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Targeted Extraction of Select Natural Products Using Supercritical CO_2

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Targeted Extraction of Select Natural Products Using Supercritical CO_2

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
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DEDICATION

To my incredible husband Brad and our children Anna Belle (5) and Ainsley Carol (2)

"Nothing in life is to be feared. It is only to be understood." – **Marie Curie**

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Abstract

This thesis examined the supercritical carbon dioxide (CO_2) extraction (SFE) of targeted secondary metabolites from selected natural products. Phytochemical characterization and determination of biological efficacy was completed using advanced analytical methods and *in-vivo* bio-assays. More than 500 SFE extracts were generated and characterized. Sample preparation (particle size, moisture content) and extraction parameters (pressure, temperature and flow rate) were investigated and the ability of SFE to extract specific biosynthetic classes of compounds was evaluated. Where warranted, pilot scale studies were conducted for larger scale biological trials.

SFE of the neotropical vine, *Souroubea sympetala*, a traditional medicine used for a culture bound syndrome related to anxiety, resulted in an extract rich in betulinic acid (5.54 ± 0.24 mg/g) determined by HPLC-APCI/MS. When subjected to validated rodent anxiety behavioral assays (e.g. elevated plus maze (EPM)) the SFE extract demonstrated significant anxiolysis (anti-anxiety) compared to the vehicle control, with a 50% increase in time spent in open arms and a 73% increase in unprotected head dips observed. The significant *in-vivo* anxiolysis observed provides scientific support for the ethnobotanical use of this traditional medicine and a promising lead for a natural health product (NHP) to treat anxiety.

Systematic variation of SFE parameters (pressure and temperature) on pyrethrum (*Chrysanthemum cinerariifolium)* oleoresin, a natural insecticide, resulted in a residue free pyrethrin concentrate, suitable for organic agricultural applications. The highest pyrethrin concentration (0.531 g/g) and highest ratio of pyrethrins I (pyrethrin I, cinerin I, jasmolin I) to pyrethrins II (pyrethrin II, cinerin II, jasmolin I) (PI:PII = 1.95) was

obtained at 40 °C; 10 MPa while 40°C; 30 MPa produced the lowest pyrethrin concentration (0.436 g/g) and lowest ratio of PI:PII (1.87). Total pyrethrin recovery was highest at 40°C; 30 MPa (8.39 g pyrethrins) vs. 40°C; 10 MPa (6.96 g pyrethrins). The insecticidal activity of the SFE extracts of the pyrethrum oleoresin was confirmed (>70% mortality at 48 h) using the Colorado potato beetle (CPB) a major pest of agriculture.

SFE of piperamides from black pepper (*Piper nigrum*), a promising botanical insecticide and synergist, was successful at pressures above 30 MPa and temperatures greater than 50°C with total yields greater than 7.87% (CV < 0.10) and piperamide concentrations greater than 150 mg/g (CV < 6.0). Flow rate, sample size, and particle size were optimized at 5 L/min, 10 g, and 1 mm mesh respectively. Insecticidal activity corroborated dose dependent piperamide bioactivity. Extract from 10 MPa; 60°C (42.38 mg/g) produced 10.0±6.8 (24 h) and 13.3±6.7% (48 h) mortality. Extract from 35 MPa; 40°C (148.56 mg/g) produced 70.0±10.0 (24 h) and 73.3±8.4% (48 h) mortality.

Northern shrimp (*Pandelus borealis*) by-products extracted by SFE (35 MPa; 40°C) generated a deep red oil rich in polyunsaturated fatty acids (PUFAs), specifically eicosapentaenoic acid EPA (7.8±0.06%) and docosahexaenoic acid DHA (8.0±0.07%). Optimization of pressure (60 MPa), temperature (80°C), moisture (13%), particle size (0.85 mm), and flow rate (9 L/min) resulted in a more efficient extraction without significantly changing concentrations of EPA and DHA. Pilot scale studies confirmed experimental data and supports further investigation of commercial production of an omega -3 (ω-3) concentrate from Northern shrimp by-products.

Résumé

Cette thèse porte sur l'extraction au dioxyde de carbone supercritique (CO_2) (SFE) d'importants métabolites secondaires appartenant à divers produits naturels. La caractérisation phytochimique et la détermination de l'efficacité biologique ont été complétées en utilisant des méthodes d'analyse avancées et des bio-essais *in-vivo*. Plus de 500 extraits SFE ont été produits et caractérisés. La préparation d'échantillons (taille des particules, la teneur en eau) et les paramètres d'extraction (pression, température et débit) ont été étudiés et la capacité de SFE pour extraire certaines classes de composés a été évaluée. Lorsqu'il y a eu lieu, des études pilotes ont été menées pour les essais biologiques à grande échelle.

L'extrait obtenu par SFE de la vigne néotropicales *Souroubea sympetala,* un remède traditionnel utilisé pour traiter un type de "syndrome relié à la culture" associé à l'anxiété, a conduit à un extrait riche acide bétulinique (5,54 ± 0,24 mg / g) comme déterminée par CLHP-APCI/MS. Lorsque soumises à des tests comportemental d'anxiété pour rongeurs (p. ex élevée labyrinthe plus (EPM)), l'extrait obtenu par SFE a démontré une réduction d'anxiété significative par rapport au contrôle, avec une augmentation de 50% du temps passé dans les bras ouverts et une augmentation de 73% d'observations d'abaissements de la tête non protégée. Cette réduction d'anxiété observée *in vivo* fournit un soutien scientifique pour l'utilisation culturelle et traditionnelle de cette médicine et offre une piste prometteuse pour le développement d'un produit de santé naturel (PSN) pour traiter l'anxiété.

La variation systématique des paramètres de SFE (pression et température) de pyrèthre *(Chrysanthemum cinerariifolium)* oléorésine, un insecticide naturel, ont abouti à un concentrer de pyréthrine sans résidus approprié pour des applications agricoles organiques. La plus forte concentration de pyréthrines (0,531 g / g) fut obtenu et le plus haut taux de pyréthrines I (pyréthrine I, I cinerin, je jasmolin) aux pyréthrines II pyréthrine (II, cinerin II, jasmolin I) (PI: PII = 1,95) a été obtenu à 40 ° C, 10 MPa alors que 40 ° C, 30 MPa a produit la concentration la plus faible de pyréthrine et le plus faible ratio de PI: PII (0,436 g / g et 1,87). La récupération totale de pyréthrine fut le plus élevé à 40 ° C, 30 MPa (8,39 pyréthrines g) vs 40 ° C, 10 MPa (6,96 g pyréthrines). L'activité insecticide des extraits SFE de l'oléorésine pyrèthre a été confirmé (70% de mortalité> 48 h) à l'aide d'un insecte ravageur, le doryphore de la pomme (OPC).

L'extrait de piperamides obtenu du poivre noir (*Piper nigrum*), un insecticide botanique prometteur et synergiste, a réussi à des pressions supérieures à 30 MPa et une température supérieure à 50 ° C avec un rendement supérieure à 7,87% (CV <0,10) et une concentration de piperamides supérieure à 150 mg / g (CV <6,0). Le débit, la taille de l'échantillon et la taille des particules ont été optimisés à 5 L / min, 10 g, et mailles de 1 mm, respectivement. Les résultats démontrent que l'activité insecticide dépend de la présence de piperamides. À 10 MPa et 60 ° C (42,38 mg / g), le pourcentage de mortalité du 2e stade OPC traités par voie topique avec un extrait de poivre (0,075%) à 24 et 48 h ont été de 10,0 ± 6,8 et 13,3 ± 6,7% respectivement. À 35 MPa et 40 ° C (148,56 mg / g) le pourcentage de mortalité à 24 et 48 h ont été 70,0 ± 10,0 et 73,3 ± 8,4% respectivement.

L'extrait des sous-produits de la crevette nordique *(Pandelus borealis)* obtenus par SFE (35 MPa et 40 ° C) a généré une huile rouge foncée riche en acides gras polyinsaturés (AGPI), plus précisément l'acide eicosapentaénoïque EPA (7,8 ± 0,06%) et l'acide docosahexaénoïque DHA (8,0 ± 0,07%). L'optimisation de la pression (60 MPa), la température (80 ° C), de l'humidité (13%), la taille des particules (0,85 mm), le débit et (9 L / min) a entraîné une extraction efficace sans modifier de façon significative les concentrations d'EPA et de DHA. Des études pilotes ont confirmé les données expérimentales et soutiennent l'idée de poursuivre l'investigation de la production commerciale d'un concentré de ω-3 obtenu des sous-produits de la crevette nordique.

Acknowledgements

This accomplishment would not have been possible without the love, guidance and support of my entire family, Dr. John Arnason and the lab group at the University of Ottawa, my colleagues at Loyalist College and John Baker at Bioniche Life Sciences.

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Acronyms & Abbreviations

ASE – accelerated solvent extraction
α – alpha
AAPCC - American Association of Poison Control Centres
ANOVA – analysis of variance
APA – American Psychiatric Association
APCI – atmospheric pressure chemical ionization
β – beta
BA – betulinic acid
BZD – benzodiazepine
BHT - butylated hydroxytoluene
CBD - cannabidiol
CI – confidence intervals
CO_2 – carbon dioxide
CVD – cardiovascular disease
CNS – central nervous system
CPB – Colorado potato beetle
CYP – cytochrome P450
DAD – diode array detector
DEET – N, N-diethyl-m-toluamide
Df – degrees of freedom
DHA - docosahexaenoic
DHP – dihydropiperlonguminine
EPA - eicosapentaenoic acid
EPM – elevated plus maze
EtOAc – ethyl acetate
EtOH – ethanol
FL – Fiducial limits
FAO – Food and Agriculture Organization
γ – gamma
GABA – γ-aminobutyric acid
GABAergic – relating to GABA transmission
GABA-T – GABA transaminase
GC – gas-chromatography
H_2O – water
HIV – human immunodeficiency virus
HPLC – high performance liquid chromatography
LC – liquid chromatography
LC/MS – liquid chromatography/mass spectrometry
LOD – limit of detection
MAP – microwave assisted process
MDP – 3,4-methylenedioxyphenyl
MeCN – acetonitrile
MeOH – methanol
Min - minute
MS – mass spectroscopy
n – number (sample size)
Na^+ - sodium ion
NRC – National Research Council
NHP – natural health product
NHPD – Natural Health Products Directorate
NMR – nuclear magnetic resonance
ω – omega
OA – oleanolic acid
OMe – methoxy (**$-OCH_3$**)
PI – pyrethrins I
PII – pyrethrins II
p – probability (statistics)
PBO – piperonyl butoxide
PL – piperlonguminine
PSMO - polysubstrate mono-oxygenase
PTFE – polytetrafluoroethylene
PUFAs – polyunsaturated fatty acids
RP – reverse phase
RT – room temperature
SB – shrimp by-products
SD – standard deviation
SE – standard error
SF – supercritical fluid
SFE – supercritical fluid extraction
SCE – supercritical extraction

SFT – supercritical fluid technology
SH – shrimp heads
SIM – single ion mode
Sox – soxhlet extraction
SFE – supercritical CO_2 extraction
SM – shrimp muscle
T_c - critical temperature critical pressure
P_c - critical pressure
TESS – Toxic Exposure Surveillance Systems
TFA – total fatty acids
THC – tetrahydrocannabinol
UA – ursolic acid
UAE – ultrasonic assisted extraction
UV – ultra violet

Units

Å – Angstrom
AU – arbitrary unit
cm – centimeter
°C – degrees Celsius
g – gram
L – litre
MPa - megapascals
µg – microgram
µL – microlitre
mg – milligram
mL – millilitre
mm – millimetre
min – minute
% - percent

Chapter 1
1.0 Introduction

1.1 Introduction to thesis

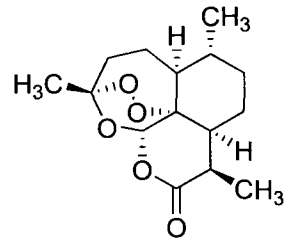
Natural products have been exploited by humans for thousands of years. Traditional medicine is probably the oldest system of botanical use and relies mainly on the many healing properties of plant secondary metabolites. Natural plant-based pesticides were used more than 1000 years B.C. by the Chinese as seed treatments and fungicides (Krief et al., 2009). More recently natural foods (terrestrial, marine based) have been associated with beneficial effects on health and the prevention of disease.

The rich history of traditional medicine, the complexity of natural product structures, and the wide array of biological properties demonstrated by natural products has provided inspiration for modern drug discovery (Kinghorn et al., 2009; Soejarto & Farnsworth, 1989; Clardy & Walsh, 2004; Cox, 1994). The isolation of compounds from plants and their derivatization is the basis of many modern pharmaceutical drugs (Cragg et al., 1997; Kinghorn et al., 2009). A recent example is the development of several semi-synthetic anti-malarial drugs from research based on artemisinin (**1**) (Figure 1) the active principle of *Artemisia annua*, a traditional Chinese phytomedicine which has been used for several millennia (Oliveira et al., 2009).

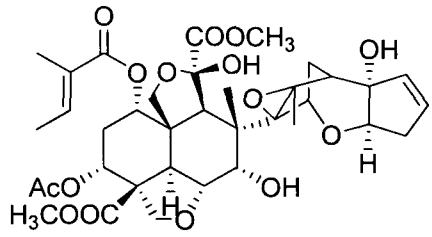
The use of synthetic pesticides (carbamates, organophosphates, synthetic pyrethroids, neonicotinoids) has contributed to the increased productivity, lower cost and intensive land use in modern agriculture. However, growing health and environmental concerns, more stringent registration procedures for pesticides and an increased consumer

adoption of organic-agricultural products has recently resulted in increased interest in botanical insecticides (Dayan et al., 2009). One example is the Indian neem tree, *Azadirachta indica* (Meliaceae) which contains the complex triterpene azadirachtin (**2**) (Figure 1) and other limonoids which are effective feeding deterrents and growth regulators to hundreds of pest insect species (Isman, 2006).

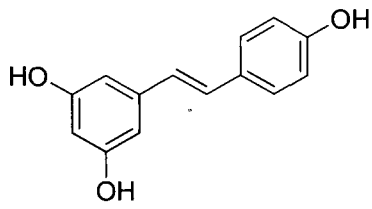
Certain diets, such as the Mediterranean diet have been associated with a decreased incidence of several chronic diseases (CVD, type II diabetes, cancer) (Block et al., 1992; De Lorgeril et al., 1999; Fung et al., 2009; Hu et al., 2001). Our increased understanding of the role of specific phytochemicals in foods includes work on resveratrol (**3**), a stilbene found in grapes and red wine (Figure 1) (Kris-Etherton et al., 2002). Reports have shown it improves the health and survival of mice on a high calorie diet (Baur et al., 2006), is associated with a decreased risk of CVD (Baur & Sinclair, 2006) and exhibits cancer chemopreventative activity (Jang et al., 1997; Kundu & Surh, 2008).



1



2



3

Figure 1: Natural products: 1. Artemisinin, 2. Azadirachtin, 3. Resveratrol

Despite increased consumer adoption of many commercial natural products (e.g. natural health product (Ipsos Reid, 2005) or botanical insecticides (Isman 2006), the majority of natural product extracts are generated using toxic and/or flammable solvents (hexane, ethyl acetate, alcohol). The health, environmental and safety challenges associated with conventional solvent extraction of natural products has resulted in the development of technologies such as ultrasonic assisted (UAE), accelerated solvent (ASE), and microwave assisted process (MAP) extraction which reduce the amount of solvent used and decrease the time required for extraction (Deevanhxay et al., 2009). Supercritical fluid (CO_2) extraction (SFE) has been studied extensively in the discipline of chemical engineering. It remains relatively unexplored for natural products. In this thesis, the use of SFE has been studied to provide an in depth assessment of the diversity of biosynthetic classes of compounds it can be effectively used with, as well as provide methods to prepare biologically active extracts for emerging products of interest.

1.2 Literature review

1.2.1 Supercritical CO_2 Extraction (SFE)

Supercritical fluid extraction (SFE) using carbon dioxide (CO_2) represents an innovative and efficient technique to produce a high quality "clean" extract with no solvent residues (Beckman, 1996; Raventós et al., 2002). These extracts can be approved for status as "organic" (=fr: biologique) providing significant market advantage. However, another reason for considering this method is its superior ability to fractionate natural product extracts to produce more effective and/or less toxic materials (Pereira & Meireles, 2009). Supercritical fluids exhibit exceptional solvent properties. Solvent clustering effects result in areas of local density much greater than the bulk density of the system. This results in liquid-like solvent solubility. This liquid-like property is complimented by the gaseous properties of surface tension, viscosity, and diffusivity which results from the distance between the clusters. The most commonly used supercritical fluid is carbon dioxide. The conditions necessary to achieve the critical parameters are mild, the critical temperature (T_c) = 304.15 K and the critical pressure (P_c) = 7.38 MPa (Appendix I). Dramatic effects in density, hence solute solubility within this region are observed with moderate changes in temperature and pressure.

SFE technology has been available for the past several decades, but due to a high capital investment and unfamiliar operating systems, the commercial applications of this technology were not recognized until fairly recently (Sahena et al., 2009; Rosa & Meireles, 2005). Safety and environmental concerns associated with organic solvents have provided an impetus for the development of clean technologies to replace many current systems. Although capital intensive, SFE using CO_2 represents a viable

alternative with several advantages: it is non-toxic, non-flammable, has GRAS (generally regarded as safe) status, is environmentally friendly, and economical to operate the equipment (Kaiser, 1996). In addition, CO_2 has a critical temperature of 31°C, which enables thermally labile biological products to be extracted without degradation. It is able to selectively extract a wide array of compounds, and recent studies point to SFE technology as a means of sterilization, useful for inactivating microbes of heat-sensitive materials (Spilimbergo et al., 2002; Zhang et al., 2006).

Temperature and pressure are the primary conditions that are modified to optimally target specific extract constituents (Hamburger et al., 2004). It eliminates the need for tedious isolations required in traditional methods and provides extracts which can be directly characterized using analytical methods without solvent removal, and better suited to include in a wide array of biological tests. Minor variations in the operational conditions of the system significantly affect the solubilizing power (density) of the supercritical fluid (SF). As a result, supercritical fluids possess the ability to selectively extract a particular solute, dependent of density.

While several conditions can theoretically achieve target densities for solubilizing a particular solute, inherent system constraints often reduce options. In addition to pressure and temperature, factors that affect SFE include flow rate, extraction time, collection technique, sample size, sample matrix, supercritical fluid used, choice of co-solvent, and the amount of co-solvent.

Supercritical CO_2 is in its simplest form best suited for the extraction of lipophilic compounds (Sahena et al., 2009). Previously, many biologically active classes of natural products in medicinal plants e.g. phenolics, alkaloids, and their glycosides were

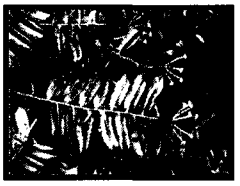
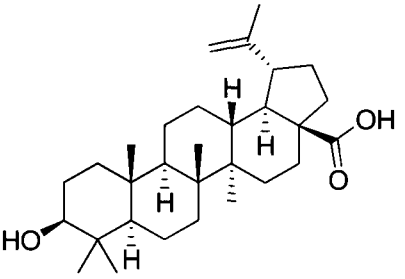
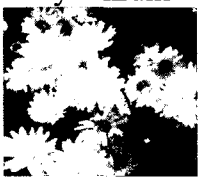
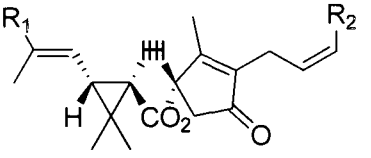
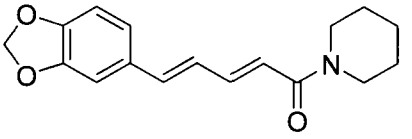
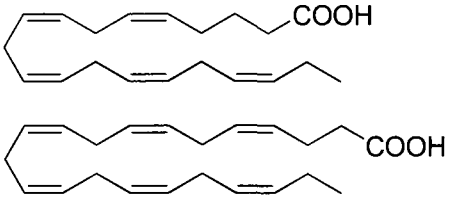
considered to have limited solubility in CO_2, as they are not readily extractable at low to moderate pressures. Engineering SFE to overcome limited solubility has been accomplished through designs which allow for increases in pressure and the seamless addition of co-solvents such as ethanol and methanol. This significantly increases the solubility of polar compounds (Shi et al., 2005). The targeted extraction of bioactive compounds from natural sources with SF CO_2 presents a unique challenge and despite dramatic increases in SFE of natural products in the past decade it remains relatively unexplored in the scientific literature (Kiran et al., 2009). Recent investigations emphasize that in addition to the technical obstacles of SFE, substantial developmental work for most natural products is necessary to develop SFE protocols which account for the often complex bio-chemical matrix of each system (Catchpole et al., 2002). A commercially promising source of resveratrol (from grape pomace) was recently targeted for extraction using SFE (Casas et al., 2010).

1.2.2 Review of Natural Products under Investigation

The selection of natural products for this thesis was based on the research interests of our laboratory and collaborators and includes: the neotropical medicinal plant sin susto (*Sourourbea sympetala* V.A. Richt Marcgraviaceae), pyrethrum (*Chrysanthemum cinerariifolium*) Benth. & Hook. (Asteraceae), black pepper (*Piper nigrum*) L. Lada (Piperaceae), and Northern Arctic Shrimp (*Pandalus borealis)* (Pandalidae). The bioactive compounds targeted for investigation using SFE are representative of different biosynthetic classes of natural products; pentacyclic triterpenoids, irregular monoterpenes, piperidine alkaloids, and polyunsaturated fatty acids (PUFAs). The

natural product extract, a representative target compound and specific bioactivity being investigated are presented (Table 1).

Table 1: Commercially promising natural products of interest and representative target biomolecules of interest.

Common name	**Species**	**Marker Bioactive Compound(s)**
Sin Susto 	*Sourourbea sympetala*	Betulinic acid 
Pyrethrum 	*Chrysanthemum cinerariifolium*	Pyrethrin I  R_1 CH_3, R_2 $CHCH_2$
Black Pepper 	*Piper nigrum*	Piperine 
Northern Arctic Shrimp 	*Pandalus borealis*	EPA and DHA 

1.2.2.1 Sin susto (Sourourbea sympetala) V.A. Richt (Marcgraviaceae)

Sourourbea sympetala V.A. Richt (Marcgraviaceae), commonly referred to as Sin Susto

(= without fear) is a neo-tropical vine deeply rooted in the ethnobotanical tradition of several indigenous populations in Central America. Schultes describes the calming effect of genus *Souroubea* in his widely cited text on Amazonian ethnobotany, "The Healing Forest" (Schultes & Raffauf, 1990). Other traditional botanicals used for *susto* have been shown to have GABAergic activity *in vitro* and reduction of anxiety-like behavior *in vivo* (Awad et al., 2007; Bourbonnais-Spear et al., 2007). An ethyl acetate (EtOAc) fraction of a *Souroubea* extract reduced anxiety-like behavior in standardized animal models, comparable to Diazapam and bioassay guided fractionation resulted in the isolation, characterization and further derivatization of several phytochemical compounds (Appendix II) (Puniani et al., manuscript in press). Betulinic acid (BA) (**4**) was identified as an active anxiolytic principal (Figure 2). Betulinic acid is also active as an apoptotic anti-cancer and anti-HIV agent (Drag-Zalesinska et al., 2009; Qian et al., 2009). An HPLC-APCI/MS (SIM) method for the quantification of betulinic acid provides an efficient, sensitive and rapid method for the analysis of this bio-active compound and several additional marker triterpenoids (Mullally et al., 2008).

