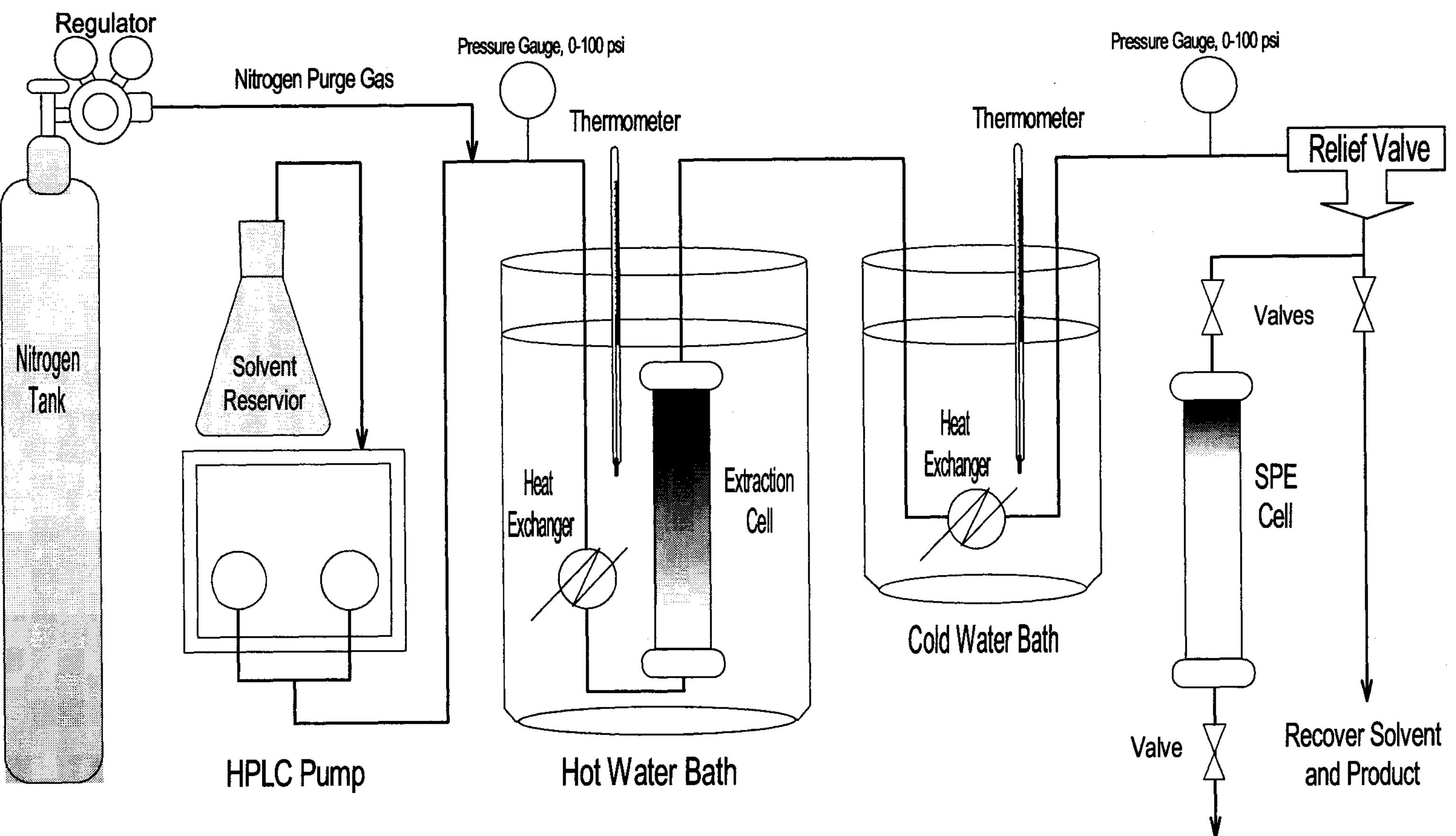


Figure 4.1 Dynamic Pressurized Liquid Extraction Experimental Setup

4.2.2. Procedures of DPLE

Hexane was selected as the extraction solvent to remove hydrophobic impurities from biomass in DPLE. The solvents used to extract taxanes from the needle powder were chloroform, dichloromethane plus a co-solvent. Methanol was chosen as the co-solvent of dichloromethane in this study. The selection of solvents was based on their physical properties including polarity, solubility, viscosity and boiling point.

Five grams of needle powder was weighed and carefully transferred into extraction cell. The height of the bed of the extraction column was set to 4.5cm by adjusting the adjustable endpiece of the column.

The hot water bath was set at a required extraction temperature; and when the temperature reached and became constant, extraction experiment was conducted.

The solvent was purged with high purity helium for 30 minutes using an HPLC online degassing system before experiment was conducted.

After the extraction cell was assembled to the extraction system, the seals of the system were tested with pressurized nitrogen. The system including the extraction cell was purged with nitrogen in order to remove oxygen that can oxidize some solutes during extraction at high temperature. The last preparation step before extraction was adjusting the relief valve to maintain the system pressure within 483-517 Pa to prevent the solvents from boiling.

The extraction cell and heat exchanger upstream of the extraction cell were immerged into hot water bath for 5 minutes until the temperature of extraction cell reached the extraction temperature. Then solvent was pumped at a constant flow rate through the system including the heat exchanger in hot water bath, the extraction cell, and the heat exchanger in cold water bath and relief valve. Time recording started when the first drop of extract came out of the system. The extract was collected in 10 ml pre-weighed and labeled test tubes. Test tube in which extract was collected was changed every 5 minutes until the end of the extraction.

After extraction, the system was first purged with high pressure nitrogen (483-517 Pa) in order to remove liquid solvent. Then the pressure of the system was reduced to ambient pressure and purged with low pressure nitrogen (less than 69 Pa) for 5 minutes to remove solvent residue.

At the final stage of extraction, the extraction cell and heat exchanger were taken out from hot water bath and cooled down in fume hood. The residue of needle powder in the disassembled extraction cell was pushed out from the column by pushing the adjustable endpiece for further analysis.

In the experiment of DPLE with NP-SPE experiment, extract was lead into NP-SPE column instead of being collected with test tubes. The NP-SPE columns were packed with 5.000 g silica gel before the experiment.

4.3. Paclitaxel Stability Study in DPLE

Five grams of <100 mesh needle powder was weighed and transferred into extraction column. The extraction column was filled with hexane and then sealed at both ends. Time recording begins when the hexane filled extraction column with needle powder was put in 90.0°C water bath.

The extraction column is put into cold water bath immediately after withdrawal from hot water bath. After cooling in cold water bath for 10 minutes, the hexane extract and needle powder were pushed out of the extraction column into 80 ml beaker. The extraction column was washed with 20-30 ml hexane and the washing hexane went into the beaker as well. Hexane was removed by placing the beaker containing biomass and hexane in fume hood for 12 hours at ambient temperature.

A magnetic bar was placed into the beaker. Then 50.0 ml HPLC grade methanol was added. The extraction was carried out under agitation at room temperature for 60 minutes. The methanol extract was pre-purified with SPE and analyzed with HPLC for paclitaxel content.

4.4. Conventional Solvent Extraction

Conventional solvent extraction experiment was carried out using apparatus shown in Figure 4.2. The agitation speed of the magnetic agitator was fixed and the size of magnetic bar was the same throughout all CSE experiment. A Buckner funnel and a filtering flask with a 70 ml test tube inside were used as vacuum filtration system.

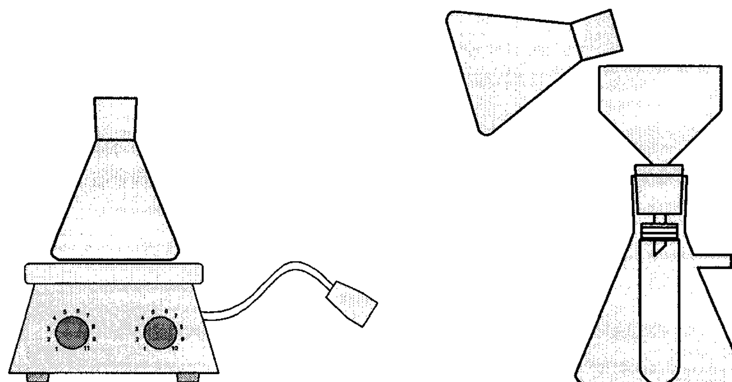


Figure 4.2 Conventional Solvent Extraction and Filtration Setup

Five grams of needle powder of *Taxus canadensis* and 3.0 g Celite® 545 as filter aid were weighed and transferred into 125 ml Erlenmeyer flask with a magnetic bar. Time recording started when 50.0 ml solvent was added to the Erlenmeyer flask and agitation began. The extract and the residue of needle powder were separated by filtration immediately after extraction. The extract was collected in a 70 ml test tube for further analysis. The extraction and filtration were carried out at ambient temperature (23-26 °C).

4.5. Solid Phase Extraction

4.5.1. DPLE with NP-SPE Experiment Procedures

Only continuous normal phase solid phase extraction (NP-SPE) was studied in the Dynamic Pressurized Liquid Extraction process.

In the continuous DPLE with NP-SPE experiment, five grams of silica gel was weighed and packed in solid phase extraction cell as illustrated in Figure 4.1. The eluate from the NP-SPE column was collected in a Petri dish and dried in fume hood for 12 hours. The

solid in the Petri dish was dissolved in methanol for HPLC analysis after filtration with 0.45 μm filter.

4.5.2. RP-SPE Experiment Procedures

Only batch reverse phase solid phase extraction was studied in conventional solvent extraction experiment. In the batch experiment, five grams of absorbent was weighed and put into 125 Erlenmeyer flask with magnetic bar. Time recording started immediately when 50.0 ml extract was added to the flask. After 90 minutes, the absorption was ended by filtration using the filtration system as shown in Figure 4.2. The filtrate was collected for further taxane content analysis with HPLC.

4.6. Analytical Methods

4.6.1. HPLC Method

The HPLC system comprised of a Millennium™ 2010 Chromatography Manager (a NEC™ 486/33i computer with Millennium™ Software 2.0), a 600E Multisolvent Delivery System, a Waters™ 717 Autosampler and a 486 Tunable Absorbance Detector. High purity helium was used for online degassing.

HPLC column (Curosil®-PFP, 250×4.6 mm) and guard column (Curosil®-PFP, 30×4.6 mm) were purchased from Phenomenex USA (Torrance, CA, USA). The wavelength of the UV detector was 227 nm. The injection volume was 10 μL. Gradient table is shown in Table 4.1.

Table 4.1 HPLC Gradient Table

	Time (min)	Flow (ml/min)	% Acetonitrile	% Water	Curve No.	Curve Description
1	0.00	1.00	25	75	6	Linear gradient
2	40.00	1.00	75	25	2	Convex gradient
3	55.00	1.00	100	0	2	Convex gradient
4	65.00	1.00	25	75	11	Maintains start condition

Taxane in samples was analyzed with HPLC. The concentrations of taxanes were given by comparing the areas of taxane peaks with those on the calibration curves prepared previously.

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

The HPLC chromatogram of mixed external standards is shown as Fig 4.3. The retention time of each taxane standard is given in Table 4.2.

Table 4.2 Retention Time of External Standards of Taxanes

Taxane	10-DAB III	Baccatin III	9-DHB III	Paclitaxel
Retention Time (min)	15.042	23.150	26.333	36.333

Taxanes in HPLC chromatogram are identified by comparing the retention times of peaks with those of external standards.

If any taxane cannot be identified clearly by its retention time because of poor separation with HPLC or variation of retention time, a few drops of acetonitrile solution of the specific taxane external standard are added to the sample and then analyzed with HPLC. The specific taxane peak is identified as the one with increased height by comparing the two chromatograms of the sample.

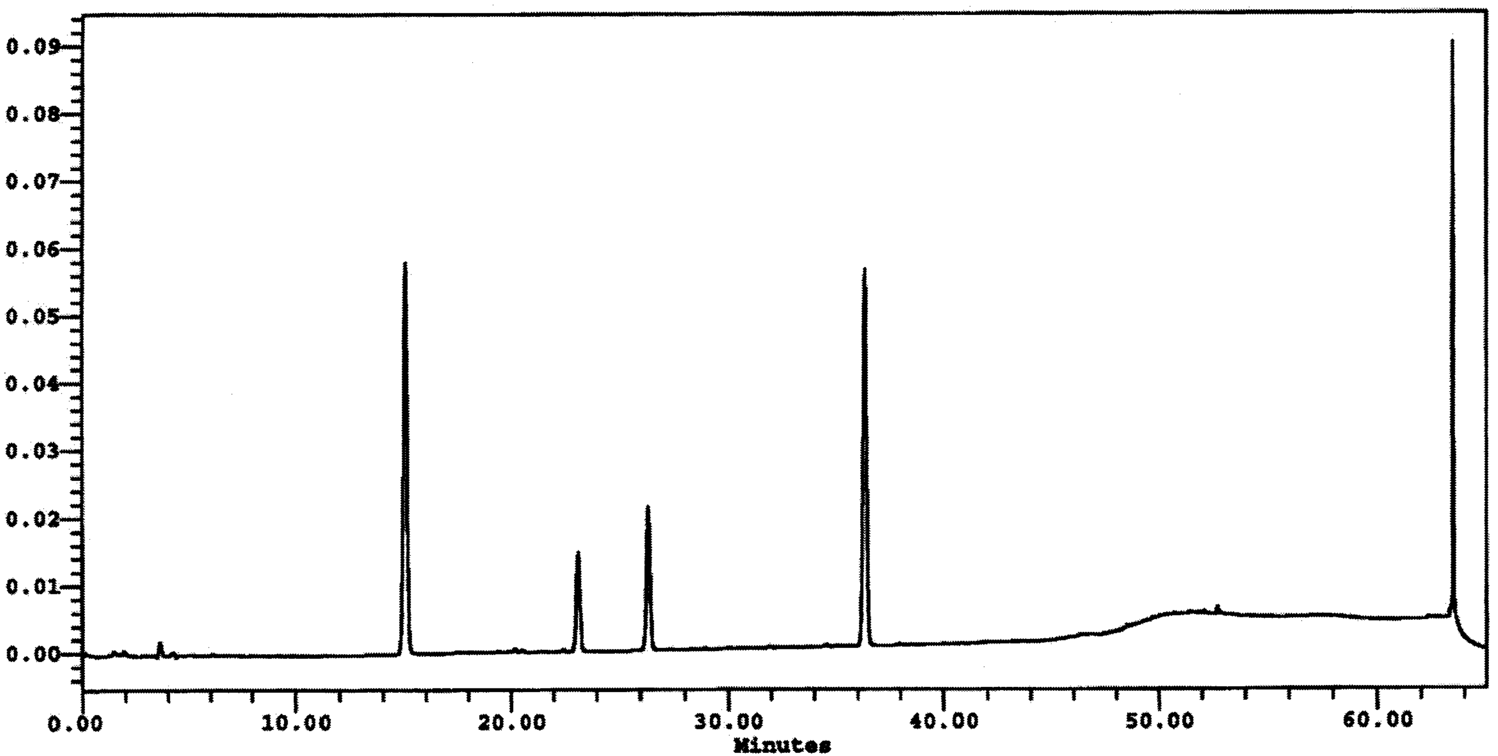


Figure 4.3 HPLC Chromatogram of Mixed External Standards. The Taxanes With Retention Time Listed From Left To Right of The Chromatogram Are: 10-DAB III, 15.042 min.; Baccatin III, 23.150 min.; 9-DHB III, 26.333 min.; Paclitaxel, 36.333 min.

4.6.2. Sample Preparation with Solid Phase Extraction for HPLC Analysis

Maxi-Clean^{TM} (C18) 300 mg SPE cartridges were purchased from Alltech Associates, Inc., Deerfield, IL, USA. The SPE procedure is described as following:

1. The cartridge was conditioned with 2 ml of methanol, then 2 ml HPLC grade water.
2. A small amount of sample in methanol was added into the same amount of HPLC grade water to make a 50% methanol/water solution.
3. The diluted sample was slowly loaded into the conditioned cartridge, and left for 30 minutes.
4. The cartridge was eluted with 2 ml 20% methanol.
5. The cartridge was eluted with 2 ml 50% methanol.
6. The taxanes were eluted with 4.0 ml 100% methanol for HPLC analysis.

The SPE method was calibrated and verified with recovery experiment. Details of recovery experiment are provided in Appendix E.