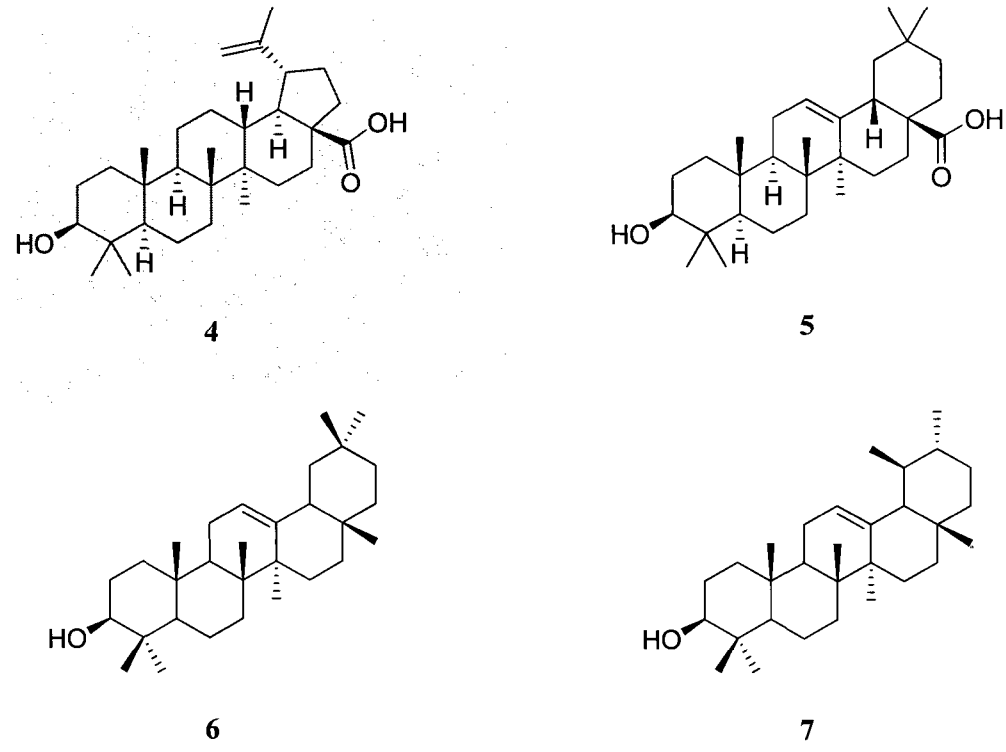


Figure 2: Pentacyclic triterpenoids, 4. betulinic acid (BA), 5. ursolic acid (UA), 6. α-amyrin (α-A) and 7. β-amyrin (β-A) used as marker phytochemicals for *Souroubea*

Further, study of the genus, Souroubea, is important because the pharmacological drugs currently used for treatment of anxiety include benzodiazepenes (e.g. Valium); which are often associated with potentially serious side effects including motor impairment, addiction and liver toxicity (Stevens & Pollack, 2005). Botanical alternatives such as Kava, St. John's Wort, Valerian and Skullcap are already being considered, as an estimated 43% of the population uses herbal products to treat anxiety (Ernst, 2006). The demand for anxiolytic botanicals has increased at a greater rate than all other categories of medicinal plants (Brevoort, 1998). This study provides a scientific basis for the ethnobotanical use of this traditional medicine and provides a promising lead for a clean, high quality, natural health product for anxiety. This is an important point to consider since 20 million people in North America are currently affected with variable forms of anxiety related illnesses (Kessler et al., 1994).

1.2.2.2 Pyrethrum (Chrysanthemum cinerariifolium) Benth & Hook. (Asteraceae)

Chrysanthemum cinerariifolium is a perennial that grows to a height of approximately 60 cm. The insecticidal active ingredients (pyrethrins) in the plant are found in the white and yellow flowers. The use of chrysanthemum flowers as insecticides dates back to as far as 400 B.C. (Krief et al., 2009). The cultivation of pyrethrum (*Chrysanthemum cinerariifolium*) from Dalmatia, now part of Croatia, started in 1847 and later expanded to Japan, which in 1886 began growing the crop. Formulations consisting of powdered flowers were used primarily to delouse armies and control insects in homes. In 1917, kerosene extracts of ground flowers were used in commercial spray formulations and by 1930 crude solvent extracts had essentially replaced powder formulations. In 1924 Staudinger and Rucizka reported on the insecticidal principles of pyrethrum: Pyrethrin I and Pyrethrin II. Following the development of analytical methods for the identification of the actives, in 1932, a fundamental study of pyrethrum was completed which technically established the pyrethrum industry (Tattersfield, 1931). Currently, the world's main pyrethrum producer is Kenya. Chysanthemum is also cultivated in Tanzania, Rwanda and more recently, in Australia.

Pyrethrin I (**8**), jasmolin I (**9**), and cinerin I (**10**) (esters of chrysanthemic acid), and pyrethin II (**11**), jasmolin II (**12**), and cinerin II (**13**) (esters of pyrethric acid) are collectively referred to as Pyrethrins, PI and PII respectively (Figure 3) The relative amounts of the six esters vary depending on the particular plant genotype, geographical source and time of harvest (Table 2) (Glynne-Jones, 2001). Drying and refining with solvents such as hexane provides a 25% to 50% pyrethrin concentrate, which is stabilized with butylated hydroxytoluene (BHT) and formulated with a synergist, most commonly

piperonyl butoxide (PBO). The neuroactive insecticidal properties of pyrethrum (Raymond-Delpech et al., 2005) and potent cytochrome P450 inhibition of PBO are responsible for its effectiveness against a wide spectrum of insect species at low doses. The low mammalian toxicity compared to insect toxicity (Duchon et al., 2009; Osimitz et al., 2009; Krief et al., 2009) and its rapid breakdown (non persistence) in the environment (Antonious et al., 1997; Angioni et al., 2005; Antonious, 2004) make pyrethrum an attractive alternative for several applications where synthetic pesticides remain in use. Pyrethrum formulations are currently registered under several trade names (e.g. Pyganic) as a broad spectrum natural insecticide.

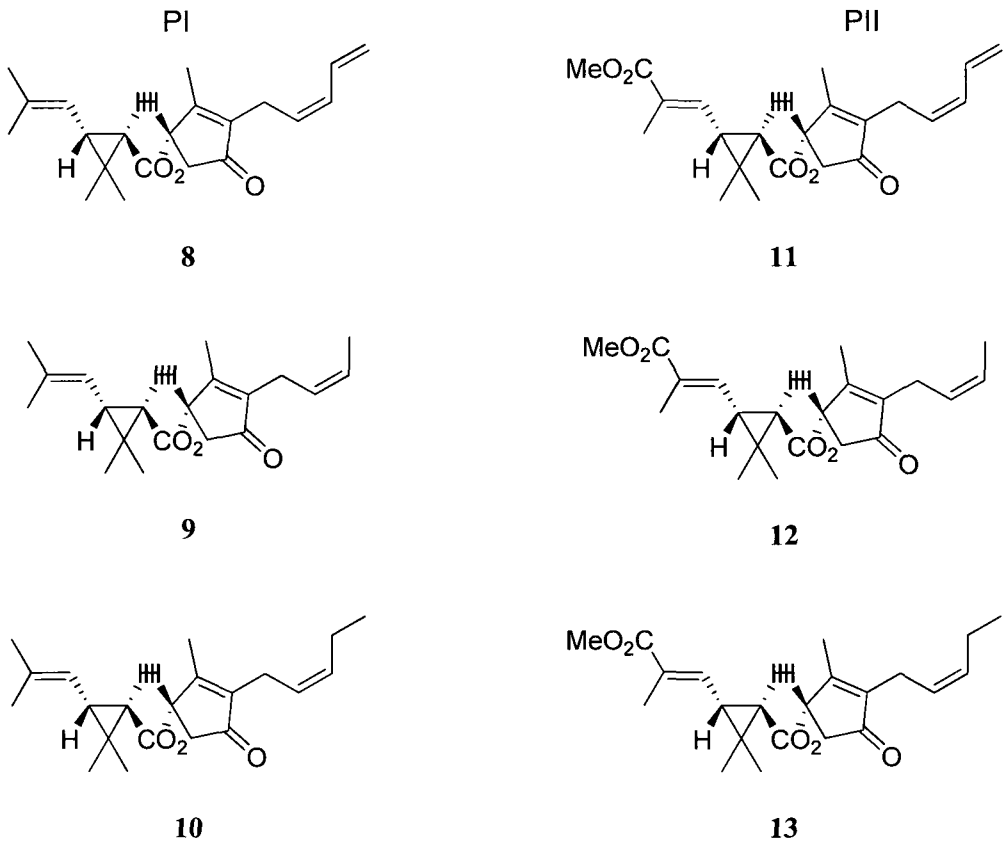


Figure 3: Structural formulas of pyrethrins: 8. Pyrethrin I, 9. jasmolin I, and 10. cinerin I, and 11. pyrethin II, 12. jasmolin II, and 13. cinerin II

Table 2: Relative proportions of the individual pyrethrins in a 50% concentrate (oleoresin) (Glynne-Jones 2001)

		Pyrethrins			
cinerin I	**jasmolin I**	**pyrethrin I**	**cinerin II**	**jasmolin II**	**pyrethrin II**
3.7%	2.0%	19.0%	5.8%	2.0%	17.5%
pyrethrins I 24.7%			pyrethrins II 25.3%		
total pyrethrins 50%					

Continued study of pyrethrum oleoresin is necessary in order to address solvent contamination and to investigate organic replacements for PBO. This research is timely because of the increasingly stringent regulations for chemical solvents, development of the organic agricultural sector and general safety concerns surrounding synthetic pesticides and synergists.

1.2.2.3 Black pepper (Piper Nigrum) L. (Piperaceae)

Piperaceae (genus *Piper*) represents a family of approximately 1000 species with a pan-tropic distribution (Scott et al., 2002). *Piper nigrum,* the most well known species of this family, is a flowering vine with fruit (peppercorn) which varies in colour (i.e. black, red, white) dependent on maturity. The rich history *P. nigrum* use as a spice and in traditional medicine has resulted in extensive investigations of its phytochemistry and biological activity (Scott et al., 2008b).

Piperamides piperine (**14**), piperlonguminine (**15**), pipercide (**16**), dihydropiperine (**17**), dihydropiperlonguminine (**18**) are commonly used as standards to determine extraction efficiency (Figure 4). The main secondary metabolite present in the peppercorn is piperine (~5.7% wt%) (Perakis et al., 2005; Scott et al., 2005a). The concentration of piperamides has been correlated to the degree of insecticidal activity (Scott et al., 2005a). Insect repellent activity has also been observed for *P. nigrum* extracts, however the duration of its effects were diminished rapidly in full sunlight (Scott et al., 2003; Scott et al., 2004). The rapid degradation observed suggests applicability for protection of stored products (e.g. grains) and small scale organic use (i.e. home and garden) rather than large scale agriculture.

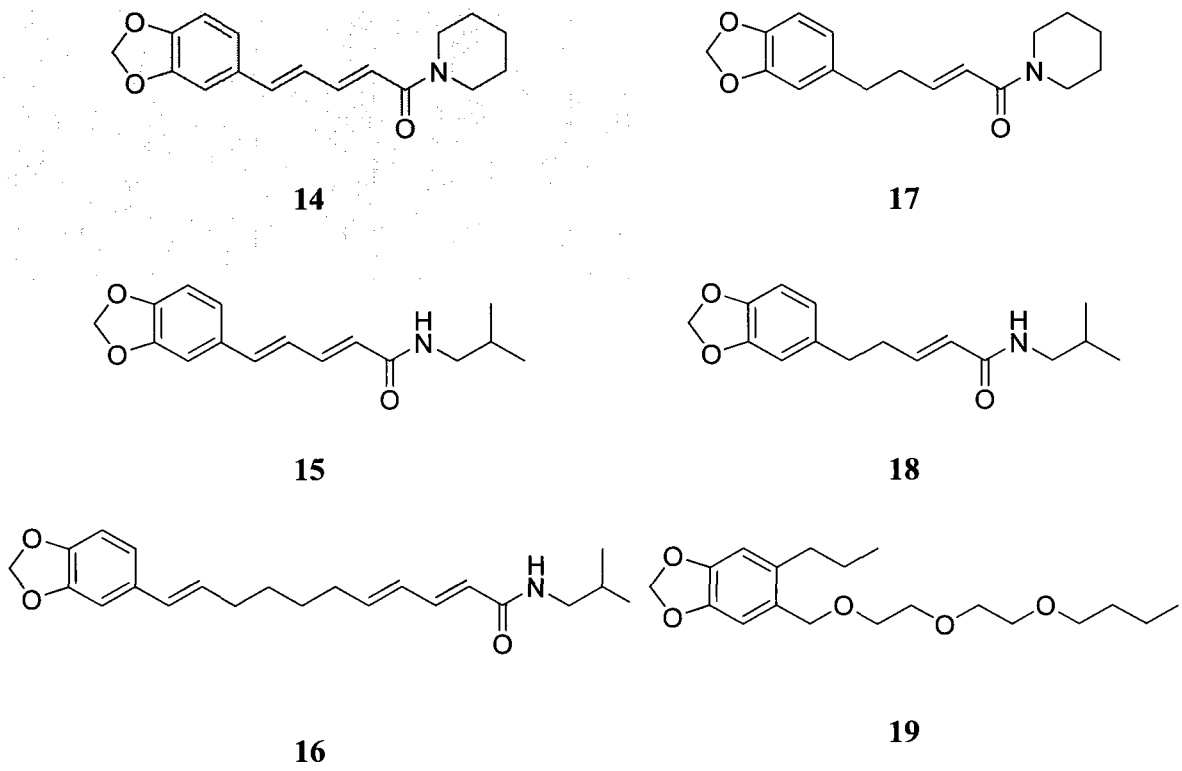


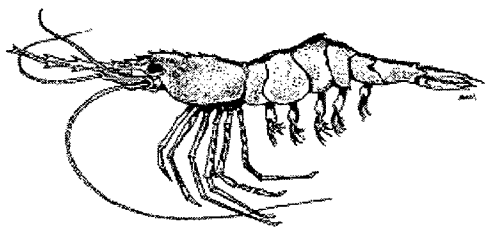
Figure 4: Structural formula of representative piperamides from *P. nigrum* and PBO: 14. Piperine, 15. Piperlonguminine, 16. Pipercide, 17. Dihydropiperine, 18. Dihydropiperlonguminine, and 19. Piperonyl butoxide

Piperamides (isobutylamides) act as contact neurotoxins to insects by a mechanism distinct from that observed for pyrethroids (Miyakado et al., 1989). The presence of the methylenedioxyphenyl (MDP) moiety, also occurring in piperonyl butoxide (PBO), enhances insect toxicity by inhibition of polysubstrate mono-oxygenase (PSMO). Further, Helen Jensen reported an exceptional synergism ratio (12) for the combination of *Piper nigrum* L. (Piperaceae) extracts and the botanical insecticide pyrethrum, which is better than that observed for PBO (Jensen 2005) .

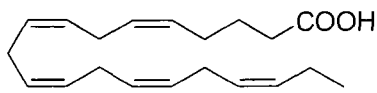
Referred to in 1.2.2.2, the study of promising botanical insecticides and synergists which are phytochemically characterized, effective, and solvent free is relevant and timely.

1.2.2.4 Northern Arctic Shrimp (Pandalus borealis) Kreyer

Northern shrimp (*Pandalus borealis* Kreyer) are cold-water shrimp commonly found at depths greater than 100 meters in shelf waters in the North Atlantic Ocean. They are protandric hermaphrodites with a lifespan of up to 10 years; growing to 13-14 cm in length. Northern shrimp have been commercially fished in Atlantic Canada since 1978 (Government of Canada, 2009). They make up greater than 70% of the cold water shrimp harvested each year (Greene et al., 2009).



Shrimp processing by-products constitute greater than 50% (wt.) of the catch and are a source of polyunsaturated fatty acids (PUFAs). PUFAs eicosapentaenoic acid (EPA) (**20**) and docosahexaenoic acid (DHA) (**21**) are typically described using systematic shorthand which indicates the carbon chain length, position and stereochemistry (*cis/Z* or *trans/E*) of double bonds. EPA; 20:5 (5c,8c,11c,14c,17c) and DHA; 22:6 (4c,7c,10c,13c,16c,19c) are also commonly referred to as omega-3 (ω-3) fatty acids which is based on the position of the double bond from the methyl terminus (Figure 5). EPA and DHA are essential fatty acids which play critical roles in the maintenance of human health. Several studies have associated diets rich in marine n-3 fatty acids with reduced risk factors (e.g. cholesterol, blood pressure) of cardiovascular disease (CVD) (Harris et al., 2008; Simopoulos, 2008; Dewailly et al., 2001).



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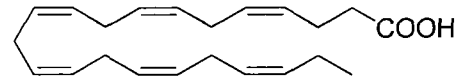

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Figure 5: Structures of marine essential fatty acids: 20. EPA and 21. DHA

Fatty fish (e.g. salmon, herring) are the best sources of ω-3 fatty acids. Extracts of by-products represent a promising source of ω-3 fatty acids which would otherwise be discarded. Extraction of high quality PUFAs, specifically ω-3 marine fatty acids, EPA and DHA using methods which consider their susceptibility to hydrolysis and oxidation, is important to the development of high quality omega-3 enriched foods and natural health products (Rubio-Rodríguez et al., 2009).

1.3 Rationale and Specific Objectives

The intent of this thesis was to develop innovative extraction and efficient characterization methods for novel biologically active fractions from selected natural products. A fundamental outcome expected from the completion of these studies was an increased understanding of targeted SFE of several biosynthetic classes of compounds. This thesis also represents applied research in providing higher quality (cleaner, well-defined) extracts better suited for commercial applications.

Specific goals cross cutting all chapters of the thesis include:

1. generation of targeted extracts of biologically active fractions using supercritical CO_2 extraction methods.
2. characterization of the biochemical profile of fractions using HPLC, HPLC-MS, GC-MS by developing analytical methods for efficient qualitative and quantitative analysis.
3. utilization of bioassay methods for biological activity characterization and to gain insight into the mode of action.
4. advancement of selected extracts to animal studies to validate *in vivo* efficacy.

Specific chapter objectives were:

1. to develop an extraction method to yield a betulinic acid enriched extract of the traditional anti-anxiety plant sin susto (*Souroubea sympetala*). A secondary objective was to determine the bioactivity of the extract in validated rodent behavioral assays for anxiety (Chapter 2).

2. to investigate the effects of SFE parameters, temperature and pressure, on the yield and individual pyrethrin profiles of pyrethrum (*Chysanthemum cinerariifolium*) oleoresin. Secondary objectives included confirmation of the insecticidal activity of the SFE extract, and examination of the potential for SFE extracts of pyrethrum oleoresin in organic agriculture applications (Chapter 3).

3. to optimize piperamide extraction from black pepper (*P. nigrum*) using SFE through modification of pressure, temperature, particle size, sample size, and flow rate. A secondary objective was to investigate the insecticidal activity of SFE extracts *in vivo* (Chapter 4).

4. to generate a high quality, residue free oil, rich in PUFAs, specifically EPA and DHA from Northern shrimp (*Pandelus borealis*) by-products using SFE. A secondary objective was to scale up optimized experimental conditions to pilot SFE for further evaluation as a commercial omega-3 rich concentrate (Chapter 5 and 6).

Chapter 2

Preface

At the time of my introduction to sin susto (*Souroubea sympetala* Marcgraviaceae): Natalie Bourbonnais-Spear and Rosalie Awad from the University of Ottawa had recently returned from an ethnobotanical study in Southern Belize. Several of the plants they collected and which were used to treat *susto* by indigenous people were found active in animal tests for anxiety. This evidence essentially linked *susto* to anxiety (Bourbonnais-Spear et al., 2007). In attempts to better understand the neurological mechanism of action, Rosalie Awad later provided evidence that the activity of the anxiolytic plants is associated with pharmacological targets in the gamma-amino butyric acid (GABA) system (Awad et al., 2009). Eva Puniani (in her PhD thesis in the Durst Chemistry lab) characterized the phytochemical profile of *Souroubea* (Marcgraviaceae) and through bioassay guided fractionation, demonstrated its *in-vivo* anxiolysis (E. Puniani, 2004). Martha Mullally's work was focused on the molecular mechanisms of *Souroubea,* and the phytochemistry of the different plant parts (wood, bark, leaves, fruit, and flowers). As a result of my background in chemistry, I worked with Martha and Ammar Saleem on development of an HPLC-MS method to quantify the active fraction, rich in pentacyclic triterpenoids which included betulinic acid. This resulted in the publication of: "**Characterization and Quantification of Triterpenes in the Neotropical Medicinal Plant *Souroubea sympetala* (Marcgraviaceae) by HPLC-APCI-MS**" and is presented in Appendix II (Mullally et al., 2008). In the course of this collaboration we reviewed the rodent behavioral assay work of Chris Cayer and Eva Puniani on *Souroubea* (Puniani et al., manuscript in press), and more recently by Chris Cayer and Martha Mullally and

planned the next phase of the study, reported here. In the present study, I developed the study design for SFE and completed the extraction in collaboration with Martha Mullally with laboratory assistance from Calum McRae and Andrew Goulah. Chris Cayer, supervised by Zul Merali undertook the *in-vivo* investigations with minor assistance from myself and Martha Mullally. The plant material was located, identified, mass collected, and dried by Mario Garcia, Marco Otarolla, Pablo Sanchez and Luis Poveda from the Universidad Nacional, Costa Rica.

2.0 Anxiolytic activity of a supercritical carbon dioxide extract of *Souroubea sympetala* (Marcgraviaceae)

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[1]These authors contributed equally to this manuscript.

Abstract

An extraction technique was developed to yield a betulinic acid (BA) enriched extract of the anti-anxiety plant *Souroubea sympetala* (Marcraviaceae). Five extraction techniques were compared: supercritical CO_2 extraction (SFE), conventional solvent extraction with ethyl acetate (EtOAc), accelerated solvent extraction (ASE), ultrasonic assisted extraction (UAE) and soxhlet extraction (Sox). The EtOAc and SFE extraction methods resulted in BA-enriched extracts, as determined by HPLC-APCI-MS. The bioactivity of the BA-enriched extracts was compared using the elevated plus maze (EPM). A 75 mg/kg oral dose of SFE extract exhibited anxiolysis as compared with vehicle controls, with a 50% increase in percent time in the open arms. No significant differences were observed between the SFE and EtOAc extracts in the EPM. The SFE extract demonstrated a dose-response in the EPM, with a trend toward decreased anxiety at 25 mg/kg, although significant anxiolysis was only observed at 75 mg/kg dose. This study demonstrates that SFE can be used to generate a betulinic acid- enriched extract that has significant anti-anxiety activity. Further, our study provides a scientific basis for the ethnobotanical use of this traditional medicine and a promising lead for a natural health product to treat anxiety.