4.6.3. Weight Analysis of Extract

Known volume of extract was added into pre-weighed Petri dish. The Petri dish is put in fume hood for 12 hours (if the extract does not contain water) or 24 hours (if the extract contains water). The Petri dish with dried solutes was then weighed. The weight of solutes was obtained by subtracting the weight of Petri dish from the gross weight.

Chapter V: Result and Discussion

5.1. Dynamic Pressurized Liquid Extraction

In Dynamic Pressurized Liquid Extraction, the selection of solvents, the amount of extraction solvents, the particle size of biomass, temperature and pressure can affect the efficiency of extraction, either on recovery rate of products or on selectivity. In addition, all these factors may have some combined relationships and affect one another.

The amount of extraction solvents in DPLE is studied in terms of flow rate and extraction time. The effects of extraction temperature and particle size of needle powder were studied under various conditions as well.

Although high pressure can facilitate extraction a little bit, it is not economically favorable to utilize high pressures in large scale PLE because of the high cost of equipment and safety issue. High pressure is not necessary in DPLE because the most important functions of high pressure in PLE are replaced in DPLE by dynamic flowing solvents except keeping solvents from boiling at elevated temperature. Therefore, pressure effect on DPLE is not studied in this work.

As elevated temperature is utilized in DPLE to facilitate releasing bioproducts from biomass, the stability of paclitaxel, which is the most important product and known as thermodynamically unstable, is therefore studied at high temperature.

5.1.1. Effect of Temperature on DPLE

Hexane and pure dichloromethane were used at various temperatures in studying the temperature effect on DPLE. The low viscosity of both hexane and dichloromethane makes them suitable for using in DPLE because of the expected low pressure drop. The residue of solvents in biomass after extraction was not a concern due to the low boiling point of these solvents.

In extraction of lipids, hexane was used in DPLE to remove hydrophobic impurities in the needle powder. High yield of extract weight with low taxanes content using a small amount of solvent is desired.

In extraction of taxanes, dichloromethane was used in DPLE. Low yield of extract weight and high recovery rate of taxanes in extract using a small amount of solvent (in terms of flow rate and extraction time) are desired.

The needle powder with 40-60 mesh particle size was used in the study of temperature effect on DPLE with hexane to remove lipids. Hexane was selected because that it is a conveniently available nonpolar solvent. Hexane was used to remove lipids from extract by means of Liquid-Liquid Extraction in previous arts.

The color of the hexane extract was dark green. The biomass left in the extraction column was light brown. This suggests that most chlorophyll on the surface of the particles of needle powder was removed efficiently in DPLE by hexane.

Elevated temperature plays a key role in both PLE and DPLE to facilitate mass transfer rate and solubility of the solvent. The viscosity of extraction solvent also decreases at

elevated temperature. Decreased viscosity of solvent is important in DPLE because low viscosity of a flowing solvent means a low pressure drop through the bed in the column. On the other hand, high extraction temperature means high operation and equipment cost. In natural product extraction, some molecules will be degraded at high temperature.

The trends that extract weight increases with temperature using hexane are observed (Figure 5.1). According to the analysis of Richter et al., (1996), this is a predictable result. In this case, this can be explained by the following:

Firstly, hexane at higher temperature has increased capacity to solubilize lipids in needle powder of *Taxus canadensis*. In lipids removal with hexane by Liquid-Liquid Extraction, hexane has limited capacity to solubilize lipids. As a result, large amount of hexane is necessary. However, in lipids removal with hexane by DPLE, amount of solvent is reduced due to its increased capacity to solubilize lipids.

Secondly, the strong lipids-matrix interactions are disrupted by elevated temperature. These interactions are caused by van der Waals forces, hydrogen bonding and dipole attractions of the solute of molecules and active sites on the matrix of needle powder of *Taxus canadensis*.

Thirdly, better penetration of matrix particles is achieved by decreased viscosity of hexane at elevated temperature.

Comparing to DPLE, in Liquid-Liquid Extraction, the ratio of amount of lipids between hexane phase and methanol-water phase is a constant at a given temperature. Therefore, one stage of Liquid-Liquid Extraction cannot remove lipids from extract completely.

The trends of extract weight increases with temperature using dichloromethane are also observed as shown in Figure 5.2. However, when temperature approached to 100°C (Figure 5.1 and Figure 5.2), the increasing trend curves become flat.

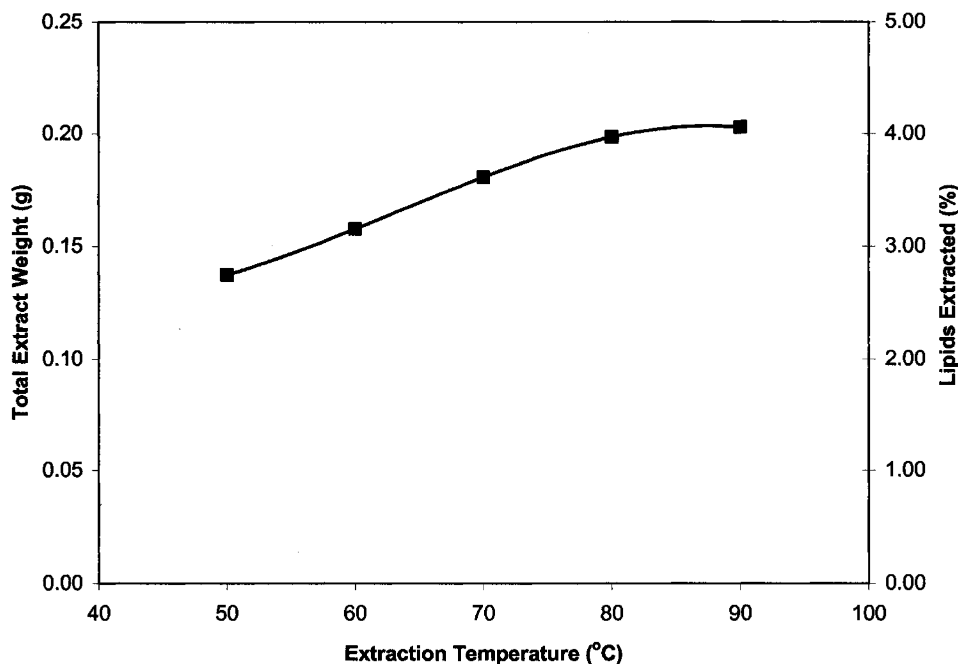


Figure 5.1 The Effect of Temperature on DPLE in Lipid Removal with Hexane. Extraction Condition: 5.000 g 40-60 mesh needle powder; flow rate 1.0 ml/min; extraction time 60.0 min.

The fact that the increasing trend curves become flat over a certain temperature indicates that there may be a "threshold" temperature for each DPLE extraction under specific extraction conditions. If the extraction temperature is below the "threshold" temperature, the efficiency of DPLE will be increased by increasing temperature. If the DPLE is carried out above the "threshold" temperature, the efficiency of DPLE will not be increased by increasing temperature. The "threshold" temperature for DPLE also depends on extraction solvents, solutes, the matrix and other properties of biomass.

Moisture content of biomass may affect extraction with nonpolar solvents. There is a large amount of hydrophilic macromolecules such as celluloses and hemicelluloses in the needle powder which can be activated as normal phase absorbents by reducing moisture and deactivated by increasing moisture (Hopkins, 1999). These hydrophilic macromolecules can absorb polar components in nonpolar solvents such as hexane and dichloromethane. This feature of needle powder is similar to that of normal phase absorbents such as cellulose, chromatographic paper and silica gel.

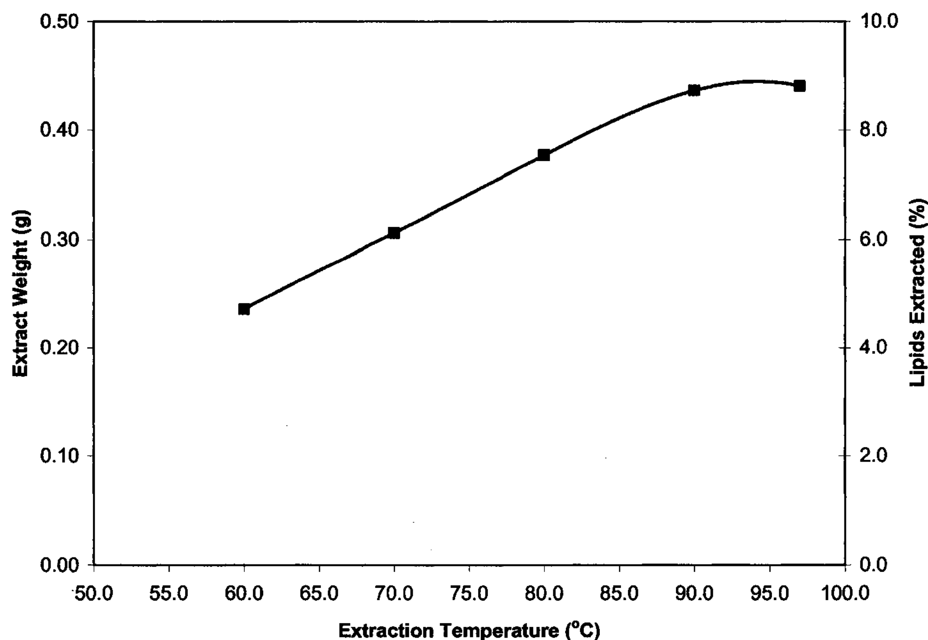


Figure 5.2 Total Extract Weight Increases with Extraction Temperature in Taxane Extraction. Extraction Condition: 5.000g <100 mesh needle powder; solvent dichloromethane, flow rate 1.0 ml/min; extraction time 60 min; the biomass in the column was pretreated with hexane at 90°C, 1.0 ml/min, and 30 min.

Among the four taxanes, 10-DAB III, Baccatin III, 9-DHB III and paclitaxel, only paclitaxel was detected in the extract of needle powder with DPLE using hexane (Table

5.1). Paclitaxel is present in the extract even when DPLE was conducted under room temperature. In the first 5 ml extract, paclitaxel content increases with extraction temperature (Figure 5.3).

After the hexane extract from DPLE was left in fume hood at room temperature for 12 hours; a small amount of green precipitate appeared on the bottom of the test tube in which the hexane extract was collected. The precipitate was separated by filtration. The filtrate was collected in Petri dish and left in fume hood for 12 hours. The paclitaxel content of both dried filtrate and precipitate was determined with HPLC.

Fortunately, there was no paclitaxel detected in the tar-like dried filtrate (Table 5.1). This means that all the paclitaxel extracted by DPLE with hexane was in the precipitate and readily separated from most lipids in hexane extract.

Table 5.1 Hexane Extract Analysis Results After 12 Hours Precipitation At Room Temperature. DPLE Condition: 1.0 ml/min, 90.0°C, 30 minutes.

	Dried Filtrate	**Precipitate**
Net Weight (g)	0.28345	0.02425
Percentage of Dry Needle Powder	5.669	0.485
Appearance	Dark brown, tar-like semi-solid	Fine green powder, readily dissolved in dichloromethane or methanol
Taxanes Content	No taxanes detected	Paclitaxel only, 80 μg

The green precipitate from hexane extract was very soluble in methanol; 70 mg of green precipitate was easily dissolved in 2.0 ml methanol. No back pressure increase was

observed in HPLC system after 6 injections of this methanol solution in HPLC analysis without any treatment. This result implies that the paclitaxel-containing green precipitate and the solutes left in the needle powder after lipid extraction with hexane were not be absorbed irreversibly on the surface of reverse phase absorbents and it is suitable for purification with reverse phase preparative chromatography. This provides another solid proof of successful lipids removal by DPLE with hexane.

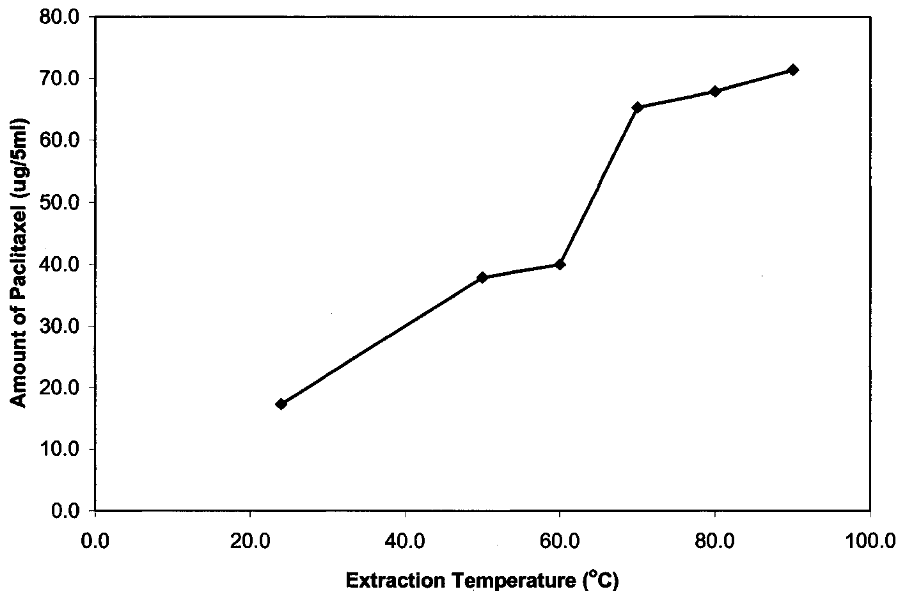


Figure 5.3 Amount of Paclitaxel in the First 5.0 ml of Hexane Extract. Extraction Condition: 5.000g <100 mesh needle powder; flow rate 1.0 ml/min.

These results suggest that DPLE with hexane is a promising replacement of Liquid-Liquid Extraction with hexane in previous arts. It exceeds other lipids removal techniques such as Liquid-Liquid Extraction (LLE) with hexane or petroleum ether in CSE based bioseparation processes on the following aspects:

1. *There are no mixed solvents generated here, therefore no mixed solvent recovery is required.* There is a large amount of mixed solvents obtained by LLE. For example, 3 mixtures of solvents are obtained in the process of removing lipids with hexane followed by taxanes extraction with chloroform from methanol-water mixture in previous arts: a), hexane with methanol and water; b), chloroform with hexane, methanol and water; c), methanol-water with hexane and chloroform. Multicomponents distillation is required to recover these mixed solvents.

2. *There is no taxane loss in the lipids removal by DPLE with hexane.* On the contrary, there is a large amount of hydrophilic taxanes, such as 10-DAB III, which can be left in the methanol-water phase in the process of extracting taxanes with chloroform or dichloromethane by LLE.

3. *There is no waste water produced in lipid removal by DPLE with hexane.* On the contrary, large amount of waste water with toxic chemical is generated from the unit operation of Liquid-Liquid Extraction in previous arts.

4. *There is at least one more valuable product available instead of a lipid waste.* The extract with hexane contains many valuable bioactive components such as vitamin E and chlorophyll. It is a potential valuable raw material for production of natural vitamin E and chlorophyll.

In taxane extraction by DPLE with dichloromethane (Figure 5.4), all the extraction columns with needle powder were pretreated in DPLE using hexane to remove most of lipids. It is also observed that the amount of both paclitaxel and 10-DAB III in dichloromethane extract increases with extraction temperature. In this batch of needle

powder, there was no Baccatin III detected. This is confirmed by the taxane content analysis of the residue of DPLE with dichloromethane.

In addition to temperature, there are many other factors that affect DPLE, such as solvent flow rate, extraction time and particle size of needle powder, even water content in needle powder and extraction pressure. Therefore, the effects of all these factors, except the effects of water content in the needle powder and pressure on extraction were considered in this work.