2.1 Introduction

Anxiety disorders, including generalized anxiety, panic disorders and phobias, are a detrimental form of mental illness that impacts an estimated 16% of people across the world (Somers, et al., 2006). In addition to being a serious form of illness on their own, anxiety disorders are commonly combined with other mental illnesses, including depression, bipolar disorder and addiction. Treatments include cognitive behaviour therapy and pharmacological interventions, but only 60% of patients are responsive to treatment (Bystritsky, 2006) and the common pharmaceuticals used to treat anxiety, benzodiazepines (BDZs), are associated with serious side effects and not recommended for chronic use (Stevens and Pollack, 2005). Anxiety disorder patients are considerable consumers of alternative treatments, 43% use herbal products to treat their anxiety (Eisenberg et al., 1998). These factors highlight a need for additional anxiolytic plants to be identified and investigated as phytomedicines to treat anxiety. As part of a natural product investigation to identify anxiolytic plants, we identified the genus *Souroubea,* a group of woody vines belonging to the neotropical family Marcgraviaceae with a tradition of use in both Belize and the Amazon to treat *susto* (fear) a folk-illness associated with anxiety (Schultes and Raffauf, 1990). We have established a link between *susto* and anxiety and fear and demonstrated that plants used by Belize healers to treat *susto* reduce anxiety and fear in rodents (Bourbonnais-Spear et al., 2007). The work described in this paper extends this line of investigation by examining the anxiety-reducing properties of *Souroubea sympetala.*

Initial investigations of *Souroubea* identified a triterpene-enriched fraction that reduced anxiety-like behaviour in rodents in a dose-responsive manner and compared favorably with the anti-anxiety drug diazepam (Durst et al., 2002). Bioassay guided fractionation identified betulinic acid (BA) (**4**) as the active principle (Figure 6). BA is a lupane-type triterpene common in the plant kingdom with demonstrated anti-cancer, anti-HIV, anti-malaria activity (Kessler et al., 2007; Cichewicz and kouzi, 2004; De Sa et al., 2009) and low toxicity (Jager et al., 2009).

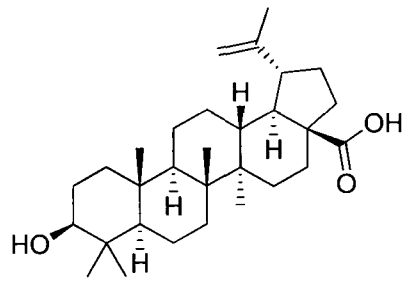

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Figure 6: 4. Betulinic acid

BA can be extracted with ethyl acetate (EtOAc), a petrochemical with both health and safety concerns. We were interested in using the emerging technique of supercritical CO_2 extraction (SFE) to generate a BA-enriched extract of *S. sympetala*. SFE is a method particularly well suited to natural health product extraction because CO_2 is non-toxic, non flammable, available in high purity (food grade), inexpensive and easily evaporated from the extract leaving no residue. Further, the tunable solvent properties of CO_2 permit selective, targeted extraction of specific chemical compound families, so it can be adjusted to selectively extract triterpeniods, like BA from plants (Hamburger et al., 2004; Mukhopadhyay, 2000; Martinez, 2008).

The purpose of this study was to produce a BA-enriched extract of *S. sympetala* for *in vivo* anxiolysis testing. We compared several extraction approaches such as solvent extraction with ethyl acetate (EtOAc), accelerated solvent extraction (ASE), ultrasonic assisted extraction (UAE) and soxhlet extraction (Sox), with SFE. The yields and BA content of each extract were compared to identify the best method to selectively generate a BA-enriched extract. The bioactivity of the extracts with highest BA content were compared in the elevated plus maze (EPM), rodent anxiety behaviour assay.

2.2 Materials and Methods

Chemicals

Analytical grade HPLC solvents were purchased from J.T. Baker (USA). Purified betulinic acid (BA) was obtained from Sigma (St. Louis, MO) for use as a standard. Extraction grade solvents (ethyl acetate, 85% ethanol) were purchased from Fisher Scientific (Ottawa, ON Canada).

Plant material

Fresh samples of wild *Souroubea sympetala* (Marcgraviaceae) were collected under permit in Tortuguero, Costa Rica. Samples were dried overnight in a commercial plant drier at 35°C and ground to 2 mm mesh. Voucher specimens were identified by two of us (L.P. and P.S.) and deposited in the JVR Herbariun, Universidad Nacional Costa Rica, and the University of Ottawa Herbarium (OH No. 19915).

Extractions

A side-by-side comparison of the extraction methods is presented in Table 3. A brief description of each method is presented below.

Conventional solvent extraction

Samples (2 g) were incubated, with shaking, in 40 mL (1:20 weight: volume) ethyl acetate (EtOAc) for 12 – 15 h at room temperature (RT). The solvent was filtered (Whatman #1) and filter cake re-extracted, twice, as above, with half as much EtOAc (1:10 and 1:5). The total solvent from the three extractions were combined for an

exhaustive extraction. For all extracts where solvent was used, the solvent was removed via rotary evaporation with a Yamato Rotary Evaporator RE50 (Yamato Scientific, Japan) at 40°C, lyophilized (Super Modulyo, Thermo Electron, USA) and stored in opaque glass vials at 4°C.

Soxhlet extraction

Samples (0.5 g) were loaded into the soxhlet thimble and extracted with 150 mL (1:300 weight: volume) 85% ethanol in a round bottom flask for 5 h.

Ultrasonic assisted extraction

Samples (0.5 g) were sonicated in a Branson 1200 ultrasonic bath (Branson Ultrasonics, Danbury, CT) for 10 min with 10 mL (1: 20, weight: volume) 85% ethanol and centrifuged at 5500 rpm for 5 min. The supernatant was filtered (Whatman #41), and the filter cake re-extracted, twice, as above. The supernatants from the three extractions were combined.

Accelerated solvent extraction

Samples (1.0 g) were packed into a 60 mL extraction cell and extracted via accelerated solvent extraction (ASE) with an Accelerated Solvent Extraction 200 Extractor (Dionex, Sunnydale, USA). The extraction was conducted with 85% ethanol at a temperature of 110°C, pressure of 120 bar (12 MPa), for two 5 min static cycles.

Supercritical CO_2 extraction

SFE extractions were performed with a SFT-250 extractor equipped with a 100 mL vessel (Supercritical Fluid Tech., Newark, DE). Samples (20 g) were extracted at 60 MPa (600 bar) and 80°C, flow rate of 3 L/min until a 450 g volume of CO_2 was consumed (25:1 solvent: biomass). Extraction efficiency was monitored at 5 min intervals.

Table 3: Summary of the five extraction methods used to generate a betulinic acid-enriched extract of *S. sympetala*.

Extraction Method	Petrochemical Solvent	Mass of Biomass (g)	Solvent: Biomass Ratio	Temperature	Pressure	Extraction Time	Rotary Evaporation & Lyophilization Required?	Equipment
Conventional	ethylacetate	2.0	20:1	20 °C	Atmospheric Pressure	3 days	Yes	Benchtop shaker
Soxhlet	ethanol (85 %)	0.5	300:1	78 °C†	Atmospheric Pressure	5 hours	Yes	Soxhlet thimble
Ultrasonic Assisted	ethanol (85 %)	0.5	20:1	20 °C	Atmospheric Pressure	45 min	Yes	Branson 1200 Ultrasonic Bath
Accelerated Solvent	ethanol (85 %)	1.0	4:1*	110 °C	12 MPa	10 min	Yes	Dionex ASE 200 Accelerated Solvent Extraction System
Supercritical Carbon Dioxide	None	20	25:1	80 °C	60 MPa	75 min	No	SFT-250 Supercritical Fluid Extractor

*An approximate value is reported because the method does not allow the determination of the absolute amount of solvent used in the extraction process. † Boiling temperature of solvent.

HPLC-APCI-MS analysis

HPLC-APCI-MS analyses were conducted as previously described (Blanchard & Blanchard, 1969). Briefly, extracts were dissolved in methanol, to a final concentration of 10 mg/mL, and filtered with a 0.2 μm PTFE filter. One μL of each extract was injected through the autosampler for each run and the elution profiles monitored via MS. A calibration curve was prepared by dissolving BA in methanol at a concentration of 2 mg/mL and diluted to a range of 1 μg/mL - 1000 μg/mL. BA was identified in the extracts by comparing the retention time and mass data with the calibration standard.

Animals

The behavioural experiments were conducted with male Sprague-Dawley rats (225 – 250 g body mass; Charles River Laboratories Inc., St. Constant, Quebec). Rats were individually housed and maintained under standard animal room conditions (clear plexiglass cages, 24 x 30 x 18 cm, 12 h light-dark cycle, 21±1°C, 60% humidity, Purina Lab Chow and tap water *ad libitum*). All experimental procedures were approved by the Research Ethics Committee of the University of Ottawa and met the guidelines set out by the Canadian Council on Animal Care. Rats (n = 66) were handled for 7 days prior to the experiment to acclimatize to the experimenter and were orally administered a 50% solution of Eagle Brand sweetened condensed milk each day to familiarize them with the feeding procedure.

Drug and plant extract administration

Anxiety-like behaviour of animals treated with the BA-enriched SFE and EtOAc extracts were compared to animals treated with diazepam (Valium, positive control) and untreated animals (vehicle control) in the EPM. The plant extract was frozen at -80°C, pulverized with an ice-cold mortar and pestle and mixed with 50% sweetened, condensed milk to a final concentration of 75 mg/kg, and stored at 4°C. Vehicle and extract-treated animals were orally administered their respective treatments daily for three consecutive days (between 10:00 - 14:00 for two days prior to testing, 60 min prior to testing). The animals were randomly assigned to one of four treatment groups: diazepam (5 mg/kg, dissolved in 40% propylene glycol, 10% ethanol, 50% distilled water), vehicle control (2 mL/kg 50% sweetened condensed milk), EtOAc plant extract (75 mg/kg) or SFE plant extract. To determine a trend in dose-response for the SFE extract, animals in this treatment group were dosed with 25 or 75 mg/kg SFE extract.

Anxiety behaviour assay: Elevated plus maze

The EPM consists of two open arms (50 x 10 cm), two perpendicular arms enclosed by 40 cm high walls, placed 50 cm above the ground, and is based on the conflict between the animal's instinct to explore its environment and its fear of exposed areas and heights. The EPM test is commonly used to assess anxiety-like behaviour in laboratory rodents (Awad et al., 2003; Chen & Ling, 2000). A video camera was mounted above the arena to permit remote monitoring and recording. Rats (n = 19 for SFE, n = 12 for EtOAc, n = 24 for vehicle, n = 11 for diazepam) were individually placed in the testing room for 1 h acclimatization. Each rat was then placed onto the open central platform of the EPM

(facing a closed arm). The behaviour was monitored for 5 min and scored as follows: (1) frequency of entries onto the open arms, (2) percentage of time spent on the open arms (time open/300 x 100), (3) frequency of entries in the closed arms, and (4) risk assessment behaviour (unprotected head dips; head protruding over the edge of an open arm and down toward the floor). Between tests, the EPM was cleaned with 70% isopropanol. The percent of time in the open arms, frequency of open arm entries, and unprotected head dips are all validated measures of anxiety-like behaviour in the EPM (Pellow et al., 1985; Griebel et al., 1997; Carobrez and Bertoglio, 2005). Increases in these measures indicate reduced anxiety-like behaviour, conversely, decreases in these parameters indicates increases in anxiety-like behaviour (File, 1992). The frequency of closed arm entries is considered an index of general motor activity of the animal and important in establishing the sedative effect of a material (Cruz et al., 1994).

Statistical analysis

One and multi-way analyses of variance (ANOVA) with Bonferroni studentized range tests were performed for mean comparisons (Zar et al., 1999). Kolmogorov-Schmirnoff and Levene's tests were used to verify the normality of distribution and the homogeneity of residual variance, respectively. All of the Fisher statistics (F), degrees of freedom (df), and p-value estimates were calculated with S-PLUS software version 7.0 (Insightful Corp., Seattle, USA). Data are reported as means ± S.E.M and the level of significance was set at $p < 0.05$.

2.3 Results and Discussion

Of the five extraction methods, SFE generated a smaller total yield but the highest concentration of BA. SFE had a mean percent yield (g extract/g dry plant material) of 5.10 ± 0.12%, significantly lower than that of ASE (15.69 ± 0.40%) and soxhlet (15.25 ± 0.57%) (Figure 7). The SFE extract had significantly higher BA content (μg BA/mg extract) than the ASE, UAE and Sox extracts ($F(5, 47)= 28.82$, $p < 0.001$). Mean BA content of the SFE extracts was 5.54 ± 0.24 μg/mg extract, higher than mean BA content for ASE, UAE and Sox (2.57 ± 0.28, 3.16 ± 0.34 and 2.37 ± 0.28 μg/mg) but not significantly different from mean BA content of the EtOAc extract (6.78 ± 0.18 μg/mg) (Figure 8).

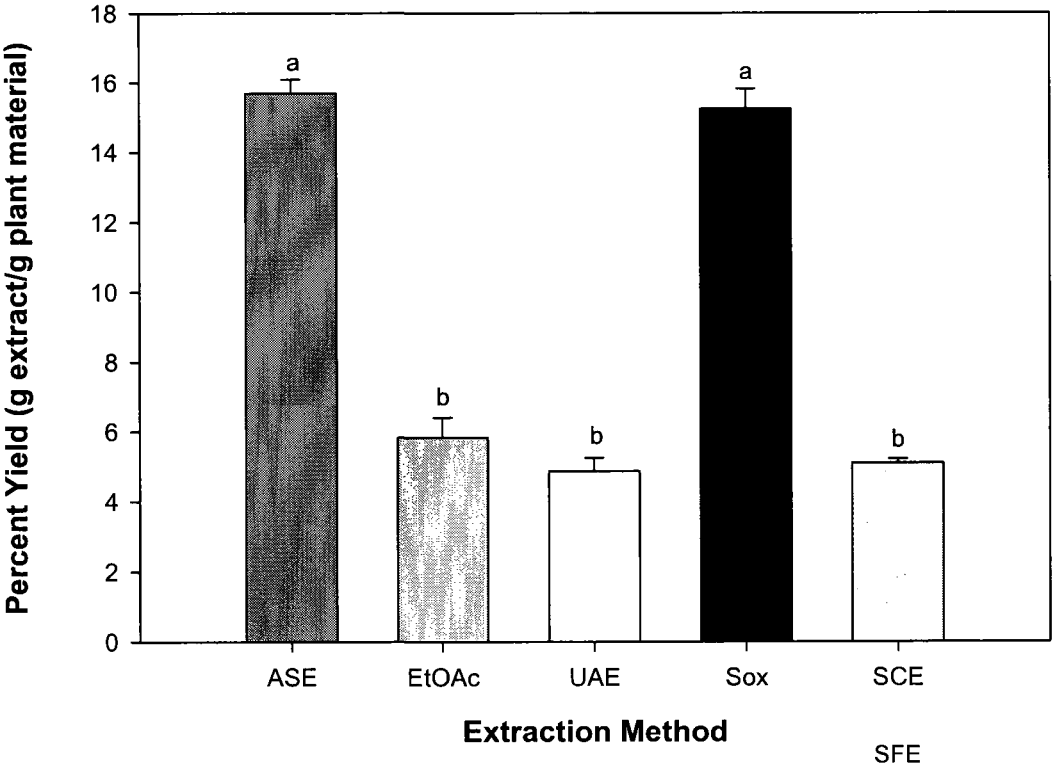

Figure 7: Comparison of percent yield for *S.sympetala* leaves extracted via Accelerated Solvent Extraction (ASE), conventional solvent extraction, with ethylacetate (EtOAc), Ultrasonic Assisted Extraction (UAE), Soxhlet Extraction (Sox) and Supercritical CO_2 extraction (SCE=SFE – in text) (n = 3). All values represent the group mean ± S.E.M. Different letters indicate statistically significant differences, $p < 0.05$.

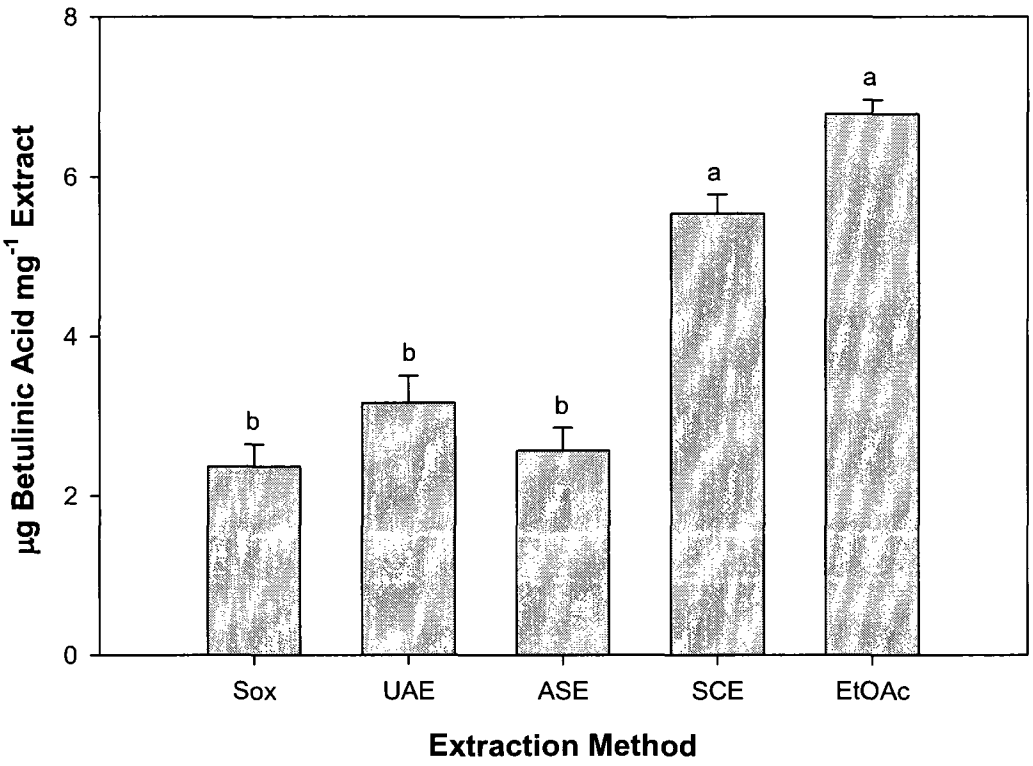

Figure 8: Mean betulinic acid content in *S.sympetala* leaves extracted via Soxhlet Extraction (Sox), Ultrasonic Assisted Extraction (UAE), Accelerated Solvent Extraction (ASE), Supercritical CO_2 extraction (SCE= SFE – in text) and conventional ethylacetate extract (EtOAc) , (n = 3). All values represent the group mean ± S.E.M. Different letters indicate statistically significant differences, $p < 0.05$.

The extraction efficiency curve for the SFE extract was generated by measuring percent yield at 5 min. intervals throughout a 75 min extraction. After 35 minutes, at a flow rate of 3 L/min., the extraction reached 91% completion and after 75 min no additional extract was generated (Figure 9A). The concentration of BA through the course of the SFE extraction was determined at 20 min intervals (Figure 9B). BA concentration increased from 4.37 μg/mg extract in the first fraction to 6.32 μg/mg and 8.12 μg/mg in the second and third fractions respectively. As the percent yield data shows, at 30 min 91% of the extract was collected. However, based on these BA tracking values, at 30 min, the BA concentration was approximately 5.35 μg/mg, only 65.9% of the BA concentration in the 60 min. fraction. This indicates that for maximum BA to be extracted the extraction conditions need to be continued until 450g of CO_2 are consumed.

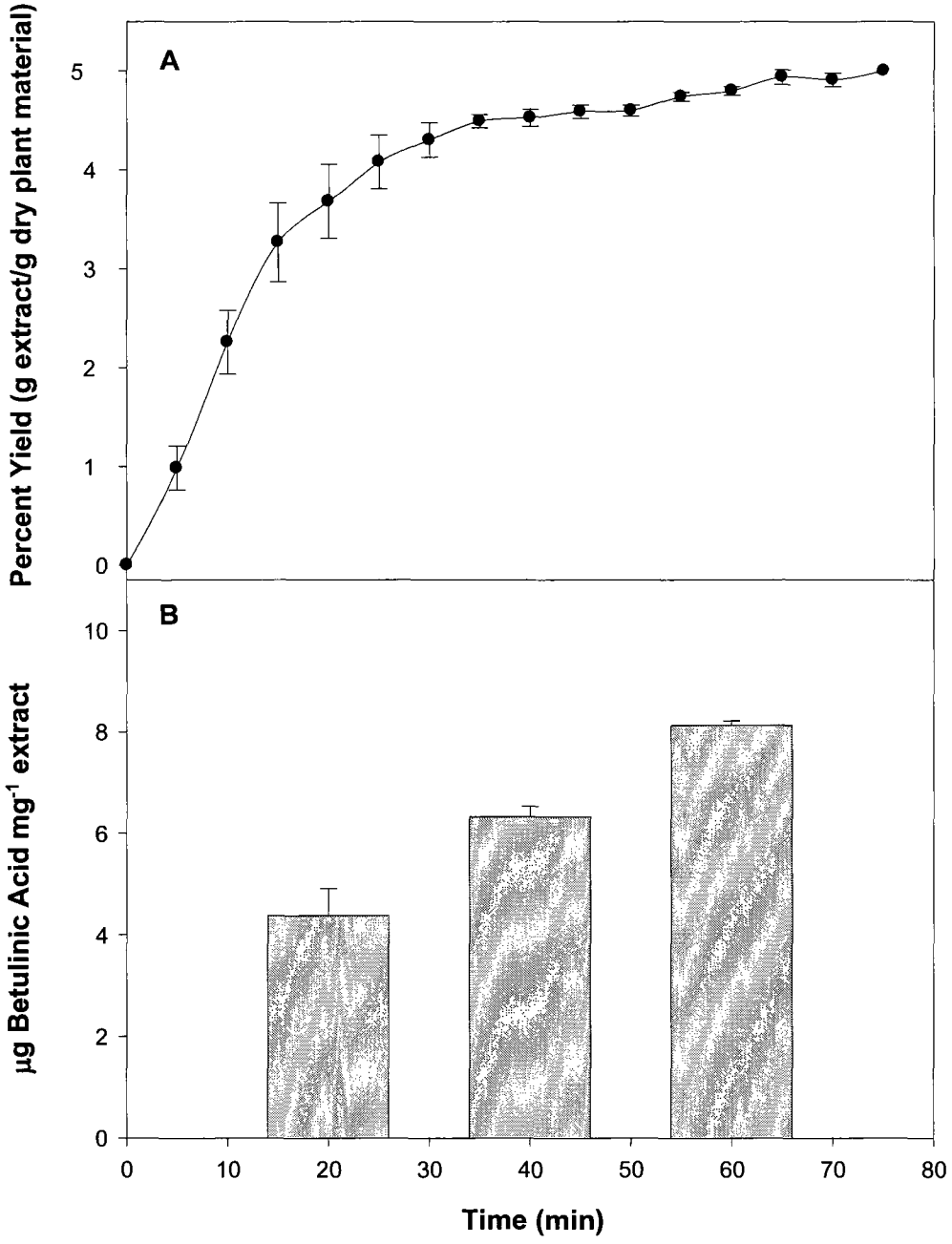

Figure 9: **(A)** Extraction efficiency curve for supercritical CO_2 (SFE) extraction of *S. sympetala.* Each point represents mean percent mass of extract collected at 5 min intervals throughout the course of the extraction ± S.E.M; **(B)** Betulinic acid content in extracts, µg/mg extract, collected at three time points in the SFE extraction (n = 3, mass of starting material = 20.0 ± 0.03, extracted at 80°C, 600 bar (60 MPa), flow rate: 3 L min^{1}, 450 g CO_2 consumed).

Despite a total lower yield, the higher BA content of SFE extracts demonstrates the selectivity of SFE extraction and that SFE can be fine-tuned to extract triterpenes, a finding consistent with previous reports (Cossuta et al., 2008). Further, compared to the EtOAc extract, which also demonstrates selectivity for BA, SFE had reduced extraction time, 75 min versus 3 days for the EtOAc extract (Table 3) and the benefit of a non-toxic, tunable solvent.

The two BA-enriched extracts, SFE and EtOAc, were compared in the EPM for their effect on anxiety-like behavior in rats. The 75 mg/kg dose of SFE extract was effective in several parameters of the EPM as compared to the vehicle control (*i.e.* percent time spent in the open arms: $F(4, 61) = 4.48$, $p < 0.01$; total time in the open arms: $F(4,61) = 4.48$, $p < 0.01$; percent time in the closed arms: $F(4,61) = 2.95$, $p < 0.05$) (Table 4). SFE dosed animals had a 50% increase in percent time in the open arms as compared to vehicle controls (Figure 10A), spent 50% more total time in the open arms, had 73% more unprotected head dips and a 42% decrease in percent time spent in the closed arms. These animals also had a significant increase in unprotected head dips as compared to EtOAc treated animals (75 mg/kg) and vehicle controls: $F(4,61) = 10.45$, $p < 0.01$ (Figure 10B). No differences were observed in any of the parameters of the EPM between the positive control, diazepam (5 mg/kg) and 75 mg/kg dose of the EtOAc extract. These results demonstrate that the SFE extract of *S. sympetala* has a significant anxiolytic effect on rodents in the EPM compared to the vehicle controls. The difference between percent time in the open arms for SFE and EtOAc extracts was not statistically significant, however the SFE extract tended to elicit greater anxiolysis; SFE-dosed animals had a 34% increase in percent time in open arms compared to EtOAc-dosed

animals. A tendency for the SFE extract to elicit greater anxiolysis was also demonstrated for unprotected head dips. Rodents administered the SFE extract exhibited greater risk assessment behaviour, unprotected head dips, than those dosed with the EtOAc extract. This selective effect on risk assessment behaviour suggests that the SFE extract may act via 5-hydroxytryptamine (5-HT) neurotransmission, as this metric of the EPM has been shown to respond specifically to ligands that bind the $5HT_{1A}$ receptor (Griebel et al., 1997). No differences were observed in the number of closed arm entries for animals treated with the plant extracts versus the vehicle controls, indicating that the extracts did not have a sedative effect.

Table 4: Comparison between the two *S. sympetala* extraction types, vehicle control (50% sweetened, condensed milk) and positive control (Diazepam) in selected parameters of the elevated plus maze paradigm, after a 1 h post-drug interval (n = 7 – 24)

Treatment	T.O.A.[a]	#O.A.E.[b]	U.P.H.D.[c]	% T.O.A.[d]	#C.A.E.[e]	% T.C.A.[f]
Vehicle control n = 24	55.5 ± 7.83	4.49 ± 0.65	3.83 ± 0.61	18.50 ± 2.61	10.58 ± 0.57	52.23 ± 3.60
Diazepam, positive control 5 mg/kg, n = 11	102.27 ± 15.56**	4.73 ± 0.79	13.63 ± 3.14**, #	34.10 ± 5.19**	9.91 ± 1.88	39.35 ± 5.53
Ethyl acetate extract 75 mg/kg, n =12	72.53 ± 13.85	5.50 ± 0.63	6.83 ± 1.27	24.17 ± 4.61	10.75 ± 0.52	41.96 ± 3.18
Supercritical CO_2 extract 25 mg/kg, n = 7	75.30 ± 19.66	6.29 ± 1.38	6.43 ± 1.94	25.10 ± 6.56	12.0 ± 0.87	43.27 ± 3.97
Supercritical CO_2 extract 75 mg/kg, n =12	110.26 ± 6.27*, **	8.08 ± 0.36	14.33 ± 1.25**, #, ##	36.75 ± 2.09**	9.25 ± 0.72	36.83 ± 1.93*

** *p*-value < 0.01 vs. vehicle control, one-way ANOVA
* *p*-value < 0.05 vs. control, one-way ANOVA
# *p*-value < 0.01 vs. ethyl acetate extract, one-way ANOVA
p-value < 0.01 vs. SFE extract 25 mg/kg, one-way ANOVA
[a] T.O.A.: time spent in the open arms (min)
[b] #O.A.E.: number of open arm entries
[c] U.P.H.D.: number of unprotected head dips
[d] % T.O.A.: percentage of time spent in the open arms
#C.A.E.[e]: number of closed arm entries
% T.C.A.[f] : percent time in closed arms

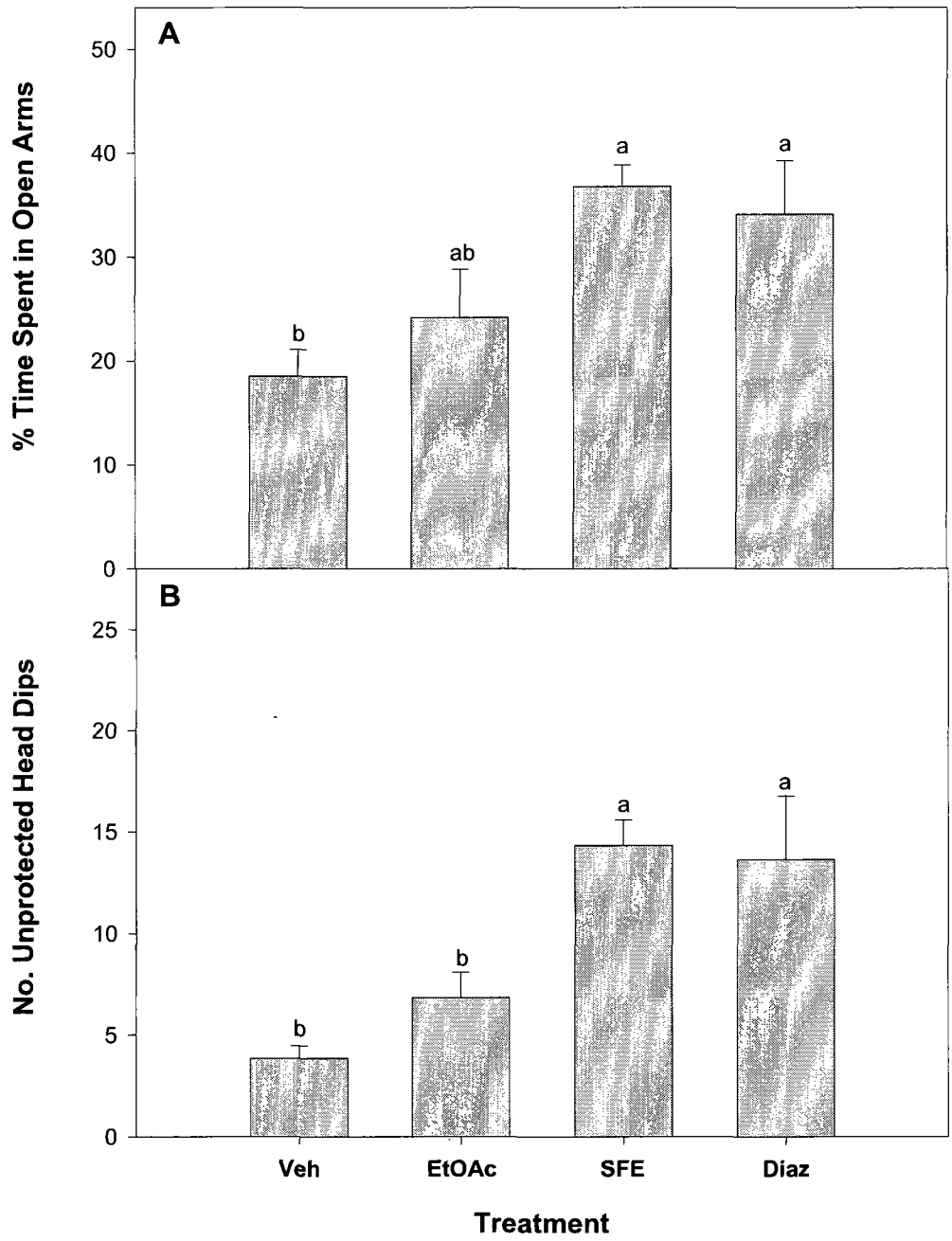

Figure 10: (A) Percent time spent in open arms of the EPM for different treatments; and (B) Number of unprotected head dips in the EPM for different treatments (doses: *S. sympetala* EtOAc and SFE extracts: 75 mg/kg, diazepam: 5 mg/kg) after a 1 h post-drug interval (n = 24 for vehicle, n = 12 for EtOAc, n = 12 for SFE, n = 11 for diazepam). All values represent the group mean ± S.E.M.; veh: vehicle control (50% sweetened, condensed milk), letters indicate significant differences, $p < 0.05$.

Increased doses of SFE extract (0, 25 and 75 mg/kg) changed parameters in the EPM in a dose response manner; higher SFE extract concentrations resulted in less anxiety-like behaviour, as indicated by increased percent time spent in the open arms. The higher SFE dose (75 mg/kg) significantly increased percent time spent in the open arms as compared to vehicle controls, ($F(4,61) = 4.48, p < 0.01$) but not as compared to the 25 mg/kg dose (Figure (11A). The higher SFE dose significantly increased the number of unprotected head dips as compared to the vehicle control and the 25 mg/kg dose ($F(4, 61) = 10.45, p < 0.01$) (Figure 11B). Previous reports of anxiolytic plant extracts in the EPM have required moderate to high doses to elicit significant anxiolysis, from 100 – 500 mg/kg for well characterized herbs, including passion flower (*Passiflora incarnate*), 375 mg/kg (Grundmann et al., 2008), blue skullcap (*Scutellaria lateriflora*), 100 mg/kg (Awad et al, 2003), kava kava (*Piper methysticum*), 120 – 240 mg/kg (Rex et al., 2002) and valerian root (*Valeriana officinalis*), 100 – 500 mg/kg (Hattesohl et al., 2008). In light of these, a 75 mg/kg oral dose of the SFE extract of *S. sympetala* is moderate and suggestive of high and selective anti-anxiety activity.

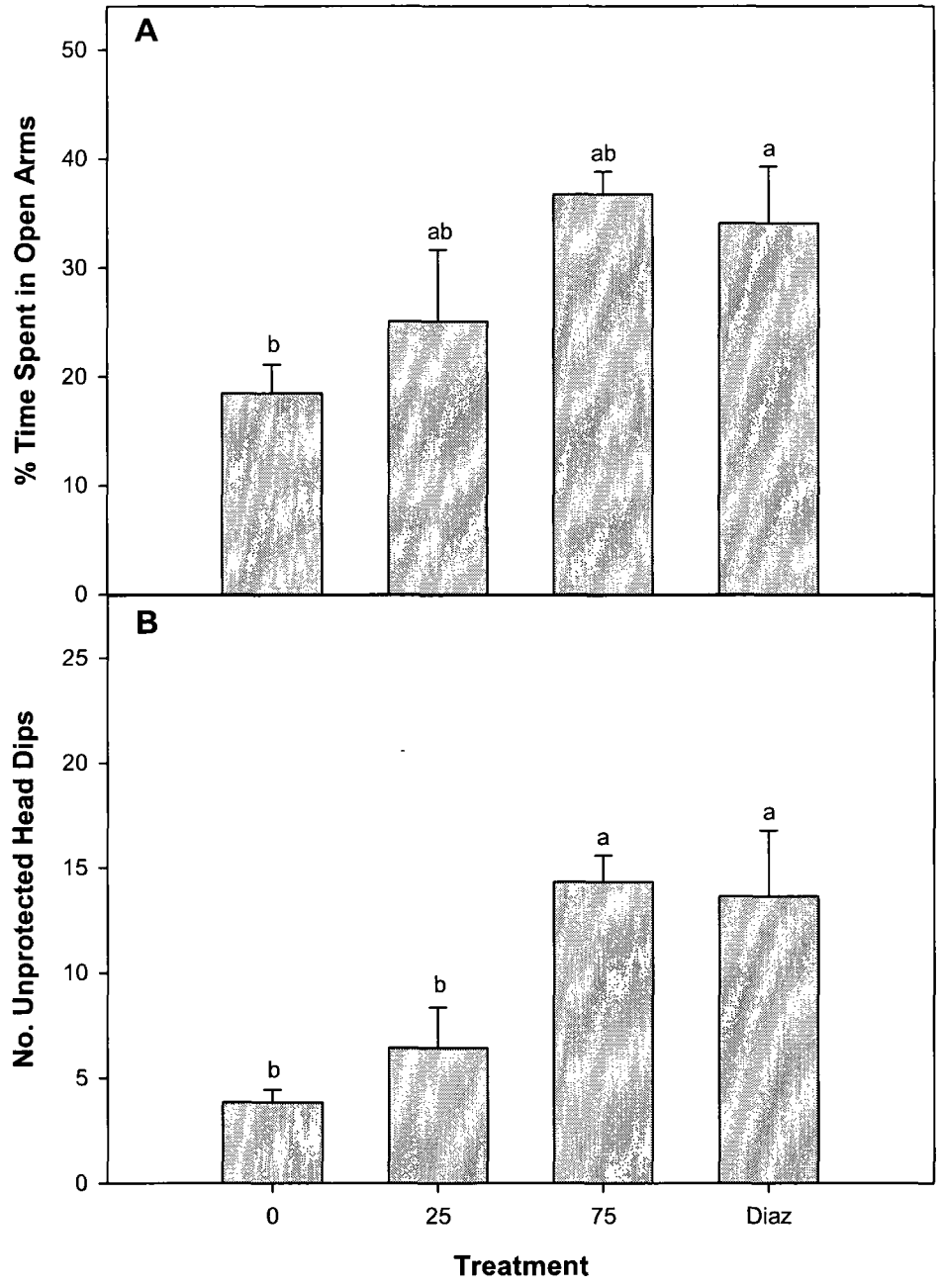

Figure 11: Dose trend for SFE extract of *S. sympetala* in the EPM. (A) Percent time spent in open arms of the EPM, $r^2 = 0.32$, $p < 0.001$; and (B) Number of unprotected head dipsfor the three doses of the SFE extract (0, 25 and 75 mg/kg) and positive control (diazepam, 5 mg/kg) after a 1 h post-drug interval (n = 7 - 24), $r^2 = 0.60$, $p < 0.001$. All values represent the group mean ± S.E.M.; control: vehicle control (50% sweetened, condensed milk), letters indicate significant differences, $p < 0.05$.

An important observation is that the SFE extract was more palatable than the EtOAc one. The SFE extracts had a very mild odour and the animals readily consumed it. In contrast, the EtOAc extracts had a strong odour, even after vigorous vacuum removal of solvent, and although animals consumed the extract, the syringe had to be placed directly in their mouths to be eaten. The increased palatability of the SFE may be due to the residue free, tasteless, and odourless properties of CO_2. Increased palatability is a benefit associated with SFE extracts used in animal trials and would also be considered beneficial in the preparation of *S. sympetala* extracts for veterinary application or for human consumption.

This report demonstrates that SFE can selectively extract triterpenoids and can be used to generate an extract enriched with the bioactive triterpenoid BA. The behavioural data indicate that *S. sympetala* is a significant anxiolytic, and demonstrate that SFE extracts of this putative natural health product are as effective, or more effective, in reducing rodent anxiety-like behaviour than a conventional solvent extract. Finally, the behavioural results corroborate the ethnobotanical identification of *S. sympetala* in the treatment of anxiety and the link between the folk illness *susto* and anxiety.

Chapter 3

Preface

The work presented in this chapter is a result of collaboration with Steve Sims and David H. Naffziger (BASFCorporation, formally Whitmire Microgen). I developed the study design and completed the SFE and HPLC work with the assistance of Jingqin Mao, Calum McCrae and Andrew Goulah. Ammar Saleem assisted with development of analytical HPLC procedures and prep-HPLC. Ian Scott Completed the insect bioassays with my assistance.

3.0 Supercritical CO_2 Extraction of Pyrethrum Oleoresin: Effects of pressure and temperature on extraction efficiency, pyrethrin profile and insecticidal activity

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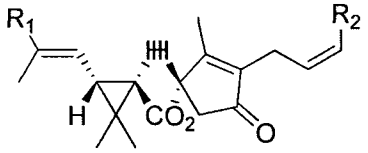
Abstract

Supercritical fluid CO_2 extraction (SFE) was used to study the yield of 6 pyrethrin insecticide components from pyrethrum oleoresin through systematic variation of pressure and temperature parameters. At 40 °C; 10 MPa the highest pyrethrin concentration (0.531 g/g oleoresin) and highest ratio of pyrethrins I (pyrethrin I, cinerin I, jasmolin I) to pyrethrins II (pyrethrin II, cinerin II, jasmolin I) (PI:PII = 1.95) was obtained while 40°C; 30 MPa produced the lowest pyrethrin concentration and lowest ratio of PI:PII (0.436 g/g oleoresin and 1.87). Total pyrethrin recovery was highest at 40°C; 30 MPa (8.39 g pyrethrins/20 g oleoresin) vs. 40°C; 10 MPa (6.96 g pyrethrins/20 g oleoresin). There was no detectable hexane in the extracts which addresses solvent contamination concerns of crude oleoresin use in organic agriculture applications. The insecticidal activity of the SFE CO_2 extract of the pyrethrum oleoresin was confirmed using a major insect pest of horticulture, the Colorado potato beetle (CPB).

3.1 Introduction

Pyrethrum, a natural product derived from flowers of *Chrysanthemum cinerariaefolium* (syn *Tanacetum cinerariifolium)* (Compositae/Asteraceae) is one of the oldest and most thoroughly studied natural insecticides (Krief et al., 2009). Pyrethrum usage in agriculture is increasing due to the concerns and regulations surrounding synthetic pesticide use. It is the most effective of the traditional botanical insecticides and is suitable for household use and organic agriculture. It is effective against a wide variety of insects and is especially valued for its knockdown effects on flying insects such as houseflies and mosquitoes (Scott 2004).

The active insecticidal principles contained in pyrethrum flowers are referred to collectively as pyrethrins (pyrethrin I (**8**) and II (**11**), cinerin I (**10**) and II (**13**), jasmolin I (**9**) and II (**12**)) (Figure 11). These are irregular monoterpenes (C10), containing an unusual cyclopentane moiety. The flowers contain 0.7-2% pyrethrins. The predominant insecticidal component of the six esters is pyrethrin I (35% of total pyrethrins). Pyrethrin II (32% of total pyrethrins) provides the rapid knockdown (paralyzing) effect. The relative amounts of each compound vary and are dependent upon the growing region (environmental factors) while the ratio of PI to PII is affected by the time of harvest (Glynne-Jones, 2001). Pyrethrins target the nervous system (specifically Na+ ion channels) of insects (Raymond-Delpech et al., 2005). While very toxic to insects, pyrethrum has a low mammalian toxicity and does not bio-accumulate, as the pyrethrins are rapidly degraded *in vivo* and in the environment by the presence of air, light and moisture (Angioni et al., 2005; Krief et al., 2009).



Pyrethrins I and II	R_1	R_2	Molecular Formula	Molecular Weight
Pyrethrin I	CH_3	$CH{=}CH_2$	$C_{21}H_{28}O_3$	328.4
Cinerin I	CH_3	CH_3	$C_{20}H_{28}O_3$	316.4
Jasmolin I	CH_3	CH_2CH_3	$C_{21}H_{30}O_3$	330.4
Pyrethrin II	CO_2CH_3	$CH{=}CH_2$	$C_{22}H_{28}O_5$	372.4
Cinerin II	CO_2CH_3	CH_3	$C_{21}H_{28}O_5$	360.4
Jasmolin II	CO_2CH_3	CH_2CH_3	$C_{22}H_{30}O_5$	374.4

Figure 12: Pyrethrins I and II

The use of conventional solvent methods for the extraction of pyrethrum for formulation reduces its acceptance for pest control in organic agriculture. Furthermore, the use of solvents such as hexane pose health, environmental and safety concerns. Supercritical fluid extraction (SFE) of pyrethrum flowers using CO_2 is an alternative to the use of toxic solvents. Non-polar supercritical SC CO_2 is highly suitable for the extraction of pyrethrin esters. In addition to the non-toxic, non-explosive properties of CO_2, it has generally regarded as safe (GRAS) status and is used under mild conditions. The critical temperature (C_T) = 31°C, and critical pressure (C_P) =7.38 MPa, used in SFE ensure a non-degraded, high quality and residue-free extract. SFE of pyrethrum flowers was first reported by Wynn et al (1995). They described a preparative SFE of pyrethrin I and pyrethrin II from pyrethrum flowers at 40°C and 80 bar (8 MPa). This study showed

that the extraction efficiencies of pyrethrins I and II using SFE were superior to conventional solvent (*n*-hexane) extraction (Pan et al., 1995). Otterbach (1999) compared ultrasonic, soxhlet and supercritical fluid extraction kinetics of pyrethrins from flowers. The total pyrethrins recovered from each method respectively were not significantly different but SFE was completed in less than half the time demonstrating the efficiency of the technology for this application (Otterbach & Wenclawiak, 1999). In a subsequent study, Otterbach further optimized the parameters for the SFE of pyrethrum flowers establishing consistent yields and increased efficiencies (Otterbach and Wenclawiak 1999).

These studies confirmed the suitability of SFE for extracting pyrethrins from flowers. However; pyrethrum is usually shipped to North America in the form of an oleoresin (containing 25-50% pyrethrins). The oleoresin concentrate is used to produce formulations with various commercial applications (i.e. agricultural crop protection, urban insect pest control), but solvent (hexane) residues persist in the crude oleoresin (Kiriamiti et al., 2003). SFE of the crude commercial pyrethrin oleoresin has only undergone preliminary studies (Kiriamiti et al., 2003), and a more thorough investigation is necessary. We initiated an advanced processing study using SFE to meet industry and consumer demand for higher quality, clean extracts, with no solvent residues. This included optimizing the SFE process for pyrethrin extraction from the oleoresin, and comparing the insecticidal activity of the pyrethrin extracts produced using the Colorado potato beetle, a major insect pest of agriculture.

3.2 Materials and Methods

SFE

Supercritical extractions using CO_2 were performed using a system (SFT-250) purchased from Supercritical Fluid Technologies (Newark, DE) equipped with a 100 mL extraction vessel and pre-heater. Crude pyrethrin oleoresin was supplied by BASF (formally Whitmire Micro-Gen). Carbon dioxide with a dip tube (CO_2 UN1013) was purchased from BOC Gases CO_2 (Belleville, ON).