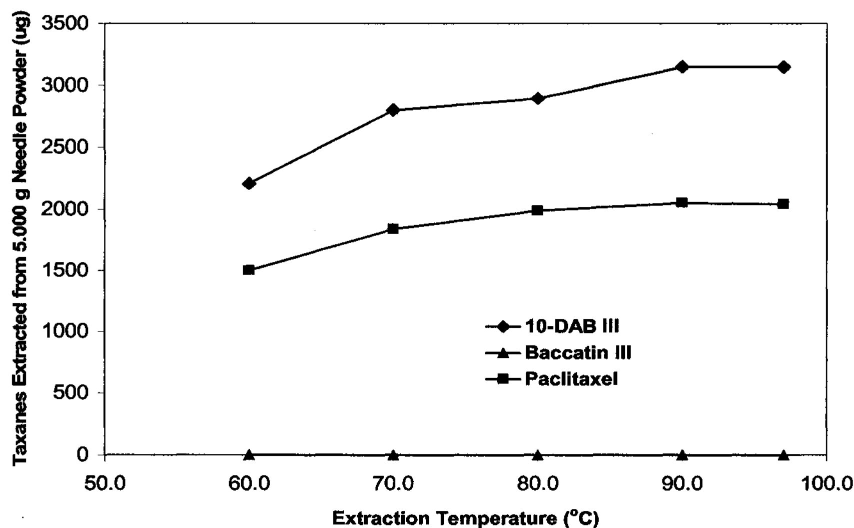


Figure 5.4 Temperature Effect on Taxane Extraction. Extraction Condition: 5.000g <100 mesh needle powder; solvent dichloromethane; flow rate 1.0 ml/min; extraction time 60 min. The biomass in the column was pretreated with hexane at 90.0°C, 1.0ml/min, and 30 min.

5.1.2. Effect of Particle Size

The particle size of needle powder plays a vital role in taxane recovery by DPLE with nonpolar solvents. The major part of needle powder is hydrophilic. For example, the fibers that constitute the cell wall are hydrophilic. Most of solutes are hydrophilic as well. This is supported with the result in this study that most of solutes in the needle powder are water soluble.

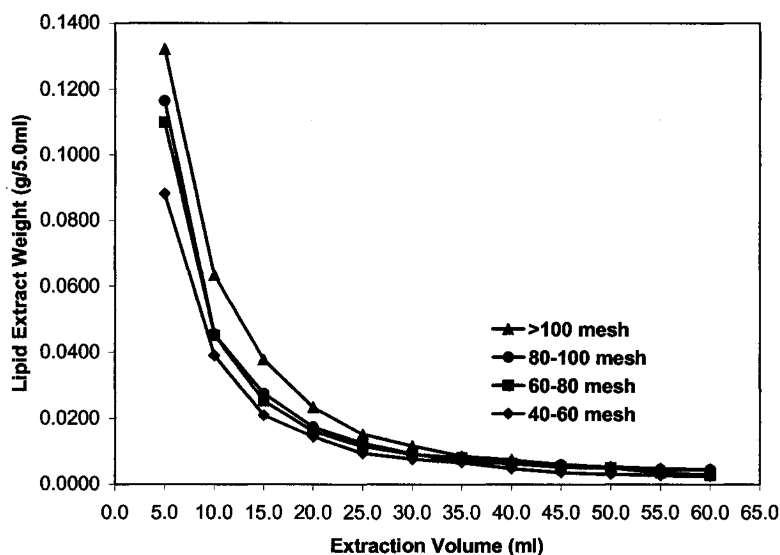


Figure 5.5 The Profile of Extract Weight of Every 5 ml Extract in Lipid Removal by DPLE with Hexane. Extraction Condition: 5.000g needle powder with various particle sizes; flow rate 1.0 ml/min; temperature 90°C.

Due to lack of affinity, nonpolar solvents have limited ability to penetrate these hydrophilic matrices. Hydrophobic solutes have inadequate ability to penetrate them as well. The only way for hydrophobic solvents to contact solutes in hydrophilic matrices is

to decrease the particle size of the needle powder. This hypothesis is confirmed experimentally in this study (Figure 5.5 and Figure 5.6).

The following particle size ranges of needle powder were used, 40-60 mesh, 60-80 mesh, 80-100 mesh and <100 mesh. Five gram needle powder was packed into each extraction column. Although the weight of needle powder used was the same in each extraction, the weights of lipids extracted were different. The weights of lipids extracted from needle powder increased with particle sizes decrease.

It can also be noticed that the differences of extract weights between different particle sizes decrease with the volume of extraction solvent increases. This is illustrated in Figure 5.5 that the weights of any two extracts of different particle sizes were getting closer and closer with extraction time prolonged.

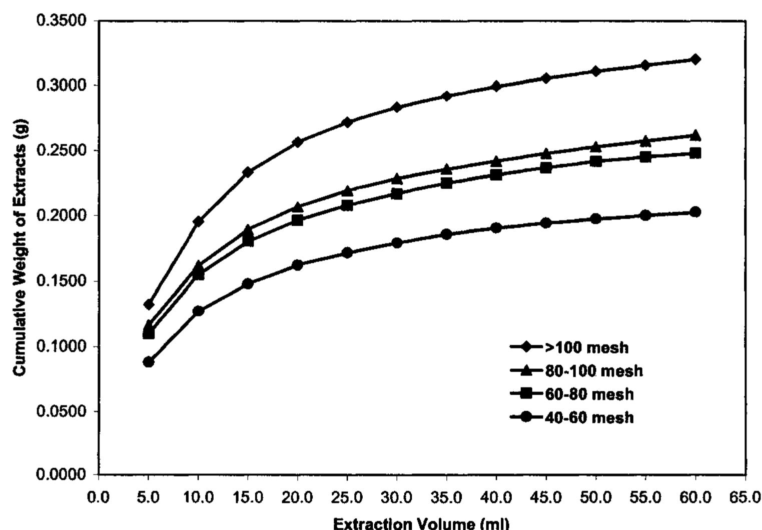


Figure 5.6 Cumulative Extract Weight Profile in Lipid Removal by DPLE with Hexane. Extraction Condition: 5.000 g needle powder, flow rate 1.0 ml/min; temperature 90°C

Although only hexane was used in DPLE to investigate the particle size effect on extraction, it is not difficult to infer that decreased particle size of needle powder can facilitate DPLE with dichloromethane as well.

However, there is a problem that the pressure drop through the DPLE column will increases with the decreased particle sizes. This is described by Darcy's Law (Belter et al., 1988):

$$\Delta p = \frac{\eta l u \phi}{d_p^2} \qquad 5.1$$

Where Δp is the pressure drop across the bed of thickness l, η is the viscosity of solvents, u is the velocity of solvents, and Φ is a constant depending on the needle powder inside the extraction column. d_p is the average diameter of particles in the extraction column.

However, when nitrogen gas at 103 Pa was applied after extraction at room temperature to purge hexane out of the extraction column in which <100 mesh needle powder was packed; liquid hexane was purged out completely within 20 second. This suggests that the flow rate of hexane inside the column was fast at that pressure, even at room temperature.

Therefore, the pressure drop in DPLE is not likely a problem due to the extremely low viscosity of hydrophobic solvents at high temperature and low solvent velocity.

5.1.3. Effect of Time and Flow Rate on Extraction

The extraction efficiency of DPLE exceeds that of PLE because fresh solvent is continuously introduced into the extraction column. Mass transfer rate inside the column is accelerated by increased concentration difference. As a result, recovery rate is amplified by DPLE.

The amount of solvent consumption in DPLE is the product of solvent flow rate and extraction time. In the experiment of lipid removal by DPLE with hexane, the effect of extraction time is studied under various temperatures at a flow rate of 1.0 ml/min as shown in Figure 5.7.

The results show that the total extracts weight increases with extraction temperature between 25°C and 90°C. The total extract weights of 80°C and 90°C were very close. This suggests that the amount of lipids will not increase significantly with the temperature over 90 °C in this situation.

It was observed that the color of hexane extract changed from dark green at the beginning to light white after 25 minutes in the experiment carried out at 90°C. This suggests that most of green lipids were removed from biomass within 25 minutes. The total weight of extracts increases slowly after 30 minutes. Extraction time of 30 minutes is adequate for green lipids removal. Therefore, for lipid removal with hexane, 30 minutes was selected as extraction time at the flow rate of 1.0 ml/min and temperature of 90°C.

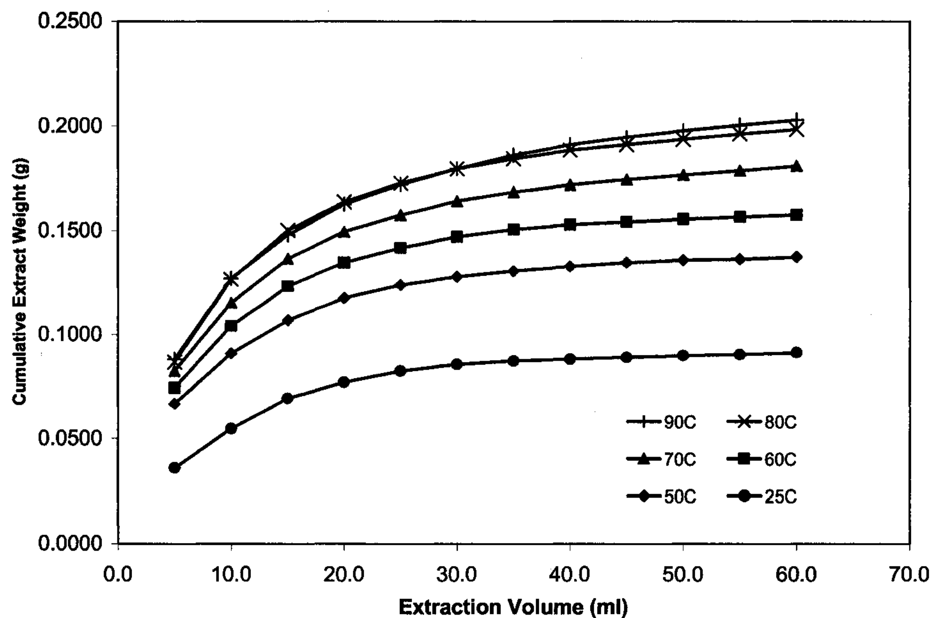


Figure 5.7 Cumulative Extract Weight Profile of DPLE with Hexane. Extraction Condition: 5.000g 40-60 mesh needle powder; flow rate 1.0 ml/min.

In DPLE, sufficient extraction time is also required for mass transfer inside the extraction column. The mass transfer time depends on the particle size, solvent viscosity and temperature. However, only solvent flow rate is studied when extraction time is fixed at 60 minutes in the following experiment (Figure 5.8).

In the experiment shown in Figure 5.8, the extraction time was fixed at 60 minutes. The amounts of taxanes extracted at various flow rates were studied. It can be noticed that sufficient flow rate is required to achieve high recovery in taxane extraction in DPLE despite the fact that the extraction time is the same (Figure 5.8).

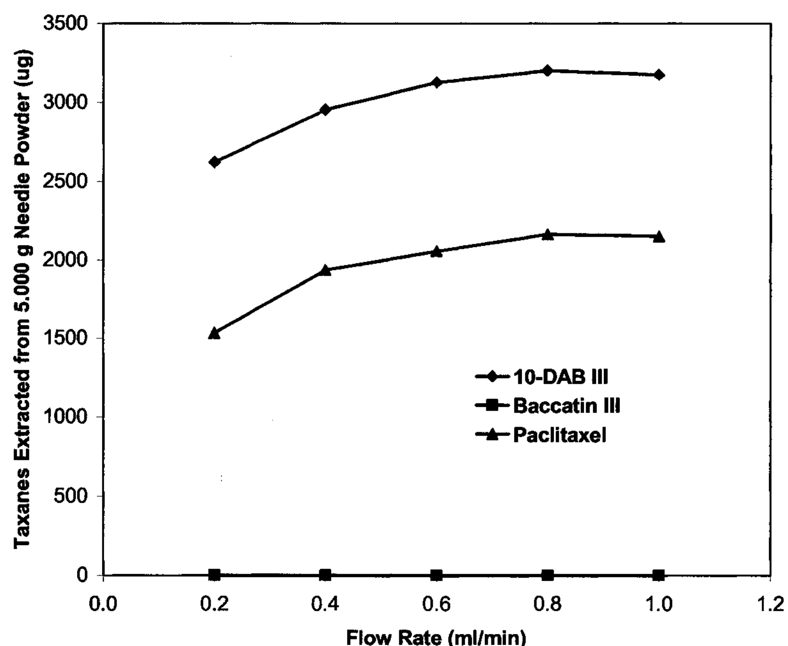


Figure 5.8 Flow Rate Effect On Taxane Extraction in DPLE with Dichloromethane. Extraction Condition: 5.000g <100 mesh needle powder; solvent is dichloromethane at 90.0°C, 60 minutes. The biomass inside the column is pretreated by DPLE with hexane at 90.0°C, 1.0ml/min, and 30 minutes.

5.1.4. Effect of Solvent Selection on Extraction

As discussed previously, the goal of lipid removal is achieved with satisfaction using DPLE with hexane. In this section, the effect of solvent selection on taxane extraction was studied. In addition to pure chloroform, pure dichloromethane and dichloromethane containing 1.0%, 2.0%, 3.0% and 4.0% methanol were used as extraction solvents. Methanol was selected as co-solvent of dichloromethane for taxane extraction.

Each of the extraction columns used here was packed with 5.000 g <100 mesh needle powder and pretreated using DPLE with hexane at the following conditions: flow rate was 1.0 ml/min; pressure was between 483 Pa and 517 Pa; extraction temperature was 90.0°C; extraction time was 30 minutes. After lipid extraction, hexane left in the biomass was purged with nitrogen.

Solvent selection has considerable effect on weight of extracts using DPLE. The results are given in Figure 5.9 and Figure 5.10. DPLE with chloroform yielded lowest extract weight. Total extract weights of DPLE with dichloromethane-methanol increased with methanol content in dichloromethane as shown in Figure 5.10, and so for the weight of each extract as shown in Figure 5.9.

DPLE gives much lower extract weight when compared with that of CSE. Considering that the taxane contents in needle powder of *Taxus canadensis* are as low as several hundreds ppm, most of the components in extracts from either CSE or DPLE are impurities. If the taxane contents of the extract of CSE and DPLE are the same, much higher selectivity can be achieved by DPLE. This is due to the fact that most of the solutes of needle powder are hydrophilic. The amount of hydrophobic impurities, such as

wax and chlorophyll, in needle powder is much smaller than that of hydrophilic impurities.

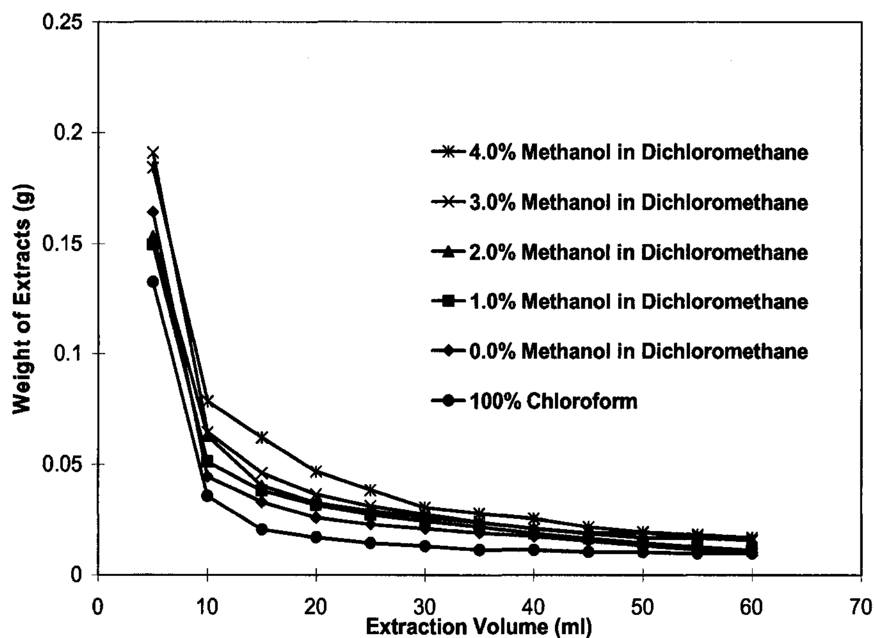


Figure 5.9 Extract Weight Profiles of Various Solvents in DPLE. Extraction Condition: 5.000g <100 mesh needle powder; flow rate 1.0 ml/min at 90.0°C. The needle powder inside the extraction column was pretreated with hexane at 90.0°C with flow rate 1.0 ml/min for 30 minutes.