Oleoresin (20 g) was poured into the extraction vessel which was filled with of 1-2 mm diameter glass beads. Filter mesh screens (5 μm) SFT (Newark, DE) were located at both ends of the vessel. Carbon dioxide was introduced and the system and the vessel was heated and pressurized to the predetermined conditions. Temperatures ranged from 40-60°C and pressures tested were 8, 10, 12, 14, 16, 18, 20, 25, 30 and 35 MPa. Dynamic extractions were performed with flow rates of 3-5 L/min. The mass of the extract, which was collected in a 4 oz; 125 mL I-CHEM Septa-Jar, and the mass of CO_2 used was determined every 5-10 minutes to exhaustion in order to calculate the extraction efficiency and solvent to biomass ratio. The test duration was ~160 min. All samples were stored under refrigeration before being brought to room temperature prior to quantitative analysis.

Analysis

Analytical grade solvents were purchased from J.T. Baker (USA). The analysis of pyrethrum extracts were performed on an Agilent 1100 high pressure liquid chromatography-diode array detector (HPLC-DAD) equipped with an autosampler

(model 410). Reverse phase separations of pyrethrins were performed on a Luna 5μ C8(2) 100 A 250 x 4.6 mm ID column (Phenomenex).

The mobile phase consisted of acetonitrile (A) and water (B). The separation of the six pyrethrins was achieved using a linear gradient of 58-75% A in 35 min at a flow rate of 1 mL/min. The column temperature was maintained at 35 °C. The UV of the samples was monitored at 250 nm. One millilitre of each sample (10 mg/mL) was filtered through a PTFE membrane before injection into the system and a 2 μL aliquot was injected through the auto-sampler for analysis. The quantification of the individual pyrethrins was based on isolated standards generated from an oleoresin SFE extract using preparative scale RP.

Bioassay

Insecticidal bioassays were performed using a Potter's tower (Burkard Manufacturing Co. Limited, Rickmansworth, Hertfordshire, UK). Second instar larvae from an insecticide-susceptible strain of the Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (CPB) were exposed to pyrethrum SFE extracts at dosages = 0.5, 0.75, 1.0, 2.5, 5.0 (x 10^{-3}) %. Five CPB larvae were treated per replicate with duplicate replicates per concentration in each trial. Trials were repeated four times for a total of 40 larvae treated per concentration. For application of the extract to the CPB, 5 mL of extract was applied using a Potter's tower and treated CPB were then transferred to an untreated potato leaf disc. Mortality of the CPB larvae was observed at 24 and 48 h. The LC_{50} (+/- 95% Fiducial Limits) value was calculated from the mortality data using probit analyses (SAS ver 9.1). Each of the three remaining pyrethrum extracts

was then tested using the LC_{50} concentration calculated for the first pyrethrum extract in order to assess whether there was a significant difference in the 48 h mortality.

3.3 Results

Extraction efficiency (Figure 13), measured as percent yield (extract mass/starting sample mass x 100) was determined at 5 min intervals to 160 min for pressures from 8-30 MPa at 40 °C. For all pressures tested, extraction increased initially in a linear fashion and then leveled off to reach a plateau without further increase with time. This plateau was reached in as little as 60 min at 30 MPa but increased to 120 min as pressure was decreased to 8 MPa. When maximum extraction yields are compared in the plateau region at 120 min., yields increased from 56.25 % to 96.20 % as pressure increased from 8 MPa to 30 MPa. Extraction efficiency tests were repeated in triplicate. At 40°C; 10 MPa, the final percent yield was 39.27±2.30%. The average standard deviation (S.D.) of the percent yield determined at 5 min intervals (0-90 min) was 2.42% (Figure 13 - inset).

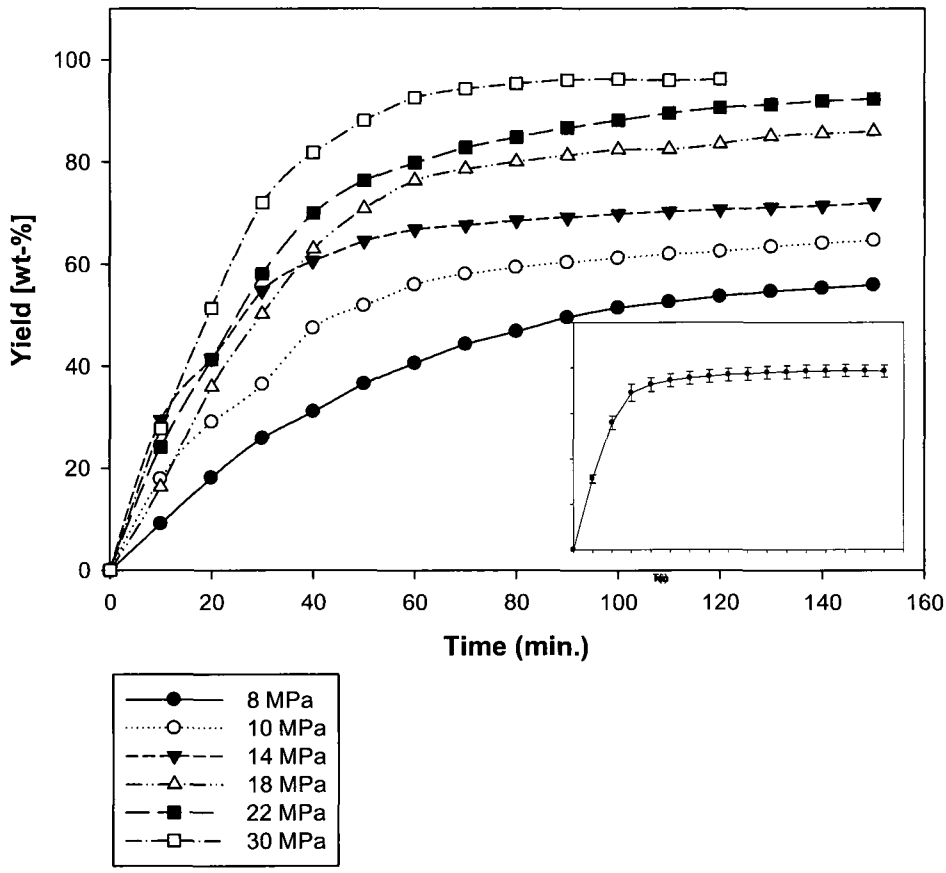


Figure 13: SFE efficiency of pyrethrum oleoresin at 40°C. Pressures 12, 16, 20, and 25 MPa were consistent with the results shown but were omitted for clarity. The inset is an extraction efficiency graph illustrating the mean+/- s.e. of triplicate experiments conducted at 40°C; 10 MPa. The coefficient of variation on the results was low; less than 0.07 based on 3 replicates.

When the total yields of PI and PII are considered (Figure 14), the highest pyrethrin concentration (0.531 g/g) and highest ratio of pyrethrins I (pyrethrin I, cinerin I, jasmolin I) to pyrethrins II (pyrethrin II, cinerin II, jasmolin I) (PI:PII = 1.95) was observed at 40°C, 10 MPa. By contrast 30 MPa produced the lowest pyrethrins concentration and lowest ratio of PI:PII (0.436 g/g and 1.87) (Figure 14). Total pyrethrin recovery was, however, highest at 30 MPa (8.39 g pyrethrins) vs 10 MPa (6.96 g pyrethrins). This may be due to the greater density (solvent power) of CO_2 at the higher pressure (30 MPa) but decreased selectivity.

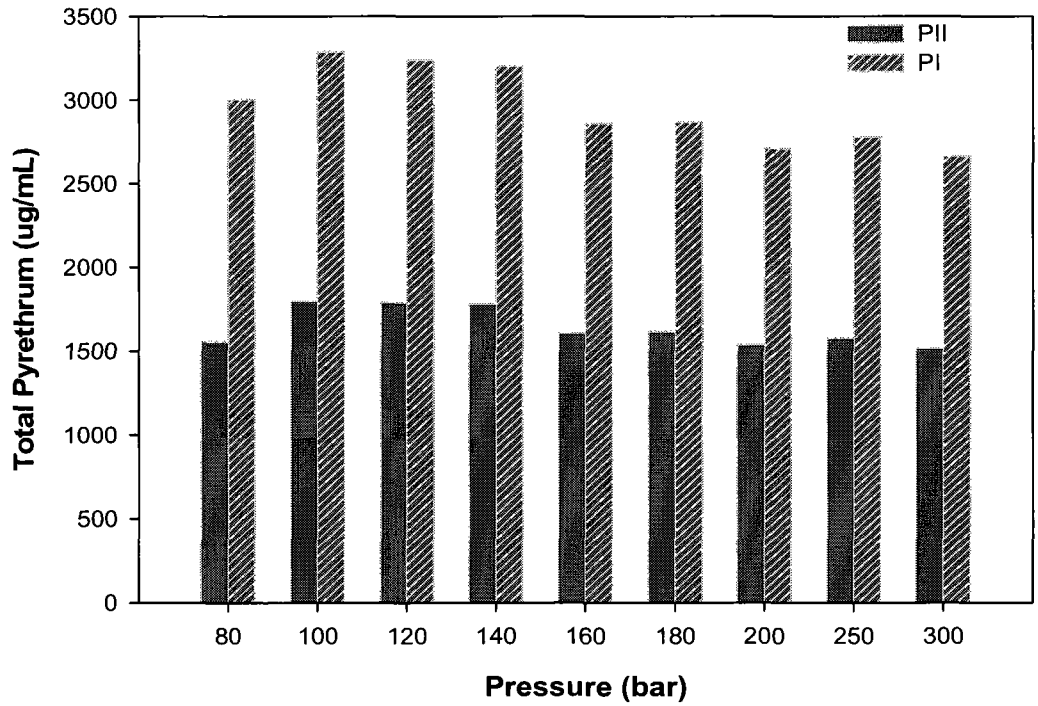


Figure 14: Effect of pressure at 40°C on total yields of PI and PII (10 bar = 1 MPa)

At 40°C and 8 MPa, where extraction is 90% complete the 6 individual pyrethrins and total concentrations were determined at 30 min intervals (Figure 15). The extract profile of pyrethrins was similar throughout the 90 min extraction. When individual pyrethrins were assessed from extraction with pressure increasing from 6 MPa to 20 MPa (Figure 16) the highest ratio of PI:PII was observed at 6 MPa (3.0). The extract containing the highest concentration of pyrethrins was extracted at 8 MPa (0.538 g/g). Subsequent fractions, extracted under increased pressures, generally had lower pyrethrin concentrations and decreased ratios of PI:PII. The concentration and relative ratios reported for 6 MPa and 20 MPa were 0.464 g/g; 3.0 and 0.251 g/g; 1.33 respectively on a second batch of pyrethrum oleoresin replicates. SFE CO_2 extractions performed at 40°C and 10 MPa were compared to soxhlet (hexane) extractions to compare relative pyrethrin profiles. Comparison of the percent of individual pyrethrins extracted by soxhlet and SFE respectively is reported in Table 5.

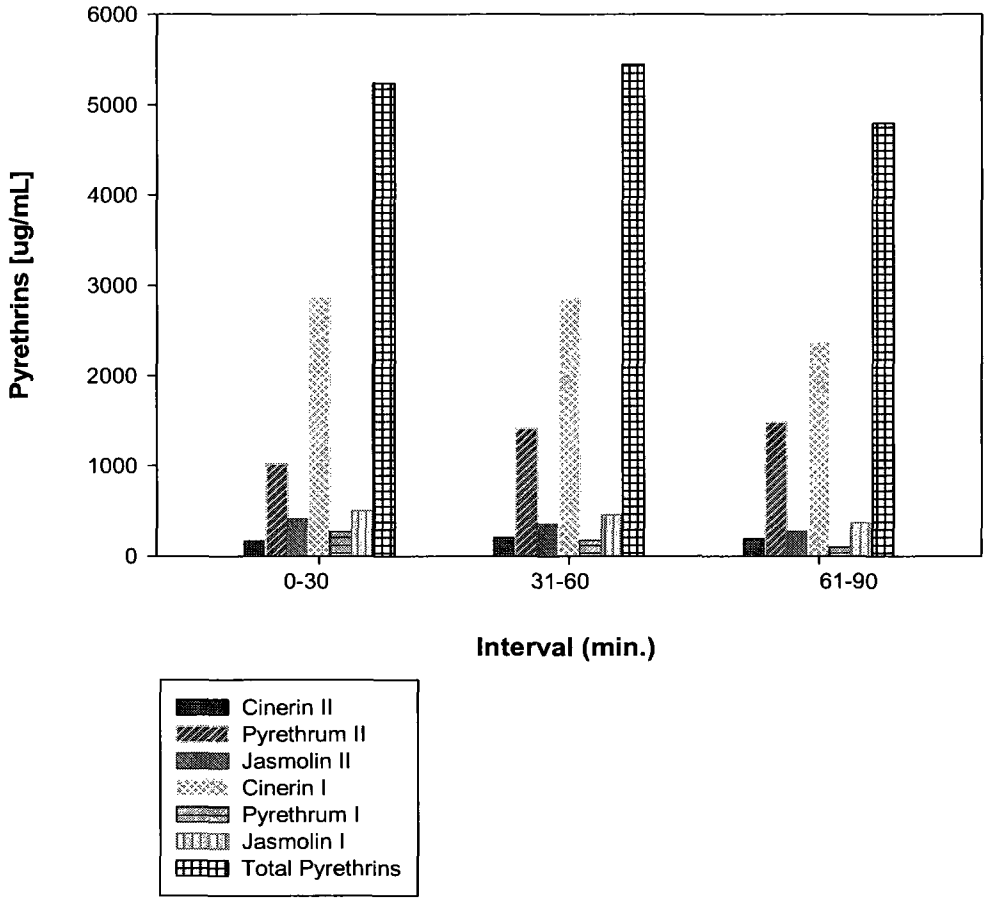


Figure 15: Pyrethrin profile in 30 min intervals at 40 C and 8 MPa

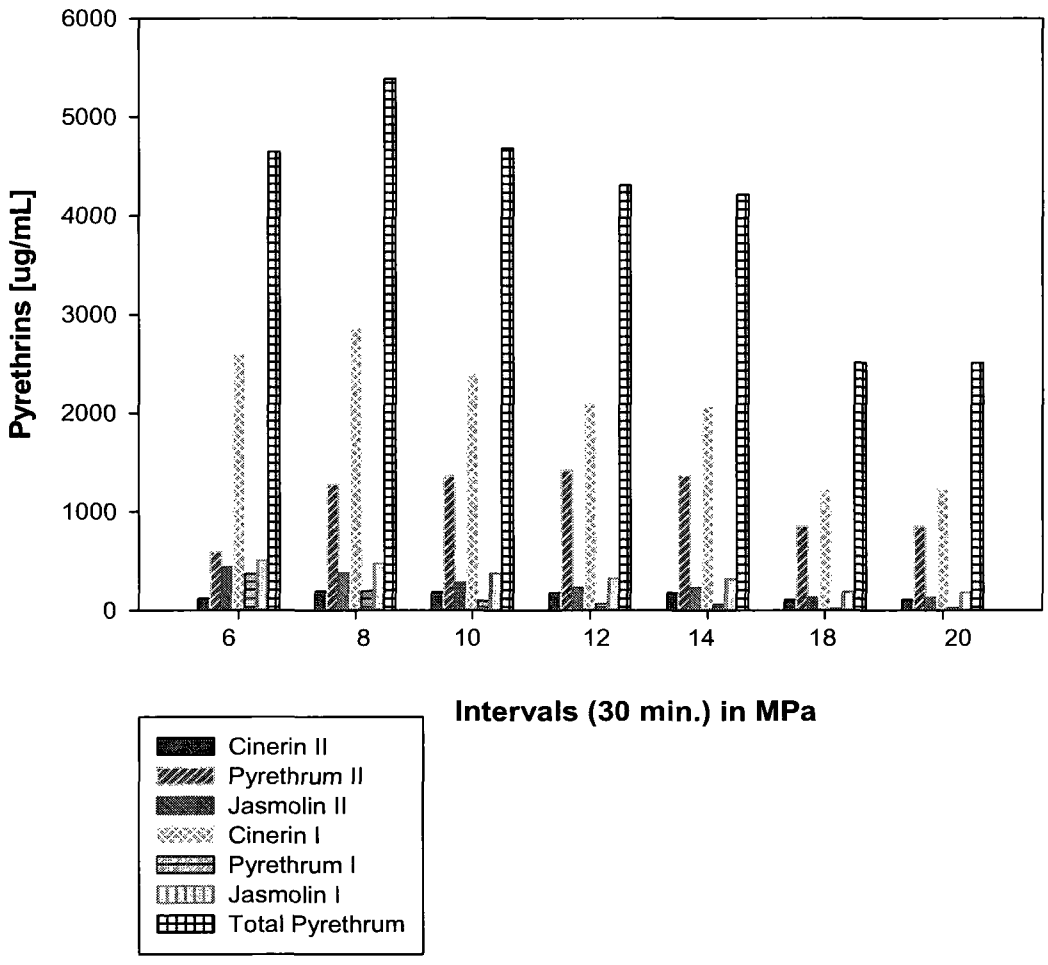


Figure 16: Effects of increasing pressure at 40°C on concentration of individual pyrethrins

Table 5: Pyrethrum profile (% pyrethrins) of SFE (40°C; 10 MPa) vs Soxhlet (hexane) extracts

	Soxhlet	**SFE**
cinerin II	6.94+/-0.07	6.65+/-0.86
pyrethrum II	19.41+/-0.13	18.18+/-0.42
jasmolin II	2.34+/-0.08	2.04+/-0.02
cinerin I	3.90+/0.03	4.49+/-0.15
pyrethrum I	16.23+/-0.15	17.33+/-0.35
Jasmolin I	1.19+/-0.01	1.31+/-0.02
pyrethrins II	28.68	26.87
pyrethrins I	21.32	23.13
total pyrethrins	50.00	50.00

Three extracts (40°C: 10 PMa, 14 MPa, and 30 MPa.) were selected for comparison of insecticidal activity. The 24 and 48 h LC_{50} values (+95% Fiducial Limits) for SFE batch 40°C: 10 MPa were 0.0023% (0.0017-0.0037%) and 0.0021% (0.0015-0.0032%) respectively (N = 240), confirming high insecticidal activity of the SFE extract. The 24 h LC_{50} value was selected as the discriminating dose to test the remaining batches. The toxicity of all pyrethrum batches was greater than 50% after 24 and 48 h (Table 6). Extraction parameters tested (10 MPa, 14 MPa and 30 MPa at 40°C) suggest that the milder conditions of 40°C and 10 MPa resulted in an extract with higher insecticidal activity (76% and 83.3% mortality at 24 h and 48 h respectively) compared to higher pressures 14 MPa (60.0% and 66.7% mortality at 24 h and 48 h respectively) and 30 MPa (67.5% and 70.0% at 24 h and 48 h respectively). There was a decrease in the ratio of PI/PII associated with increasing pressure (1.95, 1.92 and 1.87 for 40°C: 10 MPa, 40°C: 14 MPa and 40°C: 30 MPa respectively. The higher content of PI extracted at the

lower pressure corresponds to the highest percent mortality which corroborates the higher insecticidal activity of pyrethrin I. Pyrethrin concentrations were 0.531 g/g, 0.523 g/g and 0.436 g/g for pressures 10 MPa, 14 MPa, and 30 MPa respectively. The highest concentration (0.531 g/g) of pyrethrins which was extracted at the lowest pressure (10 MPa) reported the highest level of activity (76% and 83.3% mortality at 24 h and 48 h respectively). However, the highest pressure (30 MPa) with a concentration of 0.463 g/g reported higher bioactivity than 14 MPa (0.523 g/g) extract.

Table 6: The 24 and 48 h percent mortality of 2nd instar CPB treated topically with 0.0023% pyrethrum extract.

Pyrethrum Batch	% Mortality (24 h)	% Mortality (48 h)
30 MPa; 40°C	67.5±9.2	70.0±9.3
10 MPa;40°C	76.7±9.5	83.3±6.1
14 MPa; 40°C	60.0±10.3	66.7±11.2

3.4 Discussion

A maximum extraction temperature of 40°C was selected based on previous work (Otterbach & Wenclawiak, 1999) showing that 40°C is effective for SFE extraction of pyrethrins and unlikely to cause degradation. The ability to perform extractions at low to moderate temperatures makes it well suited for the extraction of natural products which are often unstable at temperatures exceeding 45°C. Increasing the extraction pressure was associated with a decrease in pyrethrins concentration but increase in total pyrethrin recovery. This is the result of an increase in CO_2 density enhancing solvation properties. Selectivity is compromised for a quantitative increase in yield as a result of stronger interactions between the solute matrix and solvent.

Hexane the most common solvent used in pyrethrum processing was detected in the crude oleoresin; but it was not detectable in the SFE extracts tested (LOD < 20 ppm) (*data not shown*). The efficacy demonstrated against the Colorado potato beetle (CPB) is similar to the insecticidal activity of extracts prepared using conventional solvent extraction. Our data demonstrate that SFE can produce a higher quality clean pyrethrin extract suitable for consumer use, as well as a value added "certified organic" natural pyrethrin extract.

Parameter optimization of pyrethrum oleoresin (25-50% pyrethrins) is significant because commercial application would involve refining pyrethrum oleoresin by continuous liquid-liquid (i.e. counter-current) methods. Continuous methods using liquid biomass are generally accepted as a more economical approach. Previously reported solid-fluid batch SFE operations from flower (~0.7-2% pyrethrins) extractions serve as an important source for comparison (H. Kiriamiti et al., 2003).

Another area for research involves the use of SFE CO_2 for fractionation of the oleoresin to either increase the concentration of pyrethrins and or to control the relative ratios of pyrethrins I and II. These extracts could be designed for use in customized formulations with enhanced efficacy or repellency. A new application from recent field trials has shown that a 50% formulation of pyrethrum gave 96 % repellency vs. 98% with DEET (Yarnell & Abascal, 2004). With reports questioning the safety of DEET this new pyrethrum data warrants further research (Koren et al., 2003). Pyrethrum has a good safety record. Osimitz et al., (2009) presented data on human pyrethrins exposure collected from the American Association of Poison Control Centres (AAPCC) and Toxic Exposure Surveillance Systems (TESS). Relatively few incidents were associated with pyrethrins and piperonyl butoxide (PY/PBO) and conclusions reached suggested that the risk of adverse effects, including allergic and asthma adverse events was very low. In addition, a thorough review of the literature using knowledge of pyrethrin chemistry and evidence based review of the literature and current extraction and refining techniques suggests that reports of hypersensitivity reactions are not substantiated (Franzosa et al., 2007). This strengthens the case for the development of formulations for human repellency against pests in the developed world, and more importantly in the developing world, where they are often vectors for diseases such as malaria. The widely accepted traditional uses of botanicals in many developing countries and subsistence farming may provide an incentive for local growers and a cost-effective option to aid in the reduction of Anthropod vector transmission for several prevalent diseases (Isman, 2006).

An additional step in developing formulations for organic agriculture involves the removal of semi-synthetic synergists such as piperonyl butoxide (PBO). Naturally

occurring synergists are available in various essential oils but require purification. Supercritical fluid (CO_2) chromatography (SFC) is an advanced method for the separation and isolation of targeted components and is well suited to producing a concentrate using methods acceptable to organic agriculture.

Chapter 4

Preface

Chapter 4 builds on botanical insecticide work completed by Ian Scott and Helen Jensen. Ian Scott (Agriculture Canada) hosted me at his lab for the Potter's spray tower tests on the Colorado potato beetle. I undertook design and execution of the SFE work with assistance from Jingqin Mao, Calum McCrae and Andrew Goulah. Ammar Saleem assisted with method development for the HPLC work.

4.0 Targeted Extraction of the Insecticidal Fraction of Black Pepper (*Piper nigrum*) using Supercritical CO_2

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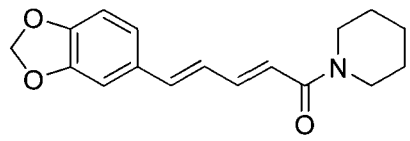
Abstract

Supercritical CO_2 extraction (SFE) was used assessed for extraction of the insecticidal piperamide fraction of black pepper (*Piper nigrum*). SFE sample preparation and extraction parameters studied included particle size (1 to 3 mm mesh), sample size (5 g to 30 g), temperature (40°C to 100°C), pressure (10 MPa to 65 MPa) and flow rate (1 L/min to 7 L/min). Pressures exceeding 35 MPa resulted in stable piperamide profiles. Extraction efficiency increased with pressure greater than 35 MPa and temperatures above 40°C. The piperamide-rich extracts generated using SFE demonstrated insecticidal activity. Mortality of Colorado Potato Beetle (CPB) treated with pepper extract derived from suboptimal extraction conditions (10 MPa; 60°C) was less than 10%. The piperamide level for the 35 MPa; 40°C was 149 mg/g whereas the piperamide level reported for 10 MPa; 60°C was significantly lower (42.3 mg/g). The 24 h mortality of 2nd instar CPB larvae topically treated with 0.075% pepper extract (35 MPa; 40°C) exceeded 70%. These results corroborate piperamide-dependent insecticidal activity. *P. nigrum* extracts generated using SFE represent an effective botanical and "organic" alternative to conventional synthetic pesticides.

4.1 Introduction

Public acceptance and urban use of synthetic pesticides has declined due to health and environmental concerns (Scott et al., 2008a). This has led to research on alternatives such as formulations based upon botanical extracts. The tropical plant family Piperaceae is a rich source of promising insecticidal phytochemicals (Arnason et al., 2002). The most recognizable species in the Piperaceae is black pepper (*Piper nigrum*). Extracts from *P. nigrum* show insecticidal efficacy against a wide range of insect pests (Scott et al., 2004; Scott et al., 2003; Scott et al., 2005; Scott et al., 2007). Black pepper extract is also an exceptional synergist in combination with pyrethrum, having a synergism ratio of 11.6 (Jensen et al., 2006b) compared to the industrial semi-synthetic synergist piperonyl butoxide (PBO) which has a synergism ratio of 4-5.

The insecticidal and synergist effects of *Piper nigrum* result from piperamides, which contribute to the natural chemical defense of this plant against insect herbivores. Bioactive piperamides are insect neurotoxins (Raymond-Delpech et al., 2005). Their synergist effects result from the inhibition of cytochrome P450 enzymes. The structures of five representative piperamides present in *P. nigrum*; Piperine (**14**), Piperlonguminine (**15**), Pipercide (**16**), Dihydropiperine (**17**), and Dihydropiperlonguminine (**18**) which are commonly used in analysis are shown in Figure 18, and compared to the commercial synergist piperonyl butoxide (PBO) (**19**). The piperidine alkaloid present in the highest concentration in *P. nigrum* is piperine.



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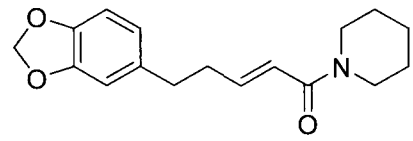

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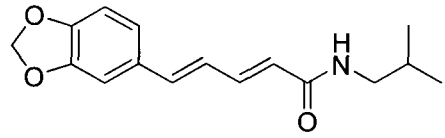

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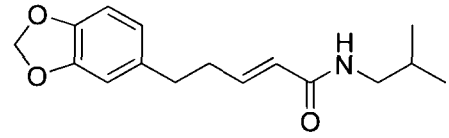

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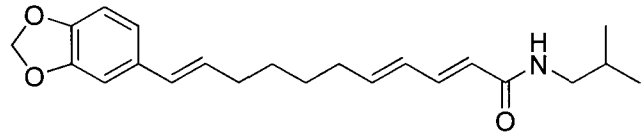

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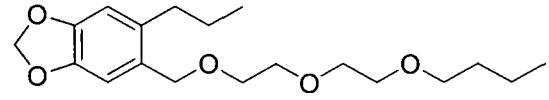

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Figure 17: Structural formula of representative piperamides from *P. nigrum* and PBO: 14. Piperine, 15. Piperlonguminine, 16. Pipercide, 17. Dihydropiperine, 18. Dihydropiperlonguminine, and 19. Piperonyl butoxide

Global use and the rich ethnomedicinal history of black pepper qualifies it as generally regarded as safe (G.R.A.S). Its short residual activity and rapid breakdown in sunlight (Scott et al., 2008b) increases its acceptability for "organic" applications. *Piper* extracts for research purposes are usually prepared using conventional solvent extraction (e.g. hexane, ethyl acetate). This limits the acceptability of the extracts as botanicals and their suitability for organic agriculture use. Supercritical fluid extraction (SFE) utilizing CO_2 provides an efficient way to generate residue-free extracts while addressing the restrictions on solvent use. SFE temperature parameters are relatively low, making it attractive for botanical applications, which often involve thermally labile actives. The critical temperature (T_c) of carbon dioxide (CO_2) is 304 K (31°C) and the critical pressure (P_c) is 7.38 MPa. Extractions within these parameters are characterized by dramatic changes in density (solute solubility) with moderate changes in temperature and pressure. These properties which can effectively utilized for targeted (selective) extractions of specific classes of biomolecules such as piperamides. SFE of *P. nigrum* essential oil has been thoroughly investigated using low pressure/moderate temperature (low density) parameters (Catchpole et al., 2003; Ferreira et al., 1993; Ferreira et al., 1999; Ferreira & Meireles, 2002; Perakis et al., 2005; Sovová et al., 1995). Optimized extraction of the piperamide fraction and confirmation of the respective insecticidal activity remains undocumented.

We report optimal parameters for SFE of insecticidal piperamides from *P. nigrum*. Our results increase the known useful range of pressure and temperature parameters (Catchpole et al., 2003; Ferreira et al., 1993; Ferreira et al., 1999; Ferreira & Meireles, 2002; Perakis et al., 2005; Sovová et al., 1995) for extractions of *P. nigrum*.

We optimize flow rate, particle size and sample size, and reportthe piperamide profiles for SFE extracts of *P. nigrum*. T insecticidal activity of the piperamide-rich extracts was confirmed using the Colorado potato beetle (*Leptinotarsa decemlineata*), a pest species resistant to most classes of synthetic insecticides.

4.2 Materials and Methods

SFE

Black peppercorns *(P. nigrum)* of Southeast Asian origin were purchased from Country Bulk (London, ON, Canada). The peppercorns were ground in batches to the desired particle size using a Thomas Model 4 Wiley Mill (Thomas Scientific) prior to extraction. SFE extractions were performed on an SFT-250 extractor (Supercritical Fluid Technologies, Newark, DE). This system was equipped with a 100 mL extraction vessel. Liquid carbon dioxide with a dip tube (grade 4.0; #24062141) was purchased from Linde (Belleville, ON). SFE sample preparation and extraction parameters studied were particle size (1-3 mm mesh), sample size (5-30 g), temperature (40-100°C), pressure (10-65 MPa) and flow rate (1-7 L/min). Samples were collected in 4 oz (125 mL) I-CHEM Septa-Jars and stored in a refrigerator at 4° C prior to analysis. The mass of extract and mass of CO_2 used was monitored at 5 min intervals to exhaustion (approximately 90 min) to determine the extraction efficiency and solvent: biomass ratio.

HPLC Analysis

Piperamide analysis was conducted with an HPLC system using a Varian Prostar model pump, model 330 UV/Vis photodiode array detector and model 410 autosampler (Varian Chromatography Systems, Walnut Creek, CA). A Varian C18 (3 μm, 100 Å, 4.6 mm, 100 mm) column was used. Analytical grade solvents were purchased from J.T. Baker (USA).

Four piperamide standards, piperine, 4,5-dihydropiperine, 4,5-dihydropiperlonguminine, and piperlonguminine were synthesized as described

previously (Scott et al, 2002). Their structures were confirmed with NMR. An HPLC method for separating the piperamides in *P. nigrum* was adapted from previous work conducted in our laboratory (Scott et al., 2005). A 10-point calibration curve was generated between 1 and 250 μg/mL for each amide. Piperine and piperlonguminine were measured at 340 nm, 4,5-dihydropiperine and 4,5-dihydropiperlonguminine were measured at 205 nm, and pipercide was measured at 275 nm. The four synthesized amides were measured at 205 nm. The optimized method used a binary gradient of acetonitrile (A) and water, beginning with 30% A, increasing to 70% at 10 min, 90% at 12 min, before returning to 30% A at 15 min Samples were injected at 2μL and the flow rate was 0.4 mL/min.

Insecticidal Bioassays

Insecticidal bioassays were performed using a Potter tower (Burkard Manufacturing Co. Limited, Rickmansworth, Hertfordshire, UK). Second instar larvae from an insecticide-susceptible strain (AAFC London ON) of the Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (CPB) were exposed to concentrations of 0.06, 0.075, 0.09, 0.1, and 0.125 % of black pepper SFE extracts. Five CPB larvae were treated per replicate with duplicate replicates per concentration in each trial. Trials were repeated 4 times for a total of 40 larvae treated per concentration. Each application consisted of a 5 mL extract solution sprayed on to the CPB larvae using the Potter tower to ensure complete coverage. Treated CPB were then transferred to an untreated potato leaf disc. Mortality of the CPB larvae was observed at 24 and 48 h. The LC_{50} (+ 95% Fiducial Limits) value was calculated from the mortality data using probit analyses (SAS

ver 9.1). Each of the three remaining pepper extracts was then tested using the LC_{50} concentration calculated for the first pyrethrum 48 h mortality values.

4.3 Results and Discussion

Effect of temperature and pressure on percent yield

Among the complete data set of 120 SFE extractions, percent yield [wt %] ranged from 0.79% at 10 MPa; 100°C to a high value of 11.56% at 45 MPa; 90°C. There was a dramatic increase in yield and piperamide content at pressures greater than 30 MPa. The average yield below 30 MPa was 2.59% producing a thin yellow oil. The average yield above 30 MPa was 7.87% producing a viscous, mustard yellow, semi-solid. The effect of 2 pressures (35 MPa and 40 MPa) on extraction efficiency is illustrated in Figure 18. Extraction yield increased rapidly with time and then reached a constant value at both pressures. At 35 MPa it took 50 min to reach exhaustive extraction, but only 25 min at 40 MPa. The average coefficient of variation for triplicate experiments was < 0.10.

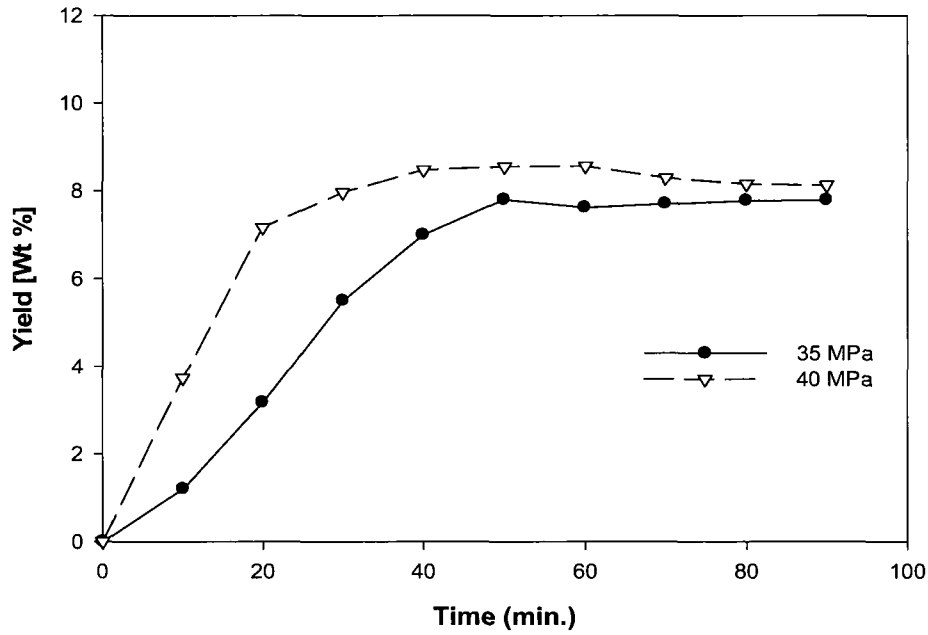

Figure 18: A representative extraction efficiency data set demonstrating the effect of pressure at 90°C

The effect of temperature at constant pressure of 40 MPa is illustrated in Figure 19. Extraction yield increases with time and reaches a constant value in each temperature trial. Temperature decreases the time at which constant extraction is achieved and increases the final yield. The greatest yield (10.5%) was generated at 100°C. The average coefficient of variation for triplicate experiments was < 0.10.

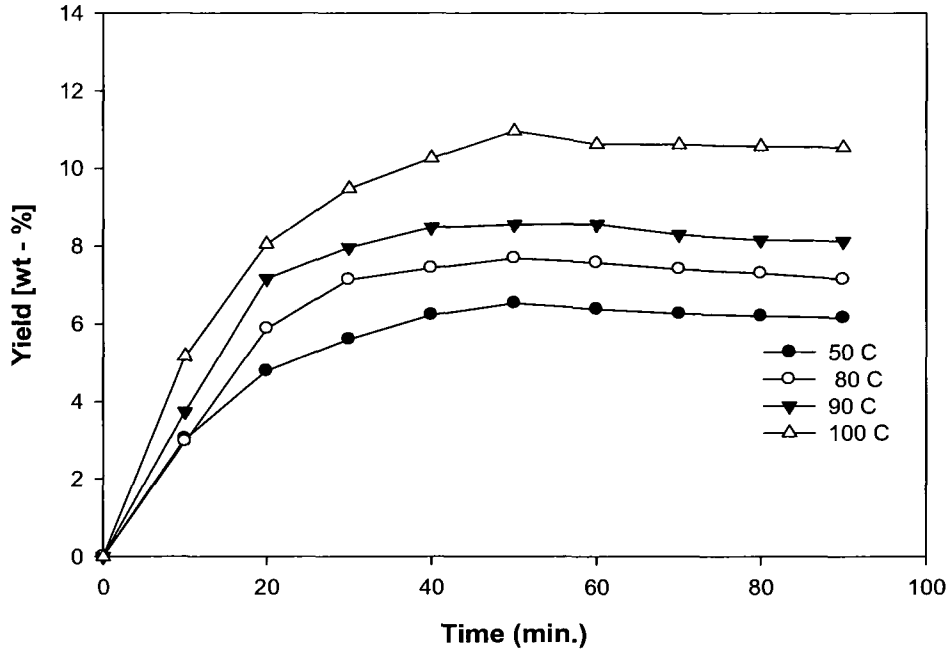

Figure 19: Effect of temperature at constant pressure of 40 MPa

Effect of flow rate, sample size and particle size on percent yield

In addition to pressure and temperature; flow rate, sample size and particle size were investigated in this study (data not shown). Flow rates were investigated to determine optimal residence time of SFE. Optimal residence time considerations optimize CO_2 usage and increase overall method efficiency. Yield steadily increased from 0.5% at a flow rate of 1 L/min until reaching to a maximum of 8.4% at 5.0 L/min. Flow rates exceeding 5.0 L/min resulted in decreases to 7.9% and 4.5 % for 6.0 L/min and 4.5 L/min respectively.

Optimal sample size for the 100 mL extraction vessel and *P. nigrum* (particle size 2 mm mesh) was 10.0 g which resulted in a yield of 8.4% versus yields of 6.4%, 6.1% and 6.2% for 5 g, 20 g, and 30 g samples respectively. This difference may have resulted

from flow variation, temperature differential, column packing channeling effects or several other factors.

Particle size reduction significantly increased the percent yield. The percent yield for 3, 2 and 1 mm mesh ground *P. nigrum* increased from 0.3 % to 1.4 % to 5.8 %. Increasing surface area dramatically affects extraction efficiency. The shape of the particle and its effect on extraction efficiency remains to be investigated.

Piperamide recovery

With respect to piperamide recovery, 4,5-DHP, PL, 3,4-DHP and piperine are representative of "total piperamides." At 10 MPa the concentration of total piperamides increased from less than 50 mg/g to greater than 150 mg/g at pressures greater than 20 MPa. SC CO_2 resulted in increased solubility of the piperamides and increased overall yield. A representative table of piperamide profile at low and high pressure is presented in Table 7. At pressures greater than 30 MPa, the level of piperamides did not vary significantly.

Table 7: Representative (mg piperamide/g extract) profile of low and high pressure SFE

Pressure MPa	Temperature °C	4,5-DHPL mg/g	PL mg/g	3,4-DHP mg/g	Piperine mg/g	$Piperamide_{tot}$ mg/g
10	60	0.26	0.33	19.91	21.88	42.38
10	100	0.17	0.56	18.27	30.96	49.96
35	40	0.36	3.35	25.64	119.21	148.56
40	100	0.65	3.78	19.43	118.47	142.33
65	80	0.34	3.57	18.29	126.13	148.34

Solvent (hexane) extractions were compared to SFE to determine piperine extraction efficiency. SFE extracted twice the piperine (*data not shown*) compared to conventional methods. Further tests are necessary to determine the significance of these results.

CPB values for the 24 and 48 h SFE *P. nigrum* extract (100°C; 35 MPa) were 0.075% (0.068-0.081% 95% Fiducial Limits) and 0.074% (0.067-0.079% 95% FL) respectively (N = 240). The 24 h LC_{50} value was selected for both extracts as the discriminating dose for comparison with the remaining batches. The percent mortality of CPB exposed to 0.075% pepper extract was lower than 50% after 24 and 48 h for 3 of the 4 extracts tested (Table 8). Greater than 50% mortality was observed after 24 and 48 h with the pepper extracted at 40°C and 35 MPa. At 60°C and 10 MPa and 100°C and 10 MPa the total piperamides were 42.4 and 5.0 mg/g respectively. At 40°C and 35 MPa and 100°C and 35 MPa the total piperamides were 149 and 155 mg/g respectively. The 3,4 DHP level was 25.0 and 19.0 mg/g in the 40°C; 35 MPa and 100°C; 35 MPa extracts

respectively. The data corroborates previous observations which correlate piperamide levels with insecticidal activity (Scott et al., 2005b).

Table 8: The 24 and 48 h percent mortality of 2nd instar CPB treated topically with 0.075% pepper extract

Pepper Batch	$Piperamide_{tot}$ (mg/g)	% Mortality (24 h)	% Mortality (48 h)
10 MPa; 60°C	42.38	10.0±6.8	13.3±6.7
10 MPa;100°C	49.96	10.0±4.5	20.0±7.3
35 MPa; 100°C	154.29	50.0	50.0
35 MPa; 40°C	148.56	70.0±10.0	73.3±8.4

4.4 Discussion

Our results confirm the insecticidal activity of the SFE extracts and corroborate data that piperine concentration is directly associated with insecticidal activity (Scott et al., 2005b). Piper secondary metabolites haveseveral modes of action: contact toxicity (Scott et al., 2004; Scott et al., 2005), inhibition of cytochrome P450 MFO's (Jensen et al., 2006a; Scott et al., 2002), repellency and antifeedant (Scott et al., 2004). The combinatorial effects of the piperamides present in extracts of *P. nigrum* present a promising alternative to combat pests which have become resistant to synthetic pesticides (e.g. carbamates) currently available. Further tests investigating the synergist effects of the numerous structurally related piperamides with pyrethrum would be useful. The rapid degradation (low environmental persistence) is a criterion necessary for organic acceptance. Although this presents a disadvantage to large crop applications it may be of particular interest for storage applications (e.g. grain bins), indoor use, indirect sunlight or soil applications on its own or combined (i.e. pyrethrum).

As a result of the greater efficiency of SFE for piperine extraction suggest that the insecticidal activity of SFE will be greaterthan solvent extract based on the increased piperine levels. This is promising as SFE is rapidly gaining industrial acceptance as an efficient and environmentally responsible method acceptable for organic applications.

P. nigrum is considered a minimum risk pesticide and is on the US EPA 25(b) list of active ingredients (listed as "white pepper") exempt from regulatory requirements, so the potential for development of this botanical as an insecticide and/or synergist is therefore favorable. Acceptance of *Piper* extract as equivalent to the 25(b) list "white pepper" ingredient remains an issue. Formulations which reduce the respiratory and

ocular irritability of *P. nigrum* would increase user acceptance. This challenge could be investigated further through fractionation technology such as SFE chromatography or formulation (encapsulation) chemistry. *Piper* extracts are more easily stored, transported and handled than ground pepper.

As a result of grassroots and federal judicial action to limit and or severely restrict chemical pesticide use, regulatory bodies in both the US and Canada have been prompted to emphasize non-conventional, low risk products and to allow bio-pesticides and botanicals to be considered separate from conventional pesticides (NRC, 2000; EPA, 2009). The national research council (NRC) has highlighted botanicals as a significant alternative for pest control (Committee on the Future Role of Pesticides in US Agriculture et al., 2000). The research surrounding *P. nigrum* (GRAS) extracted using SCE (GRAS) is consistent with a reduction in the use of synthetic pesticides. As a multi-target botanical insecticide and/or synergist combination (pyrethrum-pepper) extracted using SCE, *P. nigrum* would be suitable for the rapidly developing organic agriculture and food, and home and garden sector.

Development of botanical insecticides using SFE technology is practical because many classes of bioactive compounds using efficient methods (time saving) under mild conditions (suitable for thermally labile compounds) with low environmental impact (reduced solvent use).

Chapter 5

Preface

The next 2 chapters resulted from a project on northern natural resources initiated by Marc Allard and Guy Rochefort at Nunavik BioSciences, a subsidiary of the Inuit development corporation, Makavik. The work was undertaken in collaboration with postdoctoral fellow, Virginie Treyvaud Amiguet. Virginie developed and validated the GC method development for identification and analysis of shrimp SFE samples. I designed the second study which is an investigation of optimal conditions for extraction and scale up to pilot scale. This project was highly successful and has now launched a semi-commercial scale SFE extraction of an ω-3 rich oil from *Pandelus borealis* by-products for clinical studies.

5.0 Supercritical CO_2 extraction: a superior method for the extraction of polyunsaturated fatty acids from Northern Shrimp (*Pandalus borealis* Kreyer) processing by-products

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[1]These authors contributed equally to this manuscript.

Abstract

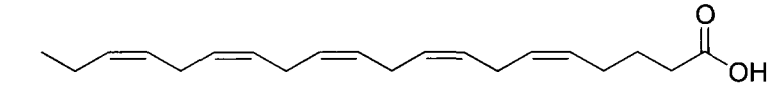
This study investigated the potential of Northern Shrimp (*Pandelus borealis* Kreyer) by-products as a source of polyunsaturated fatty acids. The by-products (heads, shell and tail) of processing account for approximately 50% of the catch. Supercritical CO_2 extraction (SFE) of the by-products at 35 MPa and 40°C generated a deep red oil, rich in polyunsaturated fatty acids specifically 7.8±0.06% eicosapentaenoic acid (EPA) and 8.0±0.07 % docosahexaenoic acid (DHA). This superior quality SFE extract, enriched in heart healthy omega-3 fatty acids, warrants further investigation as natural health product.