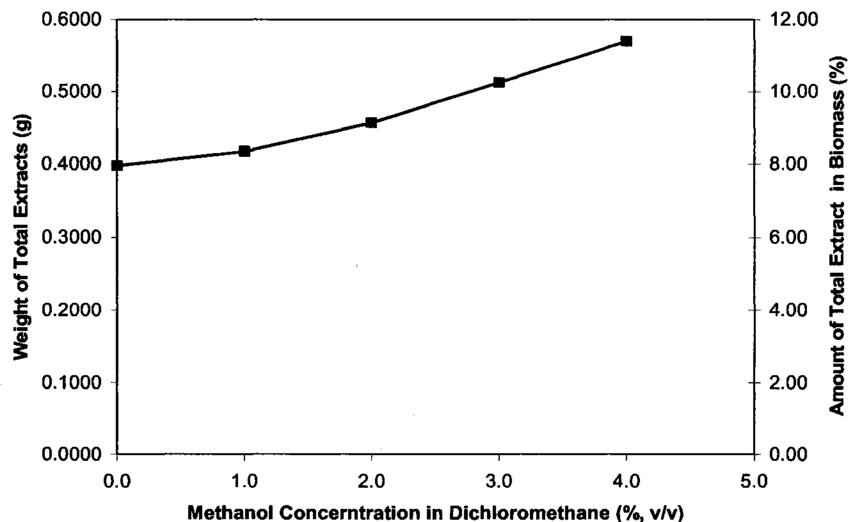


Figure 5.10 Effect of Methanol Content in Dichloromethane on Total Extract Weight in DPLE. Extraction Condition: 5.000g <100 mesh needle powder; flow rate 1.0 ml/min at 90.0°C for 60 min. The needle powder inside the column was pretreated with hexane at 90.0°C with flow rate 1.0 ml/min for 30 minutes.

Taxane contents in extracts are given in Figure 5.11, Figure 5.12 and Figure 5.13. The extraction time required for taxane extraction was obtained by analysis of the taxane contents in the extracts because that each extract corresponds a specific extraction time.

It was observed that in DPLE with pure dichloromethane, there was no Baccatin III or 10-DAB III detected in the extract after 50 minutes (Figure 5.12 and Figure 5.13). So the extraction time required for Baccatin III or 10-DAB III extraction is less than 50 minutes. It was also observed that there was only a trace amount of paclitaxel found in the extract after 55 minutes (Figure 5.11). Cumulative amount of taxanes extracted by DPLE are given in Figure 5.14, Figure 5.15 and Figure 5.16. It is found that solvent selection has strong effect on taxane extraction.

The capability for taxane extraction is increased with the methanol content in dichloromethane DPLE (Figure 5.11, Figure 5.12 and Figure 5.13). Four percent methanol in dichloromethane shows the highest capability for taxane extraction. Highest amount of total extract weight was also obtained with the same mixture.

However, in the purging stage with 103 Pa nitrogen after extraction, the flow rates of residue solvents decreased dramatically with the content of methanol in dichloromethane. The purging time required was from 20 seconds to more than 10 minutes with the methanol content increased from 0% to 4% in dichloromethane. This could cause a problem in DPLE. Therefore, higher content of methanol than 4% (v/v) is not studied in this study.

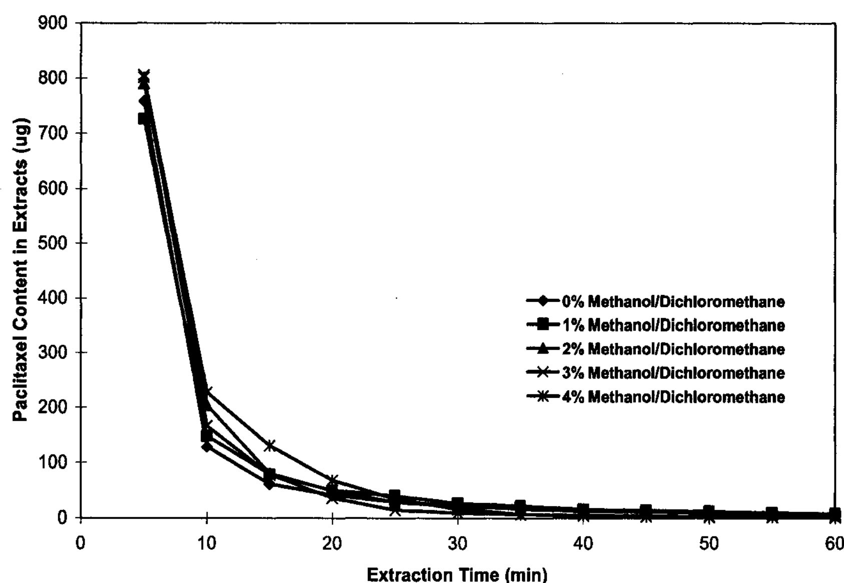


Figure 5.11 Paclitaxel Contents in Extracts of DPLE. Extraction Condition: 5.000g <100 mesh needle powder; flow rate 1.0 ml/min at 90°C. The biomass inside the extraction column was pretreated using hexane with parameters of 1.0 ml/min, at 90°C and 30 minutes.

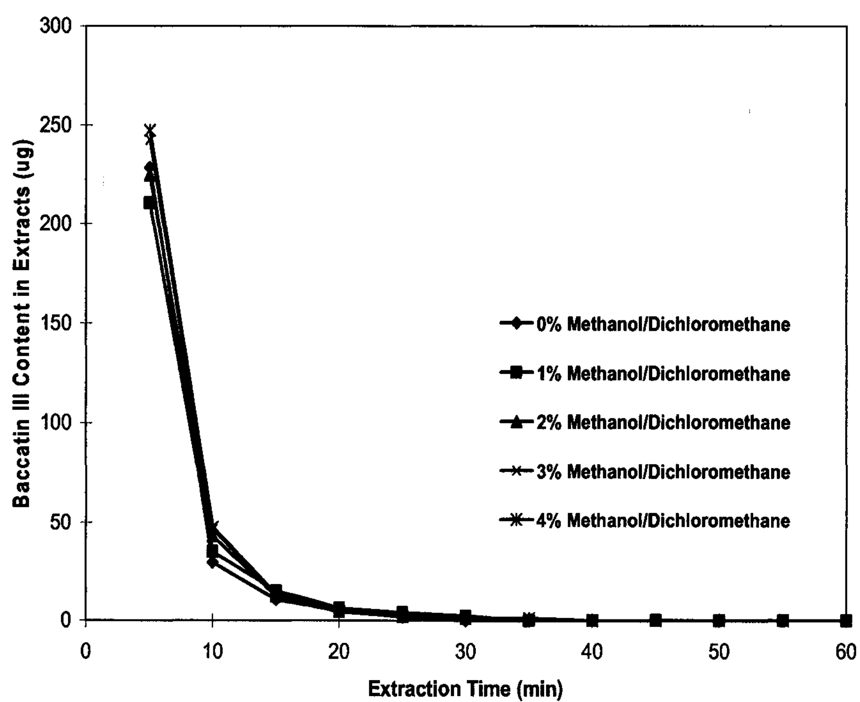


Figure 5.12 Baccatin III Contents in Extracts of DPLE. Extraction Condition: 5.000g <100 mesh needle powder; flow rate 1.0 ml/min at 90°C. The biomass inside the extraction column was pretreated using hexane with parameters of 1.0 ml/min, at 90°C and 30 minutes.

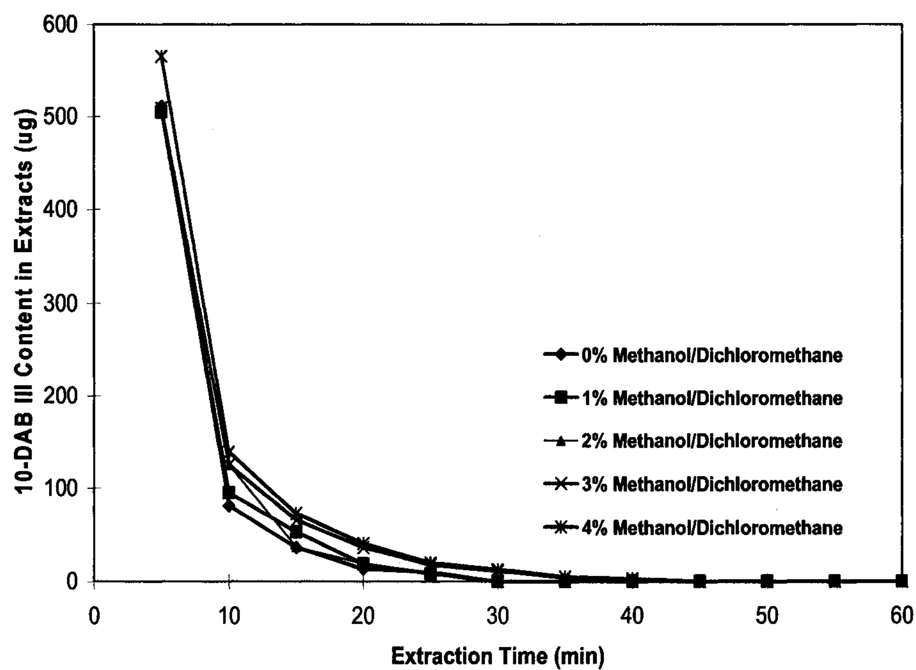


Figure 5.13 10-DAB III Contents in Extracts of DPLE. Extraction Condition: 5.000g <100 mesh needle powder; flow rate 1.0 ml/min at 90°C. The biomass inside the extraction column was pretreated using hexane with parameters of 1.0 ml/min, at 90°C and 30 minutes.

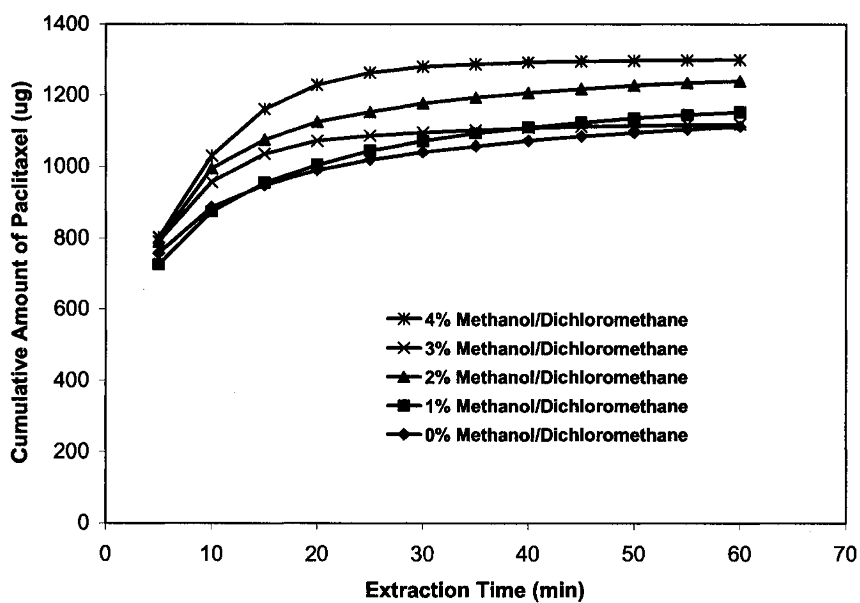


Figure 5.14 Cumulative Amount of Paclitaxel Extracted by DPLE. Extraction Condition: 5.000g <100 mesh needle powder; flow rate 1.0 ml/min at 90°C. The biomass inside the extraction column was pretreated with hexane, 1.0 ml/min, at 90°C, 30 minutes.

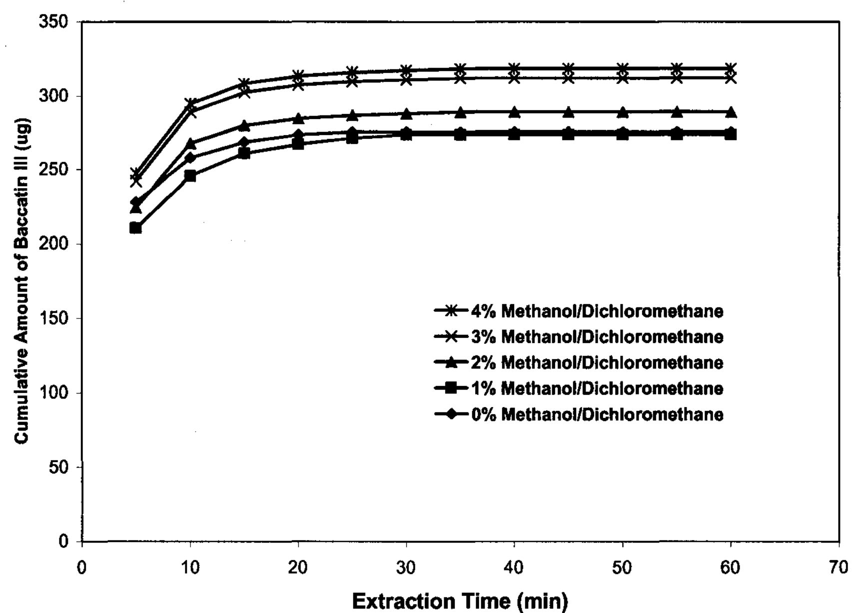


Figure 5.15 Cumulative Amount of Baccatin III Extracted by DPLE. Extraction Condition: 5.000g <100 mesh needle powder; flow rate 1.0 ml/min at 90°C. The needle powder inside the extraction column was pretreated with hexane, 1.0 ml/min, at 90°C, 30 minutes.

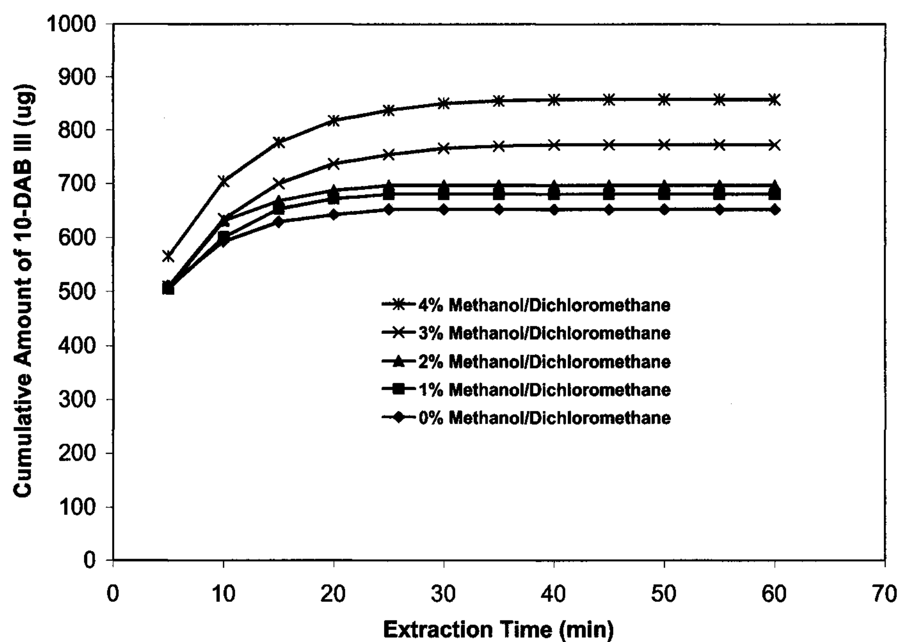


Figure 5.16 Cumulative Amount of 10-DAB III Extracted by DPLE. Extraction Condition: 5.000g <100 mesh needle powder; flow rate 1.0 ml/min at 90°C. The needle powder inside the extraction column was pretreated with hexane, 1.0 ml/min, at 90°C, 30 minutes.

Comparing to previous study on taxane recovery with PLE by Kawamura et al. (1999), the extraction conditions for DPLE are much milder than those of PLE (Table 5.2).

Table 5.2 Comparison of Extraction Conditions for DPLE and PLE

Extraction Method		**DPLE**	**PLE (Kawamura et al., 1999)**
Raw Material	Biomass	Needle Powder of *Taxus canadensis*	Bark of *Taxus cuspidata*
	Particle Size	< 100 mesh	40-60 mesh
	Water Content	NA	14%
	Drying Method	Air Dry followed by 60°C for 4 hours with ventilation	Air Dry
Extraction Conditions	Solvents	Chloroform, or 0-4% Methanol in Dichloromethane	Water, Various Organic Solvents
	Solvent in Extraction Column	Dynamic	Static
	Temperature (°C)	90	100
	Pressure (Pa)	483-517	10130000
	Extraction Time (min)	60	15

5.1.5. Reproducibility of DPLE

Two parallel experiments of DPLE with dichloromethane were performed to evaluate the reproducibility of this technique. The extraction time was extended to 180 minutes. The weight of each extract was accurately weighed and given in Figure 5.17.

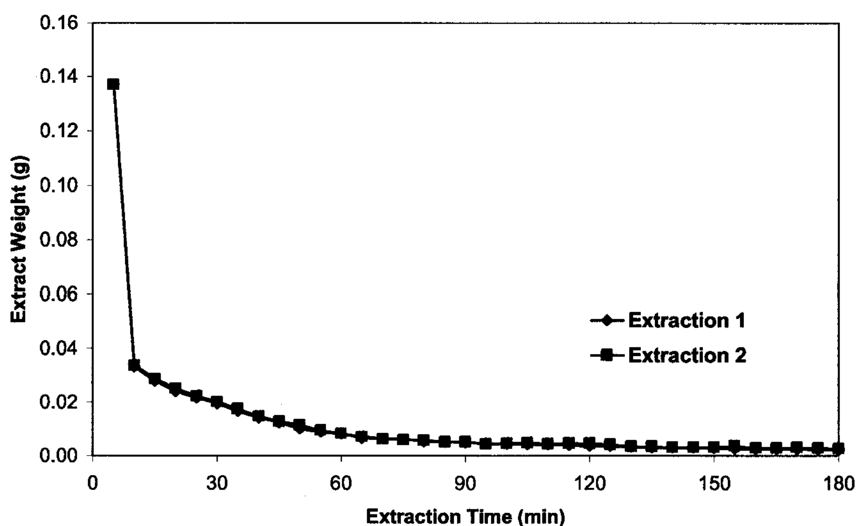


Figure 5.17 Reproducibility Study in DPLE. Extraction Conditions: 5.000g <100 mesh needle powder; flow rate 1.0 ml/min at 90°C. The needle powder inside the extraction column was pretreated with hexane, 1.0 ml/min, at 90°C, 30 minutes.

The results show that high reproducibility is achieved (Figure 5.17). The high reproducibility is mainly due to the accurate flow rate of Waters® HPLC pump and accurate timer. The homogenous needle powder contributes to the high reproducibility as well. Pressure has less important on reproducibility because that the compressibility of liquid solvents is very small.

5.1.6. Paclitaxel Stability Study in DPLE

In DPLE, elevated temperature, i.e. 90°C, is used. It is much higher than that of CSE which is usually conducted at ambient temperature. This arouses the concern of stability of paclitaxel, the thermodynamically unstable and the most valuable product in this study.

Paclitaxel is fairly stable within 60 minutes at 90.0°C in hexane as illustrated in Figure 5.18. The paclitaxel content in 5.000 g needle powder is found to decrease with time at the rate of 0.0390 ug/min. This means that approximately 0.2% of the total amount of paclitaxel is lost due to the elevated temperature in 60 minutes.

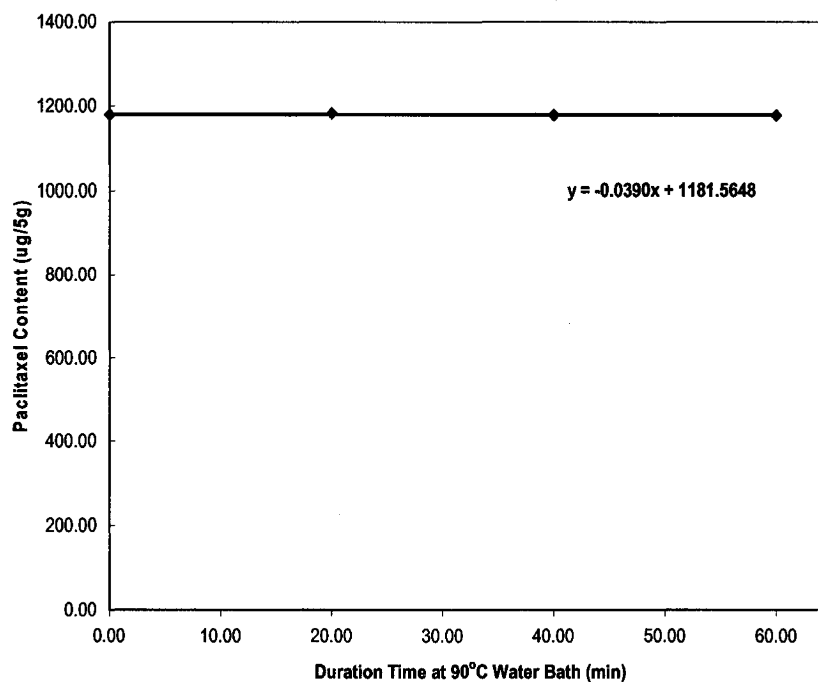


Figure 5.18 Paclitaxel Stability in Hexane at 90°C. After hexane is evaporated in fume hood at room temperature, the hexane extract along with needle powder were extracted by CSE with methanol, 5.000g/50ml, 12 hour at room temperature.

5.2. Conventional Solvent Extraction

Conventional solvent extraction (CSE) is the most popular extraction technique in natural product processing. Comparing to isolation of taxanes using DPLE with nonpolar solvents, isolation of taxanes applying CSE with solvents was studied based on the solute distribution model developed in Chapter III of this thesis and derived selectivity increasing approaches of dual-solvent extraction and extraction-SPE.

5.2.1. Selection of Extraction Time

In published CSE based bioseparation processes, the extraction time ranges from a few hours to a few days when methanol, or 95% ethanol/water (v/v) or methanol-dichloromethane was used as CSE solvents.

Forty percent ethanol/water (v/v) was selected as solvent in CSE to investigate the appropriate extraction time for taxanes. *Taxus canadensis* needle powder (<100 mesh) is used as new material in these experiment. Three grams of Celite® 545 was added to 5.000g needle powder and used as filter aid. Paclitaxel concentration in extract was analyzed with HPLC and was selected as a criterion for extraction time selection. The results are given in Figure 5.19 and Figure 5.20.

The results indicate that extraction of paclitaxel by CSE with 40% ethanol/water (v/v) was very fast. The shortest extraction time studied in this work is 2 minutes. There was already a significant amount of paclitaxel in the extract. The maximum concentration of paclitaxel in extract was observed at the extraction time of 10 minutes. A slight decline

trend of paclitaxel concentration in extract is observed when the extraction time is longer than 10 minutes.

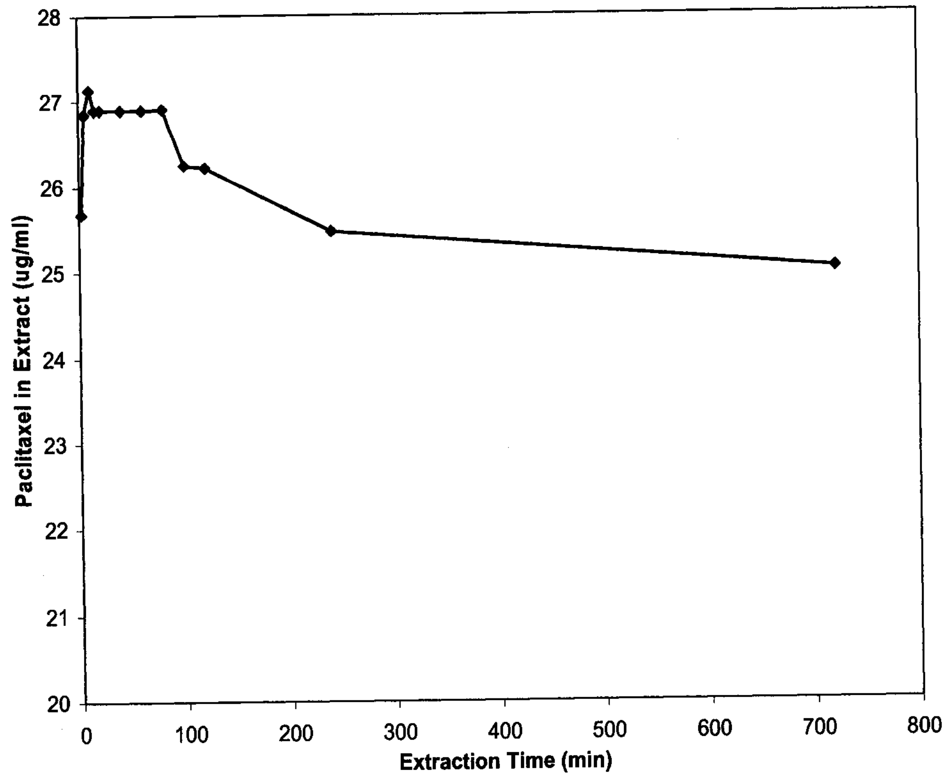


Figure 5.19 Selection of Extraction Time in CSE. Extraction Conditions: 5.000g <100 mesh needle powder; 50.0ml 40% Ethanol/Water (v/v); temperature 25°C.

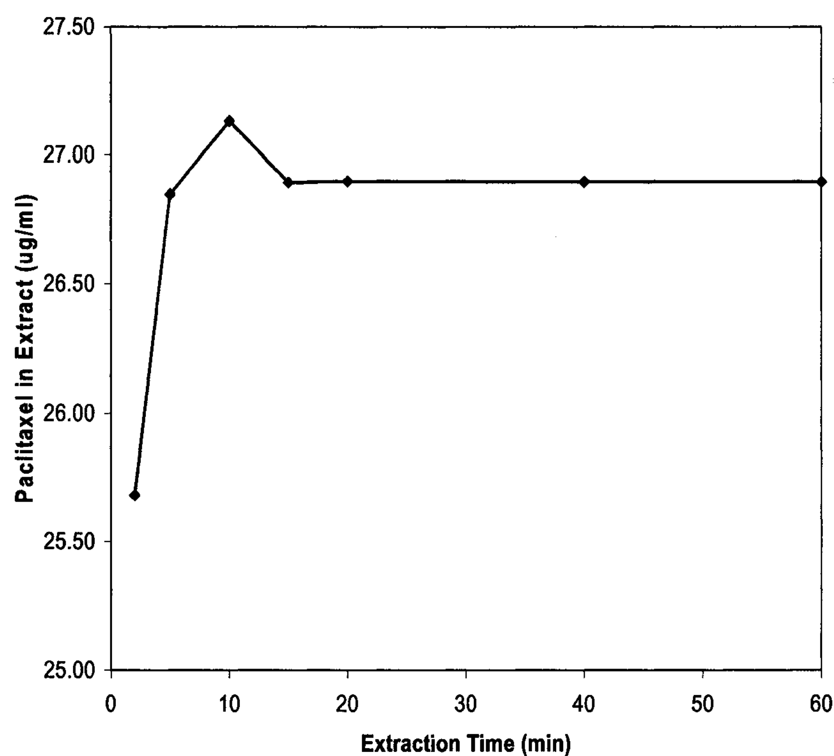


Figure 5.20 Details of Figure 5.19: Selection of Extraction Time in CSE. Extraction Conditions: 5.000g <100 mesh needle powder; 50.0ml 40% Ethanol/Water (v/v); temperature 25°C.

Two potential reasons accounting for the decline trend of paclitaxel concentration in extract are considered in this study. One is the degradation of paclitaxel. Another is the adsorption of paclitaxel by the hydrophobic lipids in biomass.

The stability of paclitaxel depends on solvents and pH of the solvent (MacEachem-Keith et al., 1997). The pH of the extract was between 5.5 and 6.0. In this study, the stability of paclitaxel was investigated and found that paclitaxel was fairly stable in ethanol/water extract (Figure 5.29). Therefore, degradation of paclitaxel is not likely the cause for the decline of its concentration in extract.

Forty percent of ethanol/water (v/v), which is used as extraction solvent, is considered as a weak solvent for hydrophobic component extraction. The hydrophobic components in the needle powder play a role as reverse phase absorbents in this weak solvent. Paclitaxel, the relatively hydrophobic component in the extract, can be absorbed back to the biomass by the hydrophobic components.

It should be noticed that the highest concentration of paclitaxel was reached at the extraction time of 10 minutes. The extraction of paclitaxel was fast and efficient at the beginning regardless 40% ethanol/water (v/v) is previously considered a weak solvent for taxane extraction. The extraction is fast and efficient so as to paclitaxel is absorbed back after it is extracted.

There is a large amount of hydrophilic impurities in the extract of 40% ethanol/water (v/v). This can be seen a large number of peaks with retention times of less than 10 minutes in the reverse phase HPLC chromatogram (Figure 5.21 and Figure 5.22). The selectivity of CSE with 40% ethanol/water (v/v) will be very poor due to the large amount of hydrophilic impurities in the extract.

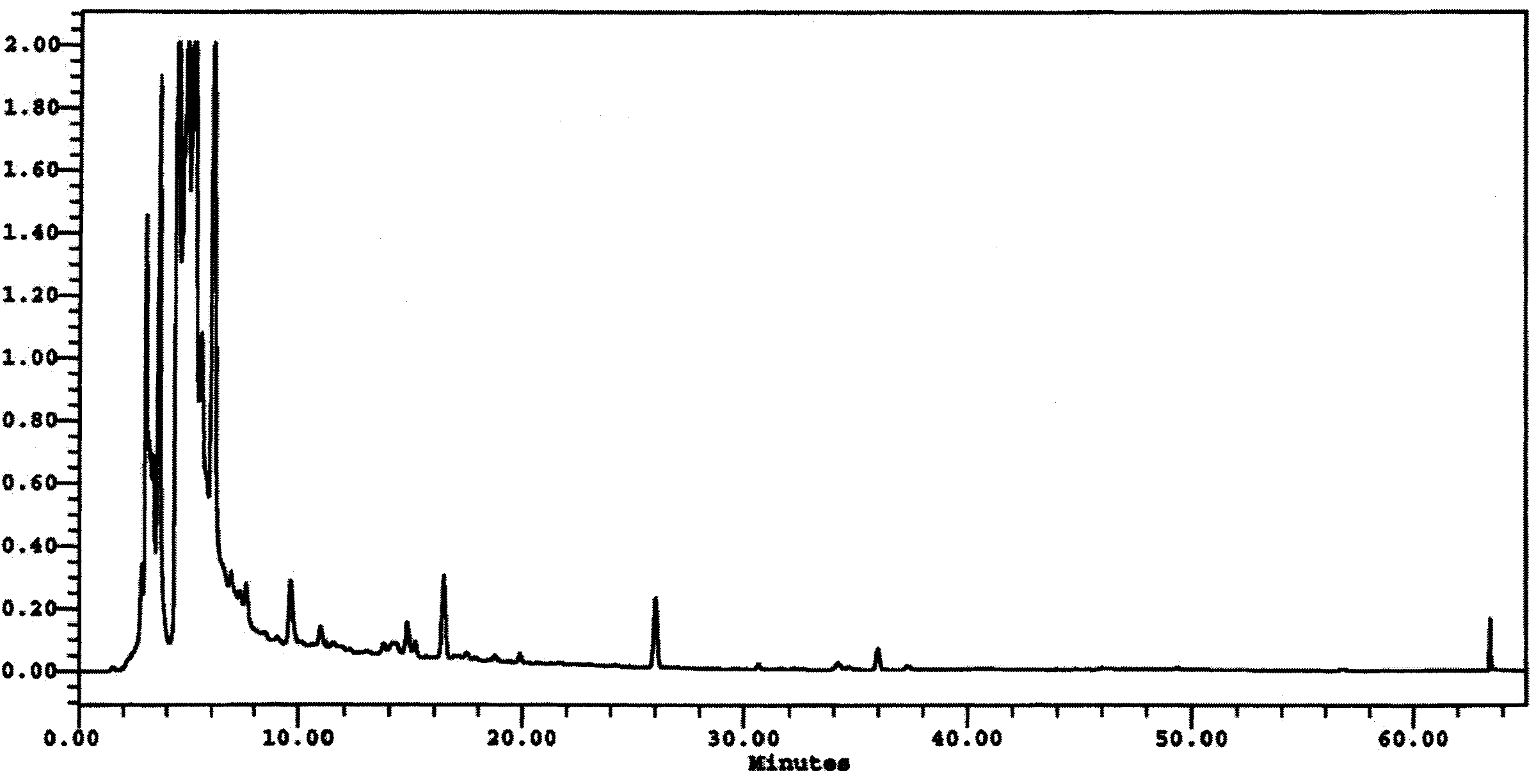


Figure 5.21 HPLC Chromatogram of 40% ethanol/water (v/v) Extract of *Taxus canadensis* Needle Powder. Extraction Conditions: 5.000g <100 mesh needle powder, 50 ml 40% ethanol/water at 25°C; extraction time 10 min.