5.1 Introduction

The Food and Agriculture Organization (FAO) 2009 market report on shrimp has indicated that the demand for shrimp in all major markets has decreased substantially and prices have declined as a result. The report encourages diversification as a means to effectively manage the current economic climate (Boisset, 2009). Lack of stability of the market prices is even more critical for small-scale producers, such as the Inuit owned Nunavut BioSciences Inc. The Inuit coastal communities in Northern Quebec and Labrador rely heavily on commercial catch of the Northern shrimp, *Pandalus borealis* Kreyer (Pandaladae). Finding an alternative income stream from previously discarded by-products presents an attractive opportunity for this sector as the head, shells and tails (by-products) constitute approximately 50% of the catch. Northern shrimp (*Pandalus borealis*) are hermaphrodites, male for approximately the first 3 years of their life (commercial small shrimp) at which point they become female (commercial large shrimp), moving to deeper waters, with the exception of the ovigerous period (March-October). The components and nutritional quality of shrimp processing by-products has been described in detail for Northern pink shrimp, *P. borealis* (Heu, Kim, & Shahidi, 2003). Review of this data revealed good levels of polyunsaturated fatty acids (PUFAs), 8.9% eicosapentaenoic acid (EPA) and 10.7% docosahexaenoic acid (DHA). Several studies have investigated shrimp shell utilization as a source of antioxidants (Seymour et al., 1996), chitin biopolymers (Pinelli et al., 1998), carotenoids (Lin et al., 2005), protein (Ferrer et al., 1996), glucosamine (Ferrer et al., 1996) and astaxanthin (Pacheco et al., 2009; Gimeno et al., 2007). There are however, no studies which have addressed whole

by-product utilization and none that have focused on PUFA extraction from this source for natural health product development.

Research on PUFA's, found primarily in fish oils, has increased substantially in recent years (Sahena et al., 2009) and many reports support their role in improving and maintaining human health. Omega-3 fatty acids have anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic, and vasodilatory properties (Simopoulos, 2007). Both preventive and curative therapies have created a considerable demand for EPA (C20:5) and DHA (C22:6) (Figure 20). EPA and DHA from fish oil have been shown in clinical studies to help support cognitive health, brain function (Haag, 2003), cardiovascular health (Kris-Etherton et al., 2003; Oh, 2005), to help reduce serum triglycerides (Sirtori et al., 1998) and, in conjunction with conventional therapy, to help reduce the pain of rheumatoid arthritis (Volker et al., 2000).



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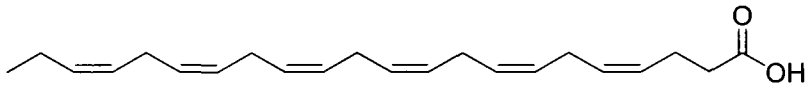

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Figure 20: Structure of polyunsaturated fatty acids, 20. eicosapentaenoic acid (EPA), 20:5 ω-3 and 21. docosahexaenoic acid (DHA), 22:6 ω-3

Fatty acids extracts are primarily generated using solvent extraction, enzymatic extraction, urea complexation, and molecular distillation. The high temperatures and toxic solvents used in these methods have increased foci towards alternative extraction

technologies. Extraction and fractionation of PUFAs from fish oil using supercritical CO_2 extraction (SFE) has generated significant interest as a means to generate cleaner (residue-free), higher quality PUFA extracts (Sahena et al., 2009). SFE using non-polar CO_2 is especially well suited to this application as the modest conditions, critical temperature (C_T) = 31°C, critical pressure (C_P) =7.38 MPa, and low oxygen setting provides an environment favorable for heat-sensitive and easily oxidized substances such as fatty acids. Additional advantages of SC CO_2 include its tasteless, odourless, non-toxic, and non-flammable properties and, it's generally regarded as safe (GRAS) status. Most recently, SFE of Antarctic krill (*order Euphausiacea*) (Corrêa et al., 2008; Létisse et al., 2006; Létisse & Comeau, 2008; Rubio-Rodríguez et al., 2008) has been shown effective in the extraction of EPA and DHA at both the research (Yamaguchi et al., 1986) and commercial scales (Harland, 2006; Watkins, 2007). SFE of Northern shrimp or its by-products has not yet been investigated.

The goal of this study was to explore SFE of Northern shrimp processing by-products which may contribute to the development of a value added product and an additional income stream for the Nunavut shrimp sector. Specific objectives included generating a supercritical CO_2 extract and evaluating the polyunsaturated fatty acids profile by GC-FID.

5.2 Materials and Methods

Chemicals

Carbon dioxide with a dip-tube (CO_2 UN1013) was purchased from BOC Gases (Belleville, ON). Methylated fatty acid standards (37 components mix, cis-11-vaccenic acid methyl ester, heptadecanoic methyl ester), copper (II) acetate monohydrate 98+% A.C.S, 0.5 N methanolic hydrochloric acid, supelclean LC-NH2 SPE tubes (1 mL) were purchased from Sigma-Aldrich, Supelco (St Louis, MO). Potassium hydroxide, hydrochloric acid and dichloromethane were purchased from Fisher Scientific.

Sample preparation

Shell-on shrimp (*Pandalus borealis*) cooked and quick frozen at sea from Newfoundland Resources Limited, were supplied by Nunavik Biosciences Inc. They were stored at -20°C. The frozen shrimp were rinsed with water, drained and thawed at room temperature for 2 hours. Heads, shells, and tails (by-products) were separated from the flesh and wrapped in cheese cloth prior to being placed on an individual stainless steel perforated racks. The racks were placed in a DeCloet drier (Tilsonberg, ON) at room temperature (23°C) which was programmed to reach a final temperature of 40°C with a ramping rate of 2°C/h (total drying time ~19 h). The moisture content was measured before and after the drying procedure using an Ohaus MB35 Halogen Moisture Balance set at 120°C. Finally, dried shrimp by-products were ground with a Cuisinart mini-prep plus processor (~2 mm mesh). Shrimp powder samples were prepared just prior to each extraction.

Supercritical CO_2 extraction (SFE)

The extractions were carried out using a research scale supercritical CO_2 extractor SFT-250 (vessel capacity 100 mL) purchased from Supercritical Fluid Technologies Inc. (Newark, DE) equipped with a pre-heater. Ten grams (10 g) of by-product powder was loaded inside the extraction vessel. Cotton was used to reduce dead volume in the extractor and two stainless steel micron filters were placed at the CO_2 entry and exit points respectively. The pressure and temperatures used were 35MPa and 40 °C and 15 MPa and 50 °C. Extractions were carried out in triplicate and yields were recorded every 10 min for 90 min to determine extraction efficiencies. The flow rate used was 3-5 L/min. The extract was collected in an I-CHEM Tall Clear WM Septa-Jar™, equipped with a vent and trap. This flow rate was confirmed by measurement of the mass of CO_2 used.

Separation of neutral lipids, nonesterified fatty acids and phospholipids

Neutral lipids (NL), nonesterified fatty acids (NEFA), and phospholipids (PL) were separated by filtration using supelclean solid-phase extraction tubes (1 mL) as described previously by Maillet and Weber (2006). Briefly, NL were eluted from the column with chloroform:isopropanol (2:1 v/v), NEFA with isopropyl ether:acetic acid (98:2 v/v) and PL with methanol. The NEFA and PL fractions were quantified by gas chromatography-flame ionization detector (GC-FID) as total fatty acid content.

Fatty acid composition

Shrimp oil was derivatized prior to GC analysis. The saponification methodology was adapted from Albrink (1959). Briefly, approximately 2 mg of shrimp oil were mixed with 0.5 mL alcoholic potassium hydroxide (6 mL of 33% potassium hydroxide in 94 mL ethanol) before being incubated in a water bath at 80°C for 1 h. Distilled water (0.5 mL) was then added to the tube which was incubated an additional 10 min. Aqueous hydrochloric acid (100 μL, 1.8 N) was added to the warm hydrolysate and vortexed to neutralize the mixture and precipitate the free fatty acids as a fine cloud. The mixture was kept at room temperature to cool prior to pipetting 1 mL of hexane into the tube. After being vortexed for approximately 30 sec., the tube was centrifuged for 5 min at 2000 rpm. The bottom aqueous phase was removed and the remaining hexane phase containing the free fatty acids was dried under nitrogen and immediately methylated following Horshi *et al.* (1973) methodology to avoid degradation. Their methylation allowed nearly complete esterification of fatty acids at room temperature in a short period of time without artifact formation or loss of polyunsaturated acids (Horshi et al., 1973). Briefly, the saponified oil was dissolved in 0.2 mL of chloroform prior to the addition of 0.2 mL of 20 mM methanolic cupric acetate monohydrate (3.9 mg cupric acetate monohydrate in 1 mL of methanol) and 1 mL of 0.5 N methanolic hydrochloric acid. The mixture was vortexed and left for 45 min at room temperature. The reaction mixture was extracted three times with 1 mL of hexane after addition of 0.4 mL of distilled water. The pooled hexane extracts were then evaporated to dryness under nitrogen. The methylated oil was re-suspended in 1 mL of dichloromethane.

The fatty acid profile of shrimp oil was analyzed by GC (Agilent 7890 GC) equipped with a FameWax column (30m x 0.32mm x 0.25μm, Restek), a flame ionization detector (FID) and a split injector. Hydrogen was used as carrier gas at constant pressure equivalent to 40 cm/sec at 130°C oven. Both injector and detector temperatures were set up at 250°C. 1 μL of sample was injected with a split ratio of 50:1 and the column temperature was heated at 130°C for 15 min, ramped at 5°C/min to 230°C and held at 230°C for 10 min. The quantification of each fatty acid was carried out by external standardization. The standard curves were forced to the origin, linear, and bracketed the samples' concentrations._Fatty acids were identified by comparing their retention times with the 37 standard FAME mix (Supelco) as well as the individual methyl ester vaccenic acid which was not present in the mix. Validation of the method gave coefficients of variation for individual fatty acid that ranged from 1.4 to 5.6%, a limit of detection of 0.01 to 0.02 μg/mL and a limit of quantification of 0.08 to 0.2 μg/mL.

Data analysis

Each sample was analyzed in triplicate and data are presented as the average in mg/g extract together with the Standard Error (SE). One-way ANOVA and *post-hoc* Bonferroni analysis was used to analyze the data.

5.3 Results and Discussion

Initial SFE of the ground shrimp by-products which included the head, shell and tails were carried out at two conditions; low pressure (15 MPa; 50°C) and moderate pressure (35 MPa; 40°C). At 15 MPa; 50°C, the oil yield increased with time of extraction and reached a plateau at a yield of 11 mg/g at 20 min with a total fatty acid content of 620 mg/g (Figure 21). The yield at 35 MPa; 40°C increased with time of extraction and reached a plateau of 137 mg/g which corresponded to 795 mg/g of total fatty acids The coefficient of variation (CV) for triplicate yield determinations at 15 MPa; 50°C was 36; the CV for 35 MPa; 40°C was 5. The lower pressure condition (15 MPa; 50°C) was previously reported as suitable for the selective extraction of polyunsaturated fatty acid ethyl esters, specifically esters of EPA and DHA from other materials (Mukhopadhyay, 2000), but is clearly insufficient for northern shrimp by-product extraction. As a result all subsequent extractions were carried out at 35 MPa; 40°C.

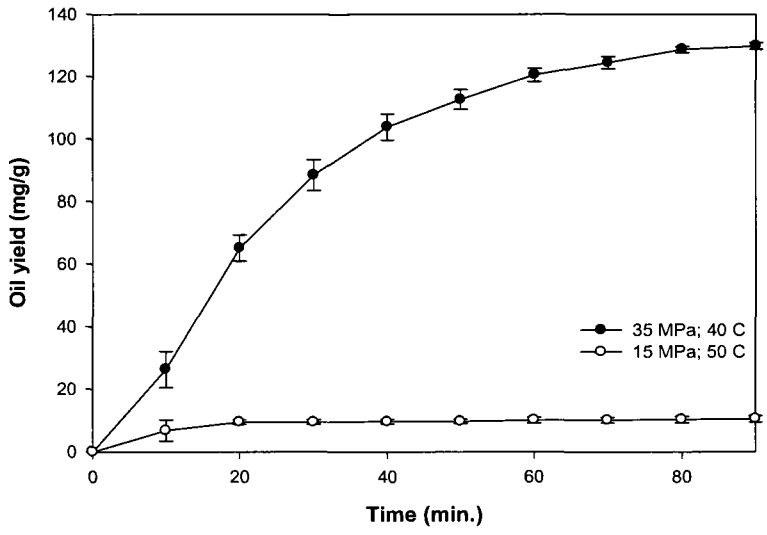

Figure 21: SC CO_2 extraction efficiency curves generated for shrimp by-products at low pressure (15MPa; 50°C) and moderate pressure (35MPa; 40°C). $CV < 21$ for triplicate experiments (36 for low pressure; 5 for moderate pressure)

For comparative purposes, solvent extractions of the ground shrimp by-products were conducted using soxhlet apparatus with both acetone and hexane. Although solvent extractions gave higher oil yields (acetone 206 mg/g, hexane 178 mg/g) than SFE (137 mg/g), the deep red oil obtained by SFE contained higher total fatty acids (TFA), 795 mg/g and ω-3's including EPA (78 mg/g) and DHA (79.7 mg/g) compared to acetone (TFA: 627 mg/g) and hexane (TFA: 705 mg/g) extractions which yielded 6.99; 6.68 and 7.20; 6.97 mg/g of EPA; DHA respectively (Table 9). The CV for triplicate analysis was less than 3.0. A representative chromatogram is shown in Figure 22. Additional solvent removal was not necessary for SFE and the total time required for the extraction was less than 90 min compared to approximately 8 h for soxhlet extraction and subsequent solvent removal. These results present the characterized SFE of Northern shrimp by-products to the literature for the first time.

Table 9: Comparative fatty acid analysis of solvent (acetone and hexane) extraction vs SFE (35 MPa; 40°C) in mg/g (oil)

Chromatogram Reference No.	Fatty acids	Solvent extraction (acetone)	Solvent extraction (hexane)	SFE (35 MPa; 40°C)
1	C14:0	32.0	35.0	43.21
2	C16:0	64.2	71.3	78.72
3	C16:1	70.8	76.8	94.15
4	C18:0	10.9	12.8	14.14
5	C18:1n9c	82.4	97.6	103.54
6	C18:1	20.7	22.1	23.85
7	C18:2n6c	6.8	22.7	8.33
8	C18:3n6	2.4	2.5	3.38
9	C18:3n3	3.9	4.7	5.08
10	C20:1	63.0	70.7	85.51
11	C20:2	2.0	2.3	2.63
12	C20:4n6	2.2	2.3	2.08
13	**C20:5n3 EPA**	**69.9**	**72.0**	**78.00**
14	C22:1n11	78.3	87.8	106.07
15	C22:1n9	43.1	46.3	56.90
16	C22:5	5.1	5.4	6.53
17	**C22:6n3 DHA**	**66.8**	**69.7**	**79.66**
18	C24:1	2.7	3.1	3.02
TFA		**627.1**	**705.1**	**794.81**

* The average coefficient of variation of triplicate sample/analysis was < 3.0.

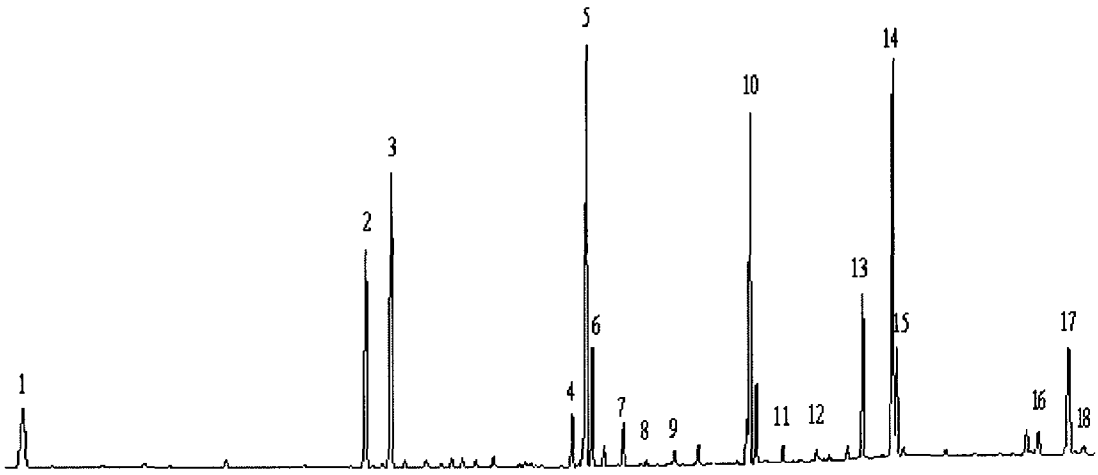

Figure 22. Representative GC-FID chromatogram of fatty acids contained in SFE of shrimp by-products. Peak 13: EPA (**20**) and peak 17: DHA(**21**).

The phospholipid content of the shrimp oils obtained by acetone and hexane soxhlet extraction was 3.6% and 3.1% respectively. With regards to phospholipids, the literature suggests that they are generally present in high levels in aquatic organisms and Yamagushi et al. (1986) also reported that they readily deteriorate and hamper the effective utilization of these oils. Furthermore, phospholipids are undesirable in the oil since they affect its stability by chelating metal ions, therefore increasing oxidative processes (Nzai & Proctor, 1998). Yamaguchi et al., (1986) reported that the SFE of Antarctic krill was effective in obtaining non-polar lipids without phospholipids. Similarly, the shrimp oils we extracted by SFE only contained traces of phospholipids with an average of 0.25% (standard error ± 0.01, n = 6).

Additional SFE extractions were completed to better understand the fatty acid distribution within the shrimp (Figure 23). Shrimp heads (SH), shrimp by-products (SB) which included the head, shell and tails, and shrimp muscle (SM) were extracted individually in order to determine the distribution of fatty acids throughout the whole shrimp. The desirable polyunsaturated fatty acids (PUFAs) were concentrated primarily in the by-products while the muscle contained less than 10% of this value. The results showed a high oil yield for SH (171 mg/g dried material), followed by SB (137 mg/g dried material) and a very low yield for SM (16 mg/g dried material) (Figure 23). The higher yield obtained with SH was easily explained by the fact that fats are localized in the shrimp head that contains almost all the organs including most of the digestive system. SB contained heads and the shells, which reduced the percentage of oil in the sample. Although SH was shown to be the optimal shrimp part to be used for oil extraction, it did not represent the actual by-product material obtained from food

processing industries and the effort that would be required to separate the shells from the heads would be time consuming and expensive.

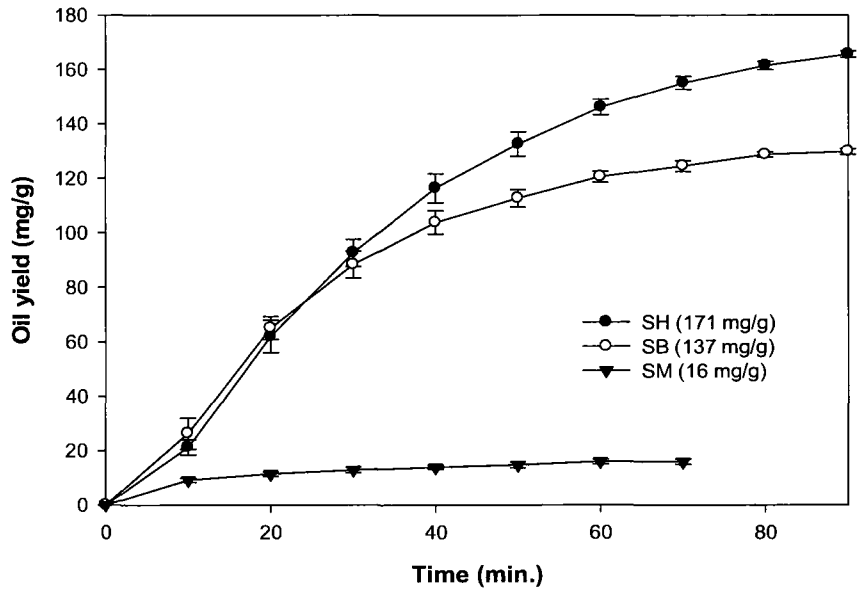

Figure 23: Comparison of the oil yields from different part of the shrimp: Shrimp heads (SH), shrimp by-products (SB), and shrimp muscle (SM). The *CV* for SH, SB, and SM were 6.35, 7.26 and 11.50 respectively.

The results summarized in Table 10 revealed that the total fatty acid (TFA), DHA and EPA contents of SH oil and SB oil were not significantly different (TFA: 795.0 mg/g and 794.8 mg/g; DHA: 78.8 mg/g and 79.7 mg/g; EPA: 77.6 mg/g and 78.0 mg/g respectively). This result was expected as the oils extracted from the two samples (SH and SB) came from the same source (head section) while the shells contained in the SB sample reduced yield by 20%. The SM sample was not only a poor source of oil, but the extracted oil contained significantly less fatty acids than SH and SB ($p<0.01$) (TFA: 574.3 mg/g; DHA: 54.2 mg/g; EPA: 56.0 mg/g).

Table 10: Omega-3 content of shrimp oil from head, residue and flesh extracted at 35 MPa and 40°C (ALA: alpha-linolenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid). Means (mg/g) ± Standard Errors (SE) are presented with n = 3. Statistical analysis was conducted for total fatty acid; EPA and DHA where a and b are significantly different with $p < 0.01$.

Fatty acids	Head		Residue		Flesh	
	Mean	±SE	Mean	±SE	Mean	±SE
Total Fatty Acid	795.0[a]	4.56	794.8[a]	4.44	574.3[b]	4.13
Omega-3						
C18:3n-3 (ALA)	5.2	0.06	5.1	0.09	3.6	0.06
C20:5n-3 (EPA)	77.6[a]	0.45	78.0[a]	0.52	56.0[b]	0.42
C22:5n-3 (DPA)	6.7	0.03	6.5	0.07	4.6	0.06
C22:6n-3 (DHA)	78.8[a]	0.48	79.7[a]	0.41	54.2[b]	0.56

Overall, the levels of PUFAs contained in the SC CO_2 extracts of *P. borealis* by-products were comparable to the levels found in traditional food sources of ω-3 (i.e. salmon) and fish oils available on the market which were extracted using conventional methods (Table 11). The marine crustacean krill, extracted by SFE has higher levels of both EPA and DHA and krill meal represents an exceptional source of EPA. Previous studies of hake and sardine by-products have demonstrated the potential of this method for generation of a value added product from a previously discarded waste stream. The yield and PUFA profiles generated by SFE for *P. borealis* by-products provide rationale that this source and method warrant further investigation as a ω-3 fatty acids natural health product with potential for commercialization. While the fatty acid composition of EPA and DHA in krill is superior; 17.4% and 12.4% respectively (Tou et al., 2007) SFE of Northern shrimp by-products is an attractive strategy as it does not compromise their primary use as food. Furthermore, by using this technology, the residual biomass is

available for further extraction of additional bioactive materials (i.e. chitin, polysaccharides), making this strategy attractive.