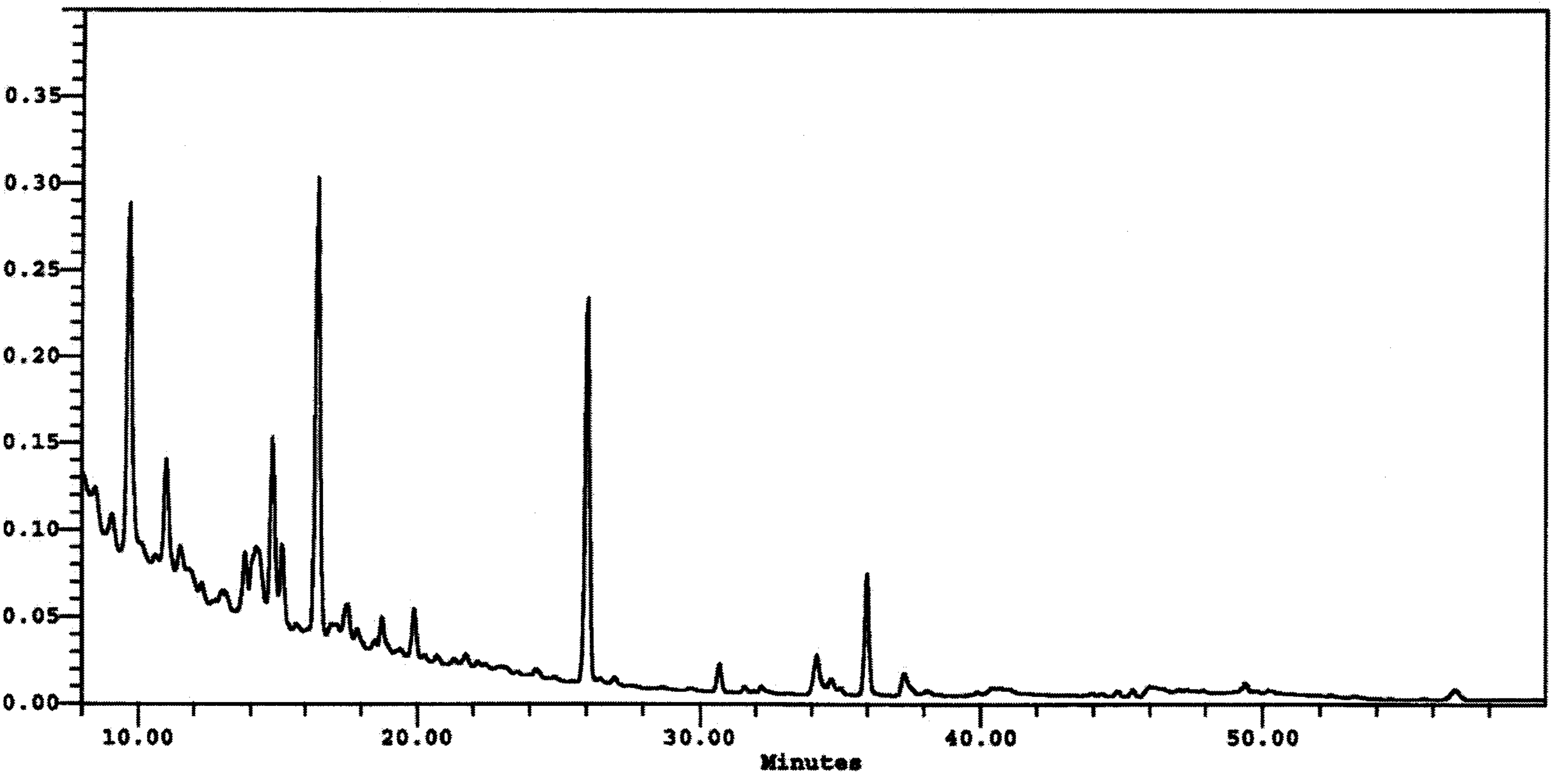


Figure 5.22 Detailed Show of HPLC Chromatogram of 40% ethanol/water Extract of *Taxus canadensis* Needle Powder. Extraction Conditions: 5.000g <100 mesh, 50ml 40% ethanol/water (v/v) at 25°C; extraction time 10 min.

5.2.2. Selection of Solvents

Low selectivity of CSE is due to the large amount of hydrophilic impurities in needle powder of *Taxus canadensis*. According to the dual-solvent extraction approach discussed in Chapter III, pure water, the most polar solvent, was used to remove the hydrophilic impurities from the needle powder of *Taxus canadensis* while taxanes are expected to be retained in it. Then organic solvents can be used to extract taxanes left in the pretreated needle powder. The selectivity of CSE is expected to be improved by applying the dual-solvent extraction strategy.

Unfortunately, a significant amount of taxanes in the extract of pure water was found as well (Table 5.3). If high recovery is desired, taxanes from pure water extract must be recovered. Therefore, dual-solvent extraction approach with polar solvents is not suitable to increase both selectivity and recovery of CSE.

Table 5.3 Taxane Contents in Pure Water Extract. Extraction Conditions: 5.000g <100 mesh needle powder and 3.0g Celite® 545; solvent 50.0 ml pure water; temperature 25°C.

Taxanes	10-DAB III	Baccatin III	Paclitaxel
Taxanes Extracted by Pure Water (μg/5.000g)	388	140	25

Considering the fact that 95% ethanol/water, methanol/dichloromethane, methanol and acetone have been intensively studied as CSE solvents, and ethanol/water is a popular solvent in herb extraction, various concentrations of ethanol/water, methanol/water and acetone/water were then tested in this study as CSE solvents for taxane extraction from needle powder of *Taxus canadensis*.

Ten minutes was selected as extraction time because the highest concentration of paclitaxel in extract was observed for that time in the previous study. The results of the experiment are given in Figure 5.23.

Considering that the volume of extracts changes very little, and was between 41.8 ml and 42.1 ml, the concentration of paclitaxel in the extracts was selected as a criterion for efficiency evaluation in CSE.

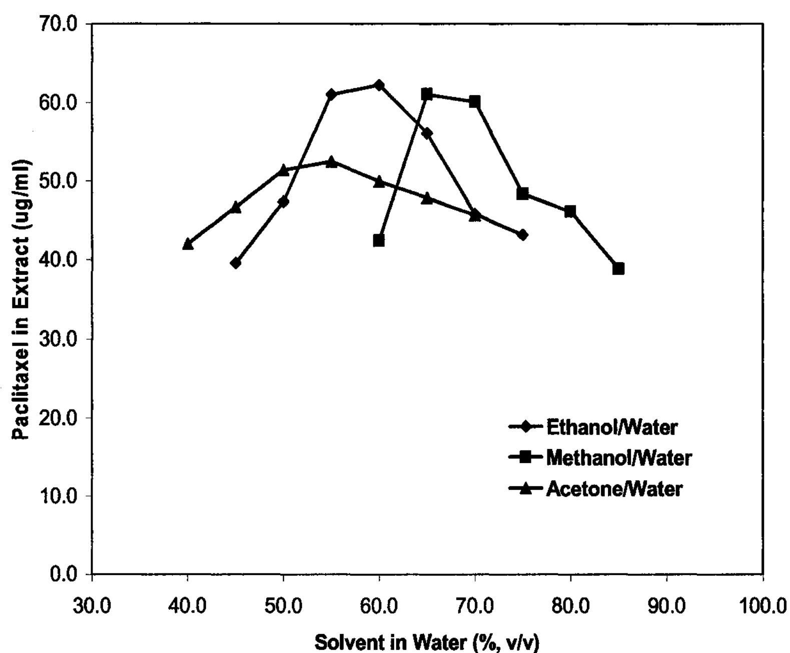


Figure 5.23 Paclitaxel Concentrations in Extracts of CSE. Extraction Conditions: 5.000g <100 mesh needle powder and 3.0g Celite® 545; the volume of solvents 50.0ml; extraction time 10.0 minutes at 25°C.

The results indicate that paclitaxel concentration in the extracts changes significantly with the solvent composition. The highest paclitaxel concentration in acetone/water extracts was observed in the 55% acetone/water (v/v) mixture. Therefore, fifty five percent of acetone/water (v/v) is considered as the best one among acetone/water mixtures in this study. Similarly, sixty percent of ethanol/water is considered as the best extraction solvent among ethanol/water solvents. Sixty percent of methanol/water (v/v) is also considered as the best extraction solvents among methanol/water solvents in this study.

Although there is not significant difference in the paclitaxel concentration between the extracts of 65% methanol/water (v/v) and 60% ethanol/water (v/v), 60% ethanol/water (v/v) is considered as a better solvent because the overall recovery of paclitaxel using CSE with 60% ethanol/water (v/v) is higher than that of using 65% methanol/water (v/v) as extraction solvent. This result is also given by the fact that paclitaxel concentration in the extract of third extraction with 60% ethanol/water (v/v) is less than that with 65% methanol/water (v/v) as shown in Figure 5.24.

Although a large amount of solvents is used in CSE, there was still a significant amount of paclitaxel in the biomass after extraction with 60% ethanol/water (v/v). This means that the recovery rate will be low in CSE with 60% ethanol/water (v/v). In addition, a large amount of Celite® 545 used as filter aid due to the filtration difficulty caused by the small particle size, <100 mesh, will generate extra solid waste in large scale extraction.

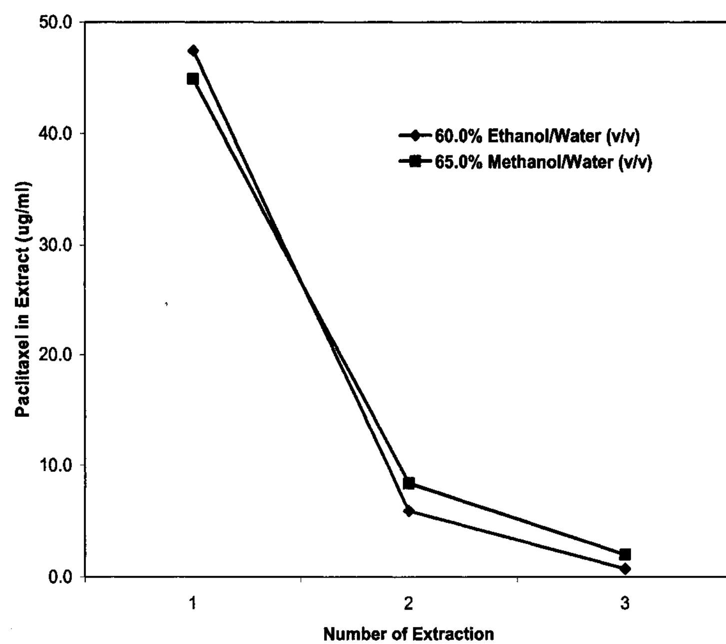


Figure 5.24 Paclitaxel Concentrations in Extracts of 3 Extraction Operations. Extraction Condition: <100 mesh needle powder; 5.000g /50.0 ml and 10.0 min for each extraction; 25°C

Paclitaxel is the most hydrophobic one among the 4 taxane products, 10-DAB III, Baccatin III, 9-DHB III and paclitaxel. Paclitaxel appears in the HPLC chromatogram at the retention time of around 36minutes. Any component with retention time longer than 36 minutes is defined as hydrophobic impurity and is not desired.

10-DAB III is the most hydrophilic one among the 4 taxane products. The retention time in HPLC chromatogram is around 16 minutes. Similarly, any component with retention time shorter than 16 minutes is defined as hydrophilic impurity.

In addition to a large amount of hydrophilic impurities in the extracts of CSE with organic solvents/water, there is a significant amount of hydrophobic impurities. This is illustrated in Figure 5.25 and Figure 5.26.

There are two unknown hydrophobic impurities with retention time 40 minutes and 46 minutes in HPLC chromatogram of 55% acetone/water extract. The amount of these impurities in extract is very large.

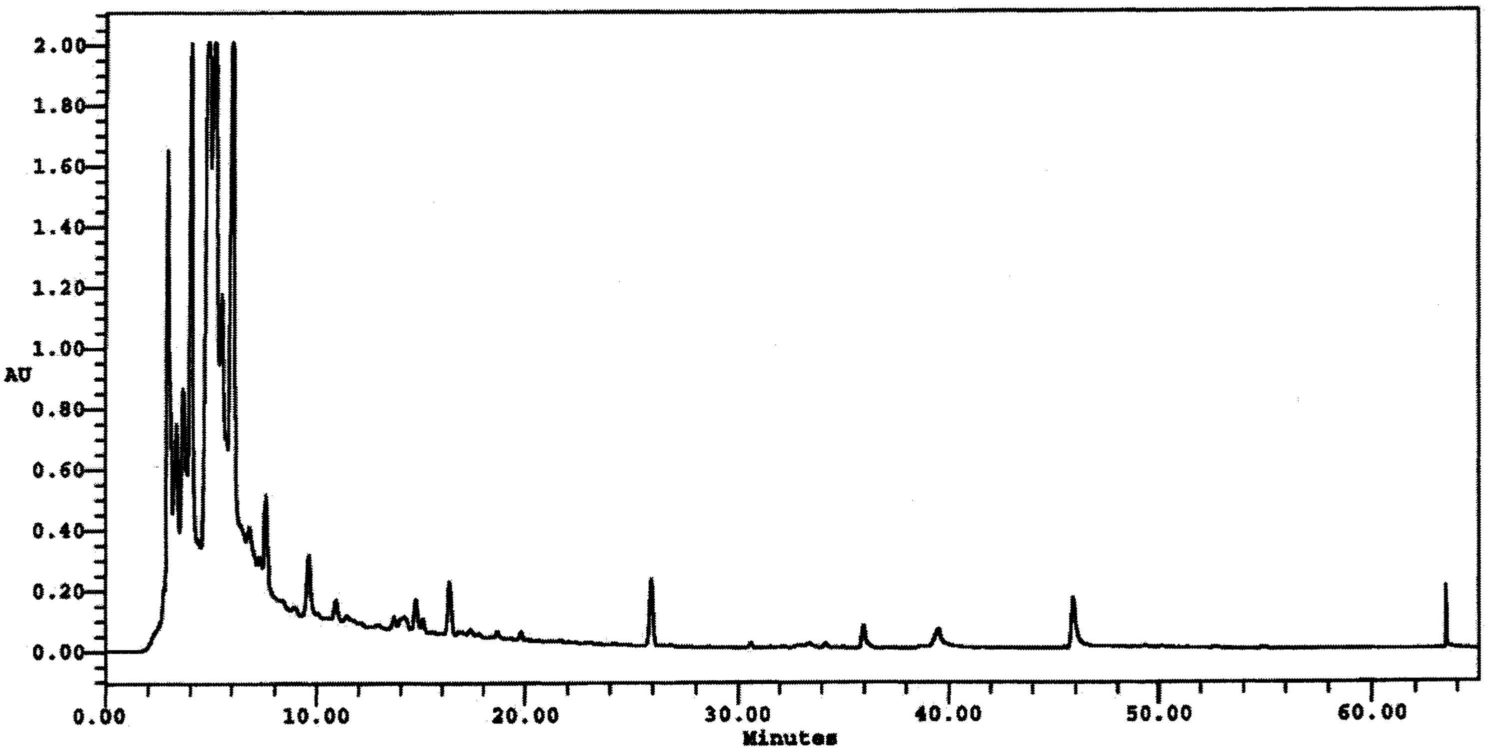


Figure 5.25 HPLC Chromatogram of the Extract of CSE with 55% Acetone/water (v/v). Extraction Conditions: 5.000g <100 mesh needle powder with 3.0g Celite® 545; 50 ml 55% acetone/water; temperature 25°C.

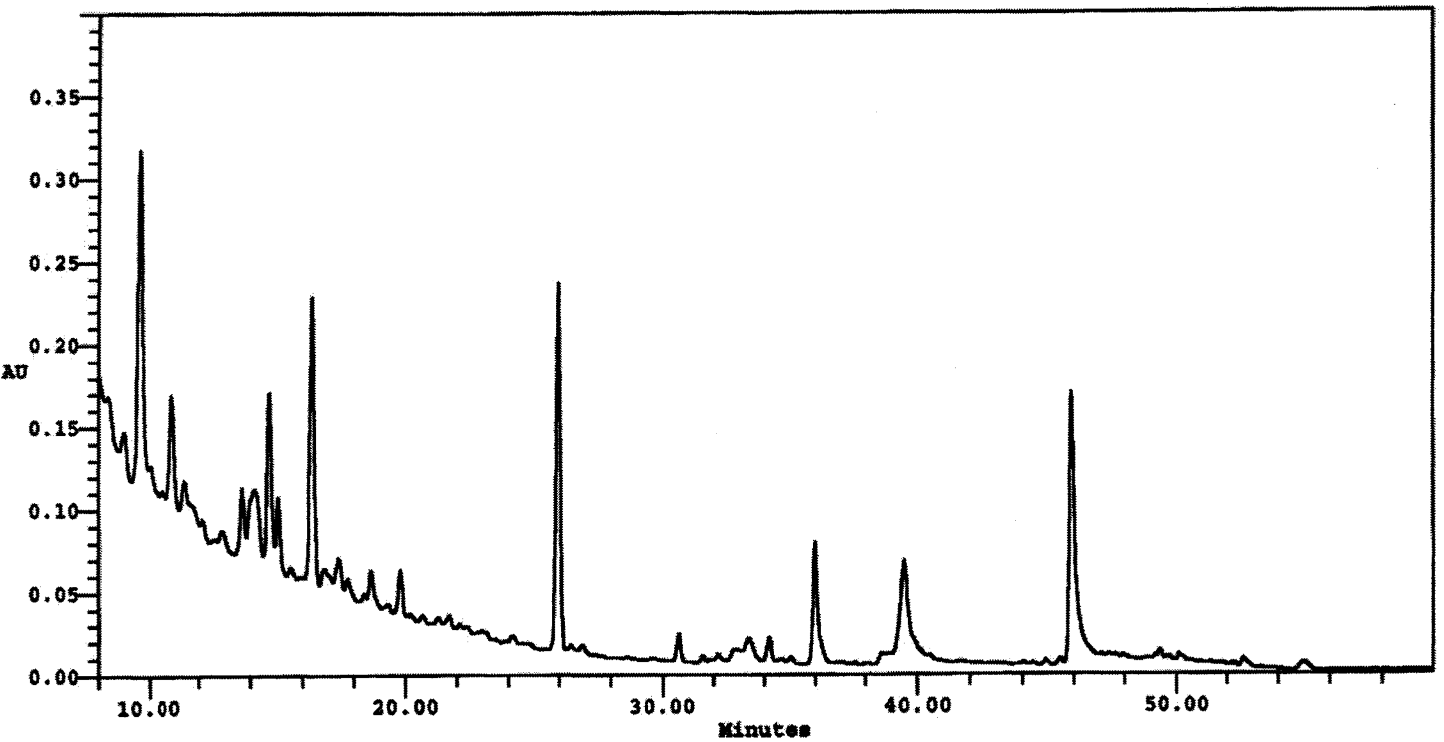


Figure 5.26 Detail show of HPLC Chromatogram of the Extract of CSE with 55% Acetone/water (v/v). Extraction Conditions: 5.000g <100 mesh needle powder with 3.0g Celite® 545; 50 ml 55% acetone/water; temperature 25°C.

5.2.3. Effect of Particle Size on CSE

Particle size of the biomass plays an important role in solvent extraction. Small particle size facilitates extraction by shortening diffusion paths. As a result, time of solute equilibrium between its inside and outside concentration of the biomass particles is shortened by reducing the particle size.

Two kinds of needle powder of *Taxus canadensis* with particle size of < 100 mesh and 40-60 mesh were used to study the effect of particle size on CSE. Forty percent of ethanol/water (v/v) mixture was used as solvent in this study. The extracts were analyzed with HPLC for paclitaxel concentration. The results are given in Figure 5.27 and Figure 5.28.

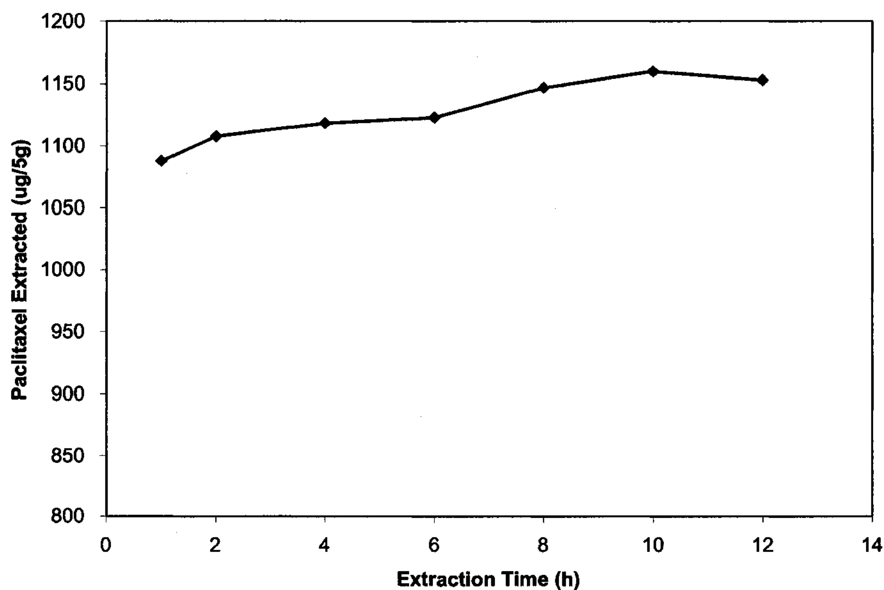


Figure 5.27 Paclitaxel Concentrations in Extracts of CSE using 40-60 mesh Needle Powder. Extraction Conditions: 5.000g 40-60 mesh needle powder; solvent 50.0ml 40% ethanol/water at 25°C.

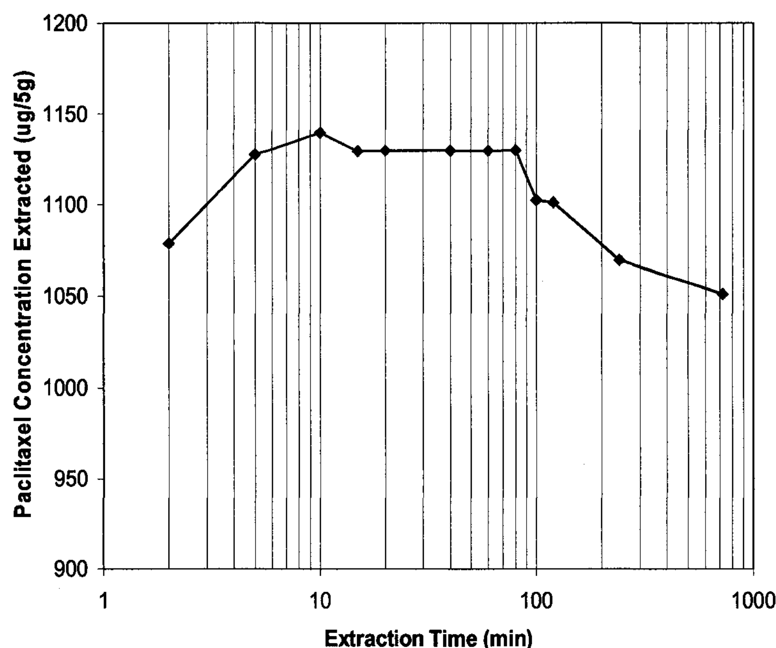


Figure 5.28 Paclitaxel Concentrations in Extracts of CSE with < 100 mesh Needle Powder. Extraction Conditions: 5.000g <100 mesh needle powder; solvent 50.0ml 40% ethanol/water (v/v) at 25°C.

Particle size has strong effect on extraction kinetics in CSE with 40% ethanol/water (v/v). Based on the results of CSE using 40-60 mesh needle powder experiment as given in Figure 5.27, the maximum concentration was observed in the extract with extraction time of 10 hours. The concentrations of paclitaxel in extracts were increasing with extraction time less than 10 hours.

However, the results of CSE of <100 mesh needle powder experiment show that the maximum concentration of paclitaxel was detected in the extract with extraction time of

10 minutes (Figure 5.28). This extraction time is significantly shorter than 10 hours when 40-60 mesh needle powder was used.

5.2.4. Paclitaxel Stability Study in CSE

Paclitaxel degradation in various solvents was reported by MacEachem-Keith et al. (1997). The declining trend of paclitaxel concentration in extracts of CSE with 40% ethanol/water was also observed in this study. This arouses the concern of paclitaxel stability in ethanol/water mixture. Therefore, paclitaxel stability is studied in this work.

The extract of <100 mesh needle powder of CSE with 60% ethanol/water was used to investigate the stability of paclitaxel. The pH of the extract in CSE using ethanol/water (v/v) mixture was measured to be between 5.5 and 6.0 (5.000g needle powder/50.0ml solvent).

The stability of paclitaxel was also studied in basic 60% ethanol/water (v/v) solution. Na_2CO_3 water solution was used to adjust pH of 60% ethanol/water (v/v) extract to 8.0-8.5.

In the paclitaxel stability study in acidic solution (pH is 5.5-6.0) or basic solution (pH is 8.0-8.5), this procedure was followed:

All samples used were from one well mixed 60% ethanol/water (v/v) extract. After filtration through 0.45μm filters, sample was injected with a 717 Autosampler at an interval of 65 minutes for paclitaxel concentration analysis with HPLC until 13 injections. The results are given in Figure 5.29 and Figure 5.30.

The results show there are no significant changes of paclitaxel concentration in ether acidic or basic solutions observed within 12 hours at room temperature. Paclitaxel is very stable in 60% ethanol-water solution at pH 5.5-6.0 (Figure 5.29), and a little bit unstable in 60% ethanol-water solution at pH 8.0-8.5.

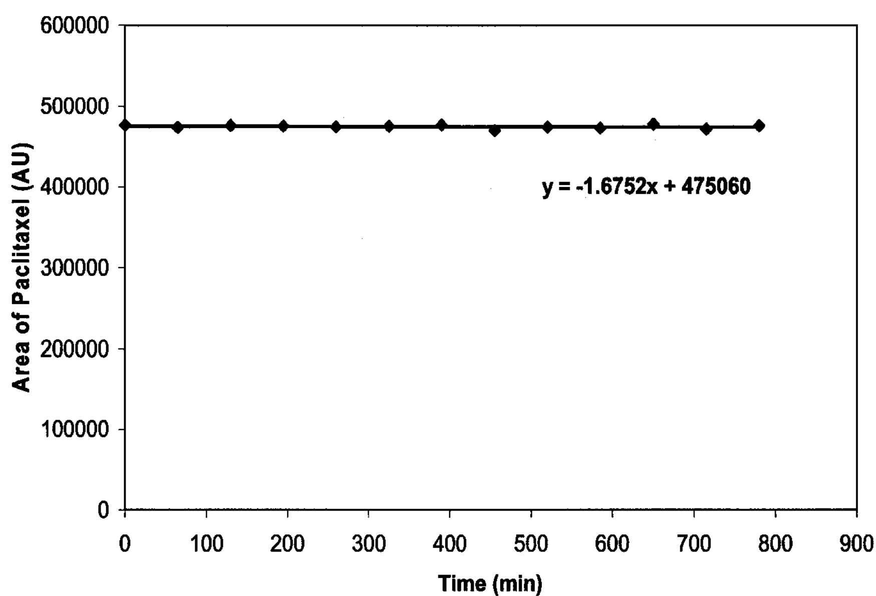


Figure 5.29 Paclitaxel Stability Study in Acidic Solution. The pH of 60% Ethanol/Water is 5.5-6.0; temperature is 25°C.

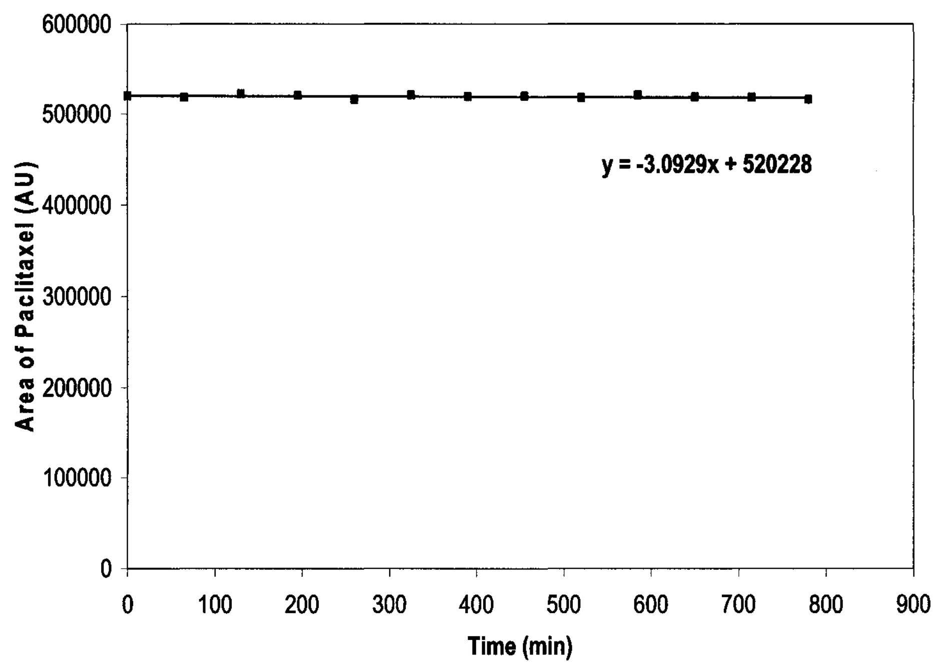


Figure 5.30 Paclitaxel Stability Study in Basic Solution. The pH of 60% Ethanol/Water is 8.0-8.5; temperature is 25°C.

5.3. Solid Phase Extraction

Solid Phase Extraction (SPE) exceeds Liquid-Liquid Extraction (LLE) for product recovery from extract in bioseparation process as discussed in Chapter II. SPE is readily integrated with extraction and preparative chromatography as discussed in Chapter III. Therefore, SPE was used to recover taxanes in extracts from either DPLE or CSE.

Only Normal-Phase SPE (NP-SPE) was studied to recover taxanes from extract of DPLE because the hydrophobic solvents used in DPLE are only suitable for NP-SPE. Similarly, only reverse-phase SPE (RP-SPE) was studied to recover taxanes from extract of CSE because the polar solvents used in CSE are only suitable for RP-SPE.

5.3.1. Normal Phase Solid Phase Extraction

Five grams of silica gel was used in NP-SPE to recover taxanes from dichloromethane extract of DPLE of 5.000 gram of < 100 mesh needle powder. The dichloromethane solution from the NP-SPE column was collected and dried in fume hood for 12 hours.

The dry residue of dichloromethane solution was dissolved in 5.0 ml methanol and analyzed for taxane content after filtration through 0.45 μm filter. There was no taxane in the residue of dichloromethane solution from NP-SPE. This result suggests that all the taxanes were recovered by NP-SPE.

NP-SPE can be followed by normal phase preparative chromatography if high purity products are desired. This can be conveniently done by adding another column packed with silica gel to the end of the SPE column and eluting the two series-wound columns.

5.3.2. Reverse Phase Solid Phase Extraction

The capability of various adsorbents for taxanes adsorption is studied in batch experiments to recover taxanes from 30% ethanol/water (v/v) and 60% ethanol/water (v/v) solutions. The selectivity of adsorption was evaluated by separation factor defined by the following equation:

$$Separation\ Factor = \frac{Percentage\ of\ Taxane\ Adsorbed}{Percentage\ of\ Solutes\ Adsorbed} \qquad 5.1$$

Both high recovery rate and high separation factor are desired in taxanes recovery experiment by RP-SPE. The results of adsorption in 60% ethanol/water (v/v) are given Table 5.4, while the results of adsorption in 30% ethanol/water (v/v) are given in Table 5.5.

Comparing the results of adsorption from solution of 60% ethanol/water (v/v) with those of the solution of 30% ethanol/water (v/v), both high recovery rate and high separation factor were achieved only in 30% ethanol-water with macropore resins. This suggests that low solvent strength facilitates adsorption and increases both recovery rate and separation factor in RP-SPE.

The separation factors for paclitaxel and 9-DHB III of macropore resins are higher than those of activated charcoal in the adsorption experiment from 30% ethanol/water (v/v) given in Table 5.5. The separation factor for paclitaxel using macropore resin SP207 is 7.1, while that using <100 mesh active carbon is merely 2.3, although the recoveries using the two adsorbents are the same as 100%.

Therefore, macropore resins are preferred in taxane recovery from low organic solvent content extracts of CSE using RP-SPE.

Table 5.4 Batch Adsorption Experiment Results in 60% Ethanol/water Solution. Five grams of adsorbents were used to adsorb taxanes in 50.0ml 60% ethanol/water extract at 25°C. Extraction Conditions: The ratio of <100 mesh needle powder and 60% ethanol/water (v/v) was 1:10 (w/v); temperature was 25°C; extraction time was 10 minutes.

Adsorbent	**Macropore Resin HP2MG**	**Macropore Resin SP207**	**20-40 mesh Activated Charcoal**	**<100 mesh Activated Charcoal**
Solutes Adsorbed by the Adsorbent	12%	14%	22%	35%
9-DHB III Recovered by the Adsorbent	17%	33%	34%	70%
Paclitaxel Recovered by the Adsorbent	40%	72%	72%	98%
Separation Factor for 9-DHB III	1.4	2.4	1.5	2.0
Separation Factor for Paclitaxel	3.3	5.1	3.3	2.8

Table 5.5 Batch Adsorption Experiment Results in 30% Ethanol/water Solution. Five grams of adsorbents were used to adsorb taxanes in 50.0ml 60% ethanol/water extract and 50.0ml water at 25°C. Extraction Conditions: The ratio of <100 mesh needle powder and 60% ethanol/water (v/v) was 1:10 (w/v); temperature was 25°C; extraction time was 10 minutes.

Adsorbents	**Macropore Resin HP2MG**	**Macropore Resin SP207**	**20-40 mesh Activated Charcoal**	**<100 mesh Activated Charcoal**
Solutes Adsorbed by the Adsorbent	12%	14%	26%	43%
9-DHB III Recovered by the Adsorbent	77%	93%	84%	99%
Paclitaxel Recovered by the Adsorbent	95%	100%	97%	100%
Separation Factor for 9-DHB III	6.4	6.6	3.2	2.3
Separation Factor for Paclitaxel	7.9	7.1	3.7	2.3

Batch adsorption is just "one plate" mode operation according to the chromatographic plate theory (Thurman, 2001). According to this theory, higher recovery rate and higher selectivity are expected if the experiment were carried out in column mode or continuous mode with the same amount of adsorbents and the same amount of extract.

Chapter VI: Conclusions and Recommendations

6.1. Conclusions

1. Solute Distribution Model of natural products in term of solvent solubility is proposed. Based on this model, two approaches, dual-solvent extraction and extraction-adsorption are proposed to increase both recovery rate and selectivity in bioseparation processes. The two approaches are applied in separation and purification of taxanes. Both dual-solvent extraction and extraction-adsorption approaches with nonpolar solvent succeed in isolation of taxanes.

2. The concept, Integrated Bioseparation Technologies, is proposed. The integrated process, DPLE-SPE-Chromatography, which is studied as an example of Integrated Bioseparation Technologies, shows promising for separation and purification of taxanes.

3. A new extraction technology for natural products recovery, Dynamic Pressurized Liquid Extraction, was invented and proved to be successful in taxanes recovery from needle powder of *Taxus canadensis*. It is a promising extraction technology in bioseparation at both large scale and lab scale. It is compatible with NP-SPE, the primary separation technique that surpasses Liquid-Liquid Extraction.

4. Based on the distribution model and approaches, Conventional Solvent Extraction was studied. Comparing to DPLE, low selectivity in CSE is due to the large amounts of hydrophilic impurities in the needle of *Taxus canadensis*.

6.2. Recommendations

1. The solute distribution in term of solvent solubility can be studied in details with Evaporative Light Scattering Detector (ELSD) in HPLC system. Reverse phase HPLC columns are suitable for hydrophilic solutes distribution study. Normal phase HPLC columns should be used to find hydrophobic solutes distribution in biomass of natural products.

2. Detailed study of DPLE is needed. More solvents should be tested for taxane recovery with DPLE. The efficiency of DPLE should be verified with more recovery experiments of various natural products.

3. More adsorbents should be tested in both NP-SPE and RP-SPE. The adsorption conditions need to be optimized.

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Appendix A: Standard Curve of Taxanes Preparation

MIXED STANDARD PREPARATION PROCEDURE:

Paclitaxel, baccatin III and 10-DAB III were weighed individually and transferred into a 100.0 ml volumetric flask. Filled the 100.0 ml volumetric flask with HPLC grade acetonitrile and shaked it to dissolve the standards.

9-DHB III were weighed and transferred into 10 ml test tube. Fill the test tube with 10.0 ml HPLC grade acetonitrile.

Mixed Standard Concentration:

Name	**Concentration (µg/ml)**
Paclitaxel	65.47
Baccatin III	24.03
10-DAB III	93.06

9-DHB III Standard Concentration:

Name	**Concentration (µg/ml)**
9-DHB III	45.0

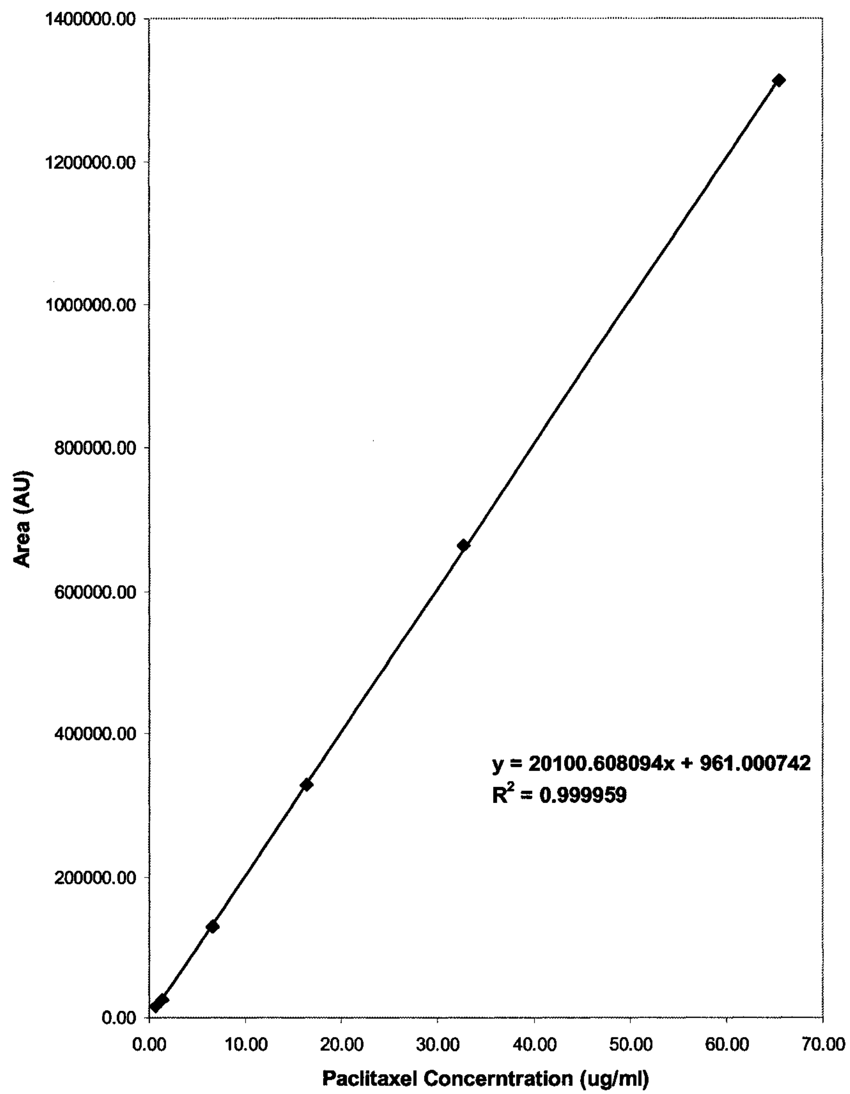
STANDARD CURVE PREPARATION:

Dilute the mixed standards with acetonitrile to make five diluted mixed standards with concentrations of 0.5, 0.25, 0.1, 0.02, and 0.01 to the original concentration.

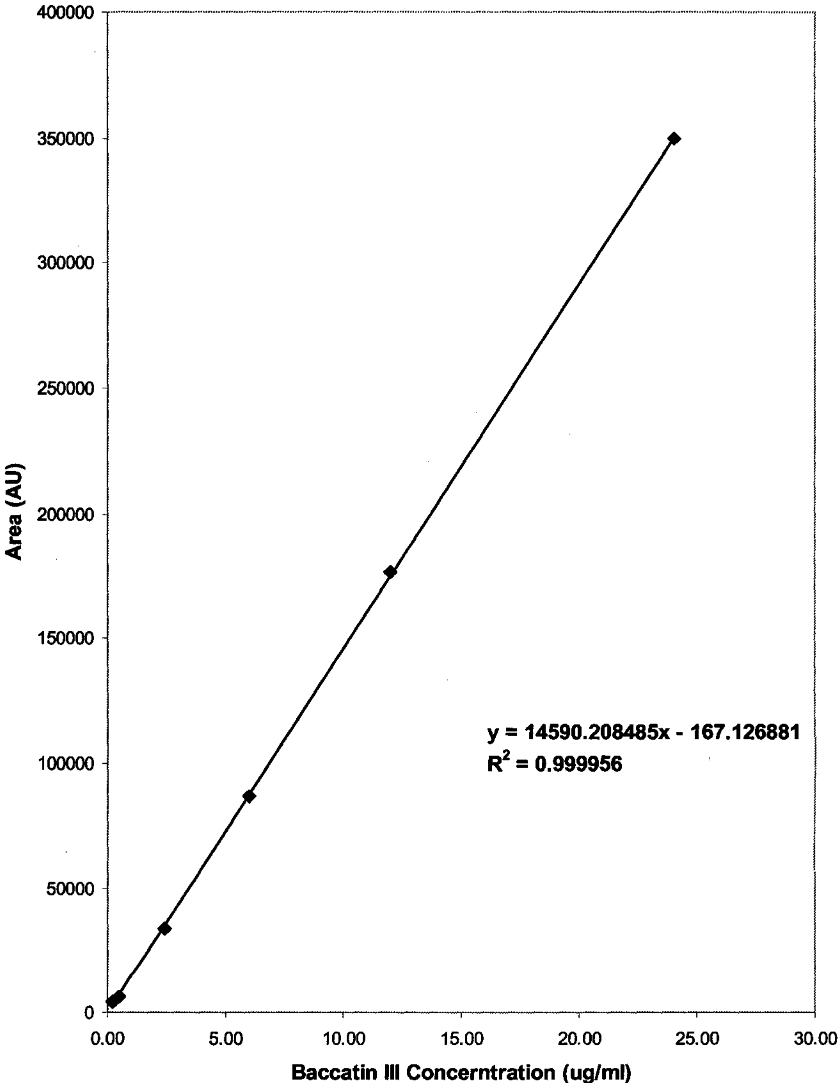
Test with HPLC, 3 injections of each concentration, 10 μL each injection in volume. Results see following table. Chart of standard curves see Appendix B, C and D.

	Concentration (ug/ml)			Average Area (AU)		
Dilution	**10-DAB III**	**Baccatin III**	**Paclitaxel**	**10-DAB III**	**Baccatin III**	**Paclitaxel**
1.00	93.06	24.03	65.47	1420625	349931	1314651
0.50	46.53	12.02	32.74	725162	176530	664957
0.25	23.27	6.01	16.37	358610	86961	328083
0.10	9.31	2.40	6.55	135185	34302	130574
0.02	1.86	0.48	1.31	26728	6213	26002
0.01	0.93	0.24	0.65	15389	4193	15591

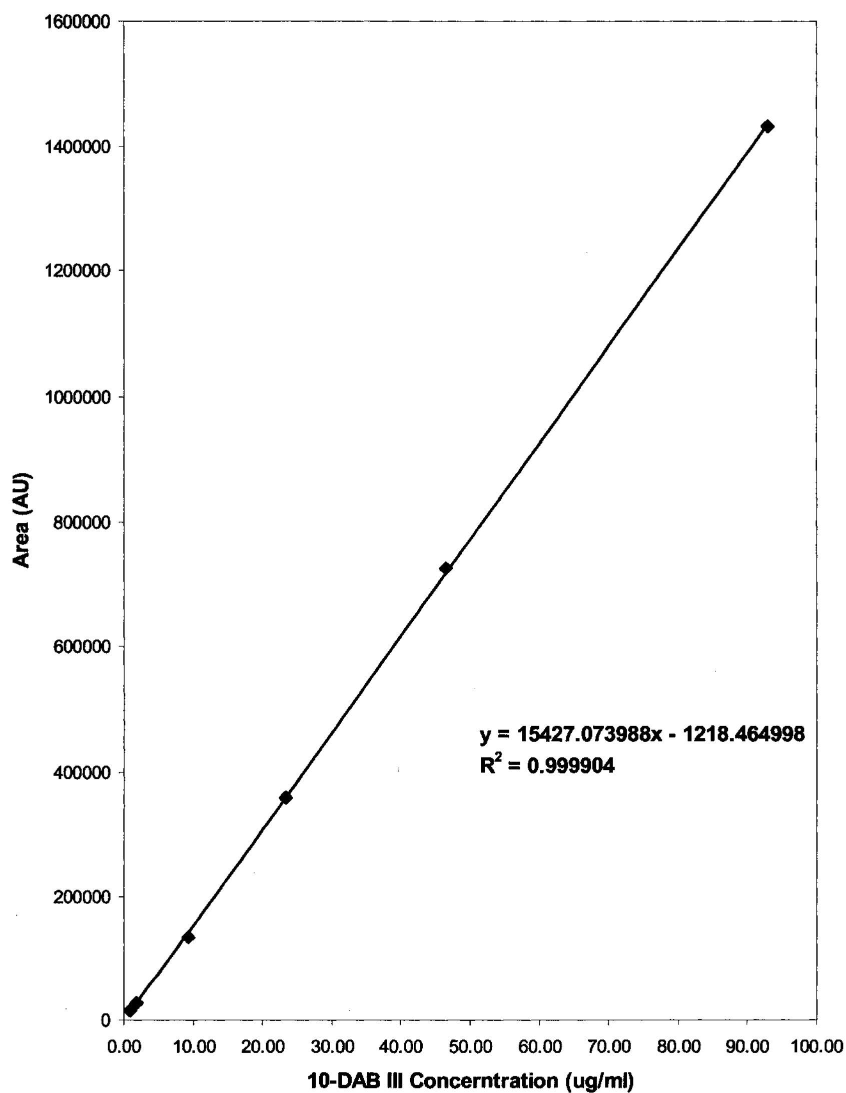
Appendix B: Standard Curve of Paclitaxel



Appendix C: Standard Curve of Baccatin III



Appendix D: Standard Curve of 10-DAB III



Appendix E: Solid Phase Extraction Validation

PROCEDURE:

Five grams of needle powder of *Taxus canadensis* (<100 mesh) was weighed and transferred into a 125 ml Erlenmeyer flask with magnetic bar. Fifty ml HPLC grade methanol was added to it. It was agitated with a magnetic agitator for 10 minutes. After 10 minutes, extract was filtrated with Buckner funnel and used to validate the SPE method.

Maxi-Clean™ (C18) 300 mg SPE cartridges from Alltech Associates, Inc. were used throughout all experiment. The procedure is described as following:

1. The cartridge is conditioned with 2 ml of methanol, then 2 ml HPLC grade water.
2. 1.0 ml of methanol extract is piped out and added 1.0 of HPLC grade water to form a diluted methanol solution.
3. The diluted solution is slowly loaded into the conditioned cartridge in 3 minutes. Stand by 30 minutes to allow the solute to be adsorbed on the surface of absorbent.
4. Elute the cartridge with 2 ml 20% methanol slowly.
5. Elute the cartridge with 2 ml 50% methanol slowly.
6. The taxanes are eluted with 4.0 ml 100% methanol for HPLC analysis.
7. Repeat step 1 to step 6 three times to obtain three samples.

The methanol extract is analyzed by HPLC without treatment. The recovery rate of SPE is calculated as 98.97% comparing with the result without treatment.

RESULTS:

Paclitaxel Content via SPE (μg/g)			**Paclitaxel Content without SPE (μg/g)**	**Recovery**
1423	1460	1442	1456	
Average: 1441			1456	98.97%