Table 11: Percent fatty acid composition of Northern shrimp by-products, Coho salmon, fish oils (salmon, tuna, shrimp), whole Antarctic krill, krill meal, by-products (hake and sardine).

Fatty acid	N.Shrimp BP[a*]	Coho salmon[b]	Salmon oil[c]	Tuna oil[c]	Shrimp oil[c]	Whole krill[d]	Krill meal[b]	Hake BP[f]	Sardine BP[g]
EPA 20:5	7.8 [a*]	7.2	6.35	6.01	6.36	11[d*] 17.4[e]	12.39	6	11
DHA 22:6	8.0 [a*]	11.1	8.87	9.39	8.85	12.4[e]	6.09	14	13.01

BP= by-products
[a]Results from this study; [*]extracted by SFE; [b](Gigliotti et al., 2008); [c](Giogios et al., 2009); [d](Yamaguchi et al., 1986) [e](Tou et al., 2007); [f](Rubio-Rodríguez et al., 2008); [g](Létisse & Comeau, 2008), (Létisse et al., 2006)

This is the first report of SFE of a PUFA rich oil from Northern shrimp processing by-products. This high quality solvent-free oil obtained by SFE contains sufficient levels of ω -3 PUFAs to warrant further investigation as a natural health product and clinical evaluation for safety and efficacy. Optimization of SFE parameters would be the next logical step.

Chapter 6

6.0 Supercritical CO_2 Extraction of Omega-3 Rich Oil from Northern Shrimp (*Pandalus borealis* Kreyer) by-products: Study of the influence of process parameters on extraction yield and oil quality at the laboratory and pilot scale

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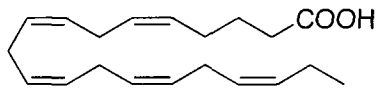
Abstract

The supercritical CO_2 extraction (SFE) of the omega-3 rich oil from North Atlantic shrimp (*Pandalus borealis* – Kreyer) by-products was optimized. Effects of sample preparation (moisture content, particle size) and process parameters (temperature, pressure, flow-rate) were investigated in terms of extraction yield and oil quality at laboratory and pilot scale. High pressure (60 MPa) and temperature (80 °C), low moisture (13%), small particle size (0.85 mm) and a moderate flow rate (9 L/min) resulted in a more efficient extraction than previously studied conditions (35 MPa, 40 °C), with findings of higher yields and comparable levels of EPA (6.08 ± 1.81) and DHA (6.43± 1.60). Pilot scale SFEs confirmed the data and support further investigation of the commercial production of an omega-3 concentrated natural health product from Northern shrimp by-products. Consumers would benefit from the health benefits associated with PUFA's and a unique opportunity would be provided for the economic development of Northern communities.

6.1 Introduction

The literature provides abundant evidence of the benefits of polyunsaturated fatty acids (PUFAs), specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Figure 24) to human health. Numerous studies reinforce the utility of omega-3 (ω-3) PUFAs for the prevention and treatment of a host of debilitating conditions which include heart disease (Siddiqui, Harvey, & Zaloga, 2008; Harris et al., 2008; Tziomalos et al., 2008; Casós et al., 2008; Pivik et al.,2009), cancer (Thiébaut et al., 2009; Serini et al., 2009), diabetes (Tziomalos et al., 2008) and mental illness (Colangelo et al., 2009).



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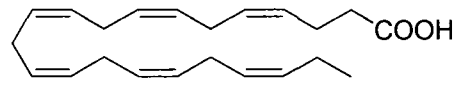

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Figure 24: Polyunsaturated fatty acids: EPA (**20**) and DHA (**21**)

Omega-6 and ω-3 PUFAs are the two major groups of essential fatty acids. They must be obtained in the diet as they are not efficiently synthesized in the body. Fatty acid intake, particularly the ratio of ω-6 to ω-3 PUFAs has changed dramatically over time and varies significantly between populations. Our neolithic ancestors, on a hunter gatherer diet, had intake ratios of approximately 1:1 while current research indicates that this ratio has vastly changed in Western societies to that of approximately 20:1 today; this dietary change has been associated with an increased prevalence of chronic diseases (Simopoulos, 2008). A number of population studies on Greenland Eskimos, the Inuit of Nunavik and more recently, a comparison of three ethnic groups in Quebec (Canada) all report that diets rich ω-3 PUFAs are inversely associated with several risk factors of

cardiovascular disease (CVD) (Dewailly et al., 2001; Dewailly et al., 2003). While omega-6 fatty acids are widespread in North American diets, the primary sources being corn, canola, safflower and sunflower oil, the primary source of ω-3 FAs is fish, which is not as prevalent or as convenient a source in the modern diet. There is no simple solution to changing what people eat and addressing this dietary deficiency. The resistance to altering habits and challenging potential inconvenience has presented for health care professionals a formidable barrier to dietary change. However, recent strategies by industry to increase the levels of ω-3 FAs in the diet (omega-3 enriched orange juice, bagels and eggs) have made significant progress. Research into alternative sources of ω-3 FAs and innovative technologies for their processing is necessary for continued success.

The industrial extraction of fish oils, rich in PUFAs has been carried out for several decades using processes including solvent extraction (hexanes, dichloromethane and acetone) and techniques including molecular distillation, high vacuum distillation and high temperature distillation. Unfortunately, these methods often are problematic for PUFAs due to their structural susceptibility to thermal and oxidative degradation. Alternative technologies which address priorities including oil quality (i.e. intact PUFAs), food safety (i.e. toxic solvent residue) and environmental concerns (i.e. solvent waste) and that are economically feasible are moving to the forefront. The past three decades have witnessed major advances in supercritical fluid technology, with applications in the pharmaceutical, nutraceutical, and food industries. Carbon dioxide (CO_2) is the most common supercritical solvent used for food applications. It is non-toxic, tasteless, odourless, available in high purity (food-grade), and possesses generally

regarded as safe (GRAS) status. Although equipment costs are relatively capital intensive, CO_2 is inexpensive and easily recycled within most system designs. The high extraction efficiencies achieved and the value-added products generated using this technology are increasingly attractive to both industry and consumers. The specific suitability of SFE for fatty acids is high. SC CO_2 readily solvates non-polar compounds and the modest critical conditions (C_T = 31°C, C_P,=7.38 MPa) employed and low oxygen environment are favorable conditions for sensitive PUFA's, resulting in residue-free, high quality extracts. Several reports have been published in the past decade on the extraction and fractionation of PUFAs from fish and fish by-products and most recently from Antarctic krill (Corrêa et al., 2008; Létisse et al., 2006; Létisse & Comeau, 2008; Rubio-Rodríguez et al., 2008).

In Northern Quebec/Labrador the harvesting of shrimp is a major industry and in Newfoundland >150 000 tonnes of shrimp are collected annually. Economic realities have challenged the shrimp industry to investigate the potential of food by-product utilization. Our laboratory recently first completed and reported the complete fatty acid composition of northern shrimp (*Pandalus borealis* Kreyer) harvested from the North Atlantic as well as the analysis of the residue by-products from food processing, consisting of the discarded shrimp heads, tails and shells that account for greater than 50% wt.% of the total catch (Greene et al., 2009). The total fatty acids (TFA), EPA and DHA content of the by-products was reported for soxhlet extraction by acetone and hexane. They were 607 mg/g, 70 mg/g, 67 mg/g and 705 mg/g, 72 mg/g, 70 mg/g respectively. Comparatively, SFE generated a superior extract, with equivalent values of fatty acids, 795 mg/g TFA, 78 mg/g EPA and 80 mg/g DHA, and further, the extractions

were completed in a fraction of the time and without any solvent residue present in the final product.

The goal of the present study was to investigate the best conditions for extraction of Northern shrimp by-products (residue) through SFE. Specific objectives were optimization of SFE conditions including temperature, pressure, flow rate, particle size and moisture content. Measurables included yield, extraction efficiency and fatty acid profile. Pilot scale-up of the optimized research conditions was investigated to aid in determination of the potential for the commercial development of a value-added natural health product.

6.2 Materials and methods

Raw material preparation

Shrimp (*P. borealis*) were supplied by Nunavik BioSciences Inc from a commercial catch off northern Labrador. They were cooked and quick frozen by Newfound Resources Limited. The frozen shrimp were rinsed with water, drained and thawed at room temperature (RT) for 2 h. Heads, shells and eggs (combined referred to as residue) were separated from the flesh and wrapped in cheese cloth prior to being placed in a DeCloet dryer, programmed to ramp from RT (23°C) to 40°C at a rate of 2°C/h. Total drying time was approximately 19 h. The moisture content was measured before and after drying using an Ohaus MB35 halogen moisture balance set at 120°C. The dried shrimp residues were ground with a cuisinart mini-prep plus processor and/or a Wiley mill to the predetermined mesh size. All shrimp were processed just prior to extraction.

Supercritical fluid extraction

Carbon dioxide with a dip tube was purchased from BOC Gases (CO_2 UN1013). At the laboratory level, supercritical CO_2 extractions were performed on an SFT-250 (Supercritical Technologies, DE) equipped with a 100 mL extraction vessel and pre-heater. All samples were collected in 4 oz (125 mL) I-CHEM Septa-Jars and stored under refrigeration prior to quantitative analysis. Pilot plant extractions were conducted using a semi-commercial extractor (mini fractionation system designed and manufactured by Eden Labs LLC., Supercritical Solutions LLC., and Accudyne Systems Inc). The system was equipped with a main extractor vessel (4L, maximum 690 bar, Grayloc Corporation), a system chiller (model #9506) equipped with a polyscience temperature

controller, an air driven fluid pump (Haskell) and high pressure stainless steel pipe-work components (HIP) (Appendix I).

Experimental design

Sample preparation parameters which were modified include particle size and moisture content. For the particle size study, samples were ground and the residue was passed through sieves of varying size. Three particle sizes were investigated, 4.75, 2.00 and 0.85 mm mesh respectively. For moisture studies, the shrimp were removed from the oven at predetermined time (4 h, 6 h 8 h) and moisture was determined. Process parameters which were studied included temperature, pressure and flow-rate. Ten grams of sample were introduced into the extraction vessel and extracted for 90 minutes at either 35 MPa, 40 °C, or 60 MPa, 80°C. Flow rates investigated were 3 L/min, 6 L/min and 9 L/min. The flow rate was monitored by CO_2 usage (total: 540 g) and extract mass was recorded in 10 minute intervals to assess extraction efficiency. For pilot extractions , the extractor was loaded with 400 g of shrimp residue powder and the extractor was set up as follows: Experimental pressure: 600 bar, Experimental temperature: 80°C, Pre-heater temperature: 100°C, Separator 1 pressure: 70 bar, Separator 1 temperature: 70°C, Separator 2 pressure: 35 bar, Separator 2 temperature: 70°C. The CO_2 consumption reached approximately 15.5 to 17 kg/run which translates to a solvent to feed ratio of approximately 35:1.

Chemicals and GC analysis

Methylated fatty acid standards (37 components mix), *cis*-11-vaccenic acid methyl ester, copper II acetate monohydrate 98+ % ACS, and 0.5 N methanolic hydrochloric acid were

purchased from Sigma-Aldrich (Supelco) (city province). Potassium hydroxide, hydrochloric acid and isooctane certified ACS were purchased from Fischer Scientific. Analysis was completed using an Agilent 7890 gas chromatograph coupled with a flame ionization detector (GC-FID) using a Famewax column (30 m x 0.32 mm x 0.25 um) (Restek). A fast efficient method with hydrogen as a carrier gas was used according to method described previously.

6.3 Results and Discussion

As a follow-up to an observation in a previous study conducted by our laboratory, we first investigated the variation in the size of shrimp on the yield of PUFA's. The present study demonstrated that commercial large (>29 mm) shrimp, which often contained eggs, (approx 120/box) have a significantly higher percent of oil than commercial small (<29 mm) shrimp (approx 180+/box) (Figure 25). All direct comparisons throughout the study were conducted by batch.

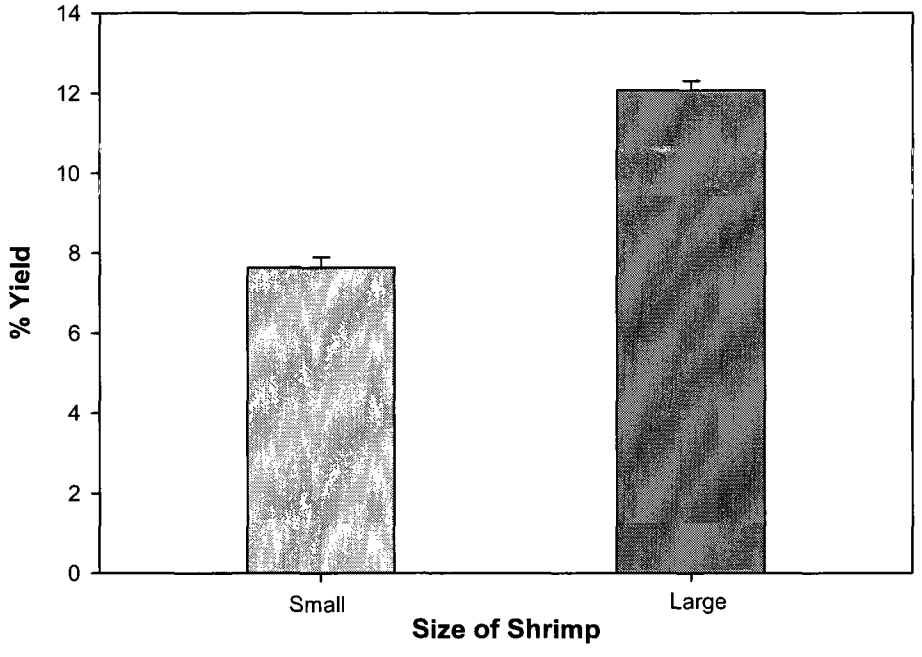

Figure 25: Effect of shrimp size on oil percent yield

Effect of Sample Preparation on SFE

It was found that grinding the dried residue to the smallest particle size (0.85 mm mesh) had the greatest extraction efficiency and overall percent yield. The extract yields for particle sizes of 4.75, 2.0 and 0.85 mm mesh were 6.7%, 7.9% and 12.6% respectively (Figure 26 and 27). This is due in large part to the increased surface area which facilitates extraction kinetics. Smaller particle sizes reduce the distance that the solute travels to reach the bulk CO_2 fluid phase. The initial linear portion of the extraction efficiency curve is indicative of the fast extraction period where the surface oil of particles is being extracted. A subsequent non-linear period which is characterized by increasingly reduced extraction rates is the result of the gradual, concentration dependent process of CO_2 diffusion into the particle and CO_2 + oil diffusion out of the particle.

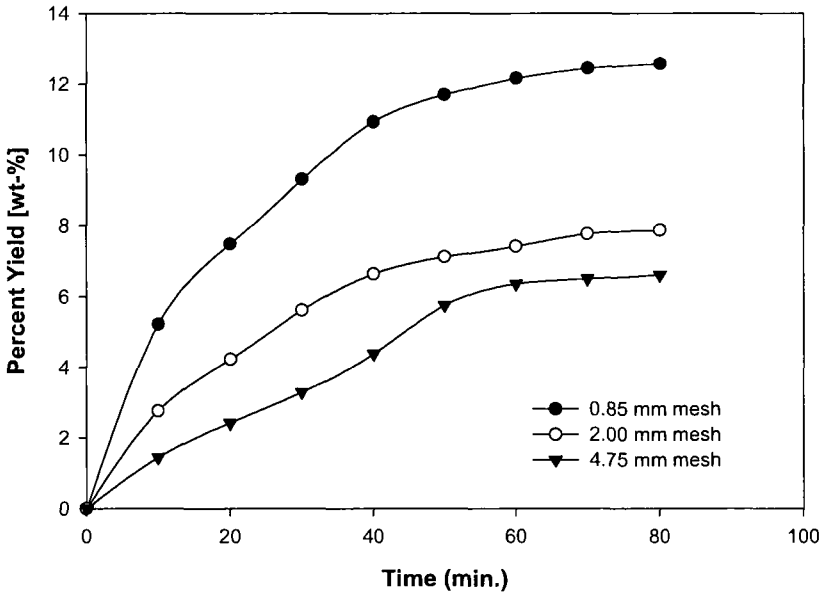

Figure 26: Effect of particle size on extraction efficiency

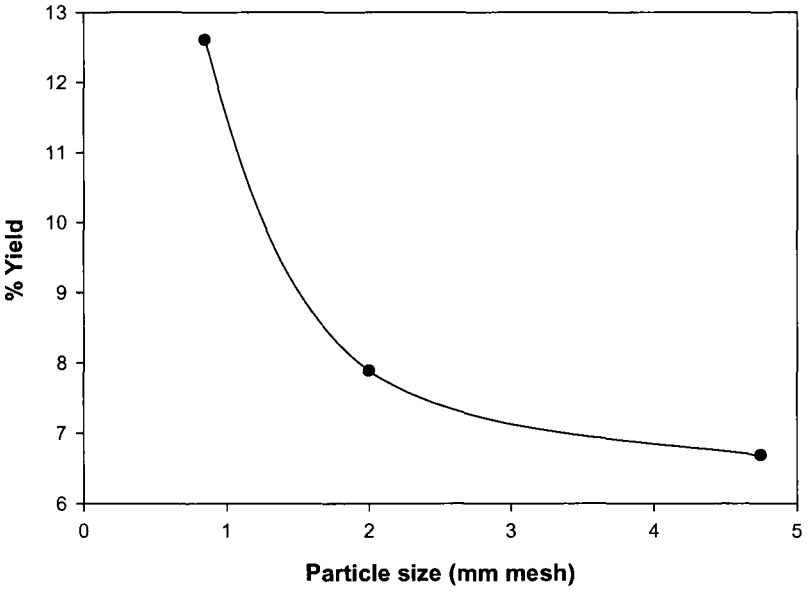

Figure 27: Effect of particle size on extraction efficiency (trend)

For moisture studies, the shrimp were removed from the oven at predetermined times (4 h, 6 h, 8 h). The moisture levels were 39%, 26%, and 13% respectively. Moisture had a detrimental effect on the extraction efficiencies and final yields (Figure 28 and 29). The yields increased with decreasing levels of moisture. At 13% moisture the yield was 8.6%, while at 26% and 39% moisture the percent yields were 7.6 and 6.4% respectively. While water may prove a useful co-solvent for the extraction of polar compounds, the presence of water impedes the extraction of the relatively non-polar PUFAs. In addition to the higher yields reported for the lower moisture contents, this result provides favorable stability implications for the final product.

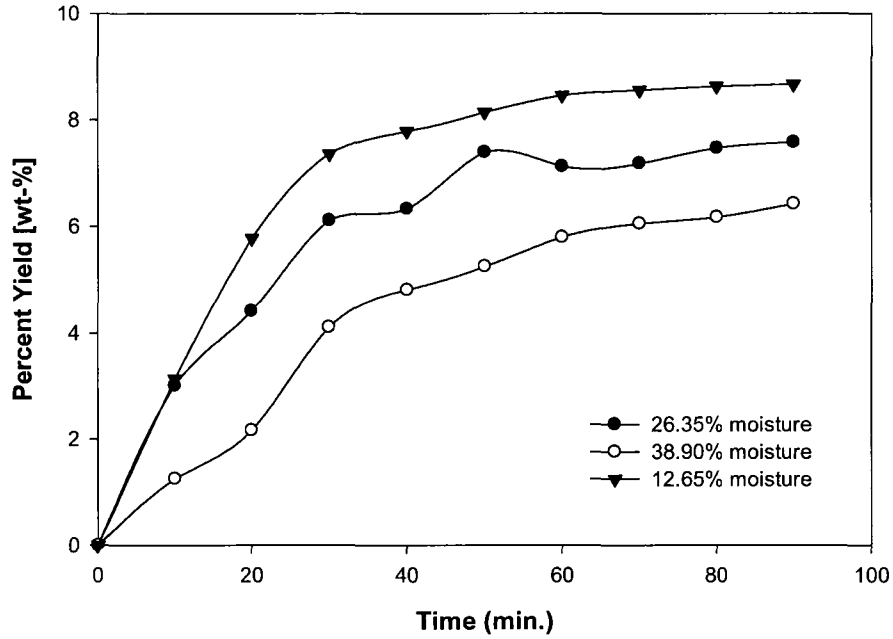

Figure 28: Effect of moisture on extraction efficiency

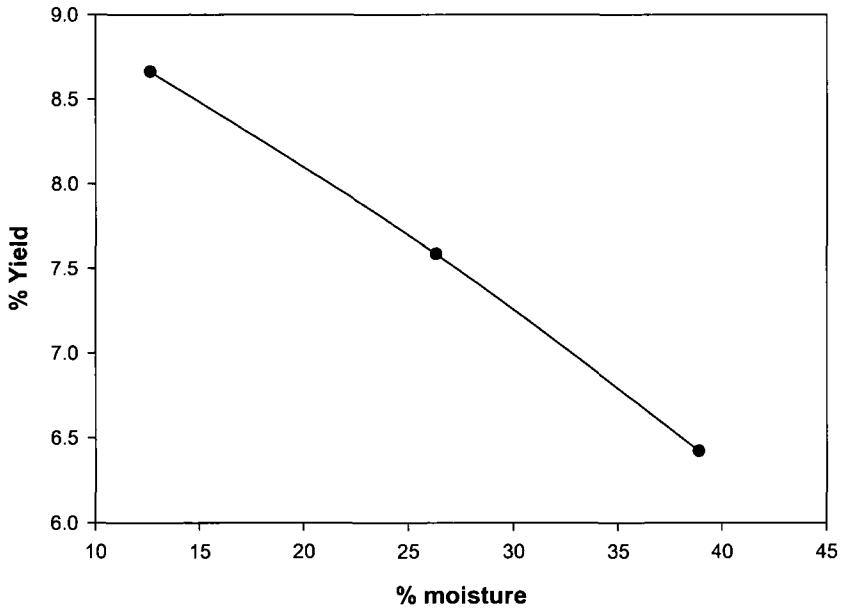

Figure 29: Effect of moisture on extraction efficiency (trend)

Extraction Parameters

Temperature and Pressure

This study investigated a high pressure (60 MPa) and high temperature (80°C) parameter set and compared it to previously studied conditions, 35 MPa and 40°C. The average yield of the moderate pressure (35 MPa) and low temperature (40°C) extraction was 1.29 g ± 0.16/10 g sample (12.89% yield); CV<7.6. The average yield of the high pressure (60 MPa) and high temperature (80 C) was 1.38 g ± 0.08/10 g sample (13.83% yield); CV<3.6 (Figure 30). The flow rate of 3 L/min corresponded to CO_2 usage of approximately 60 grams every 10 minutes. Greater than ninety percent of the yield was obtained in less than 60 min using less than 360 g of CO_2 which corresponds to a solvent to feed ratio of approximately 35:1. At 35 MPa; 40°C and 60 MPa; 80°C, the PUFA rich oil extracted was 129 mg/g and 138 mg/g respectively. The EPA and DHA levels contained in the respective extracts were not significantly different. In the 35 MPa oil the concentrations were 7.26 ± 0.270 and 7.37 ± 0.218 respectively, not significantly different from the concentrations of the oil extracted at 60 MPa which were 7.46 ± 0.180 and 7.39 ± 0.084 respectively.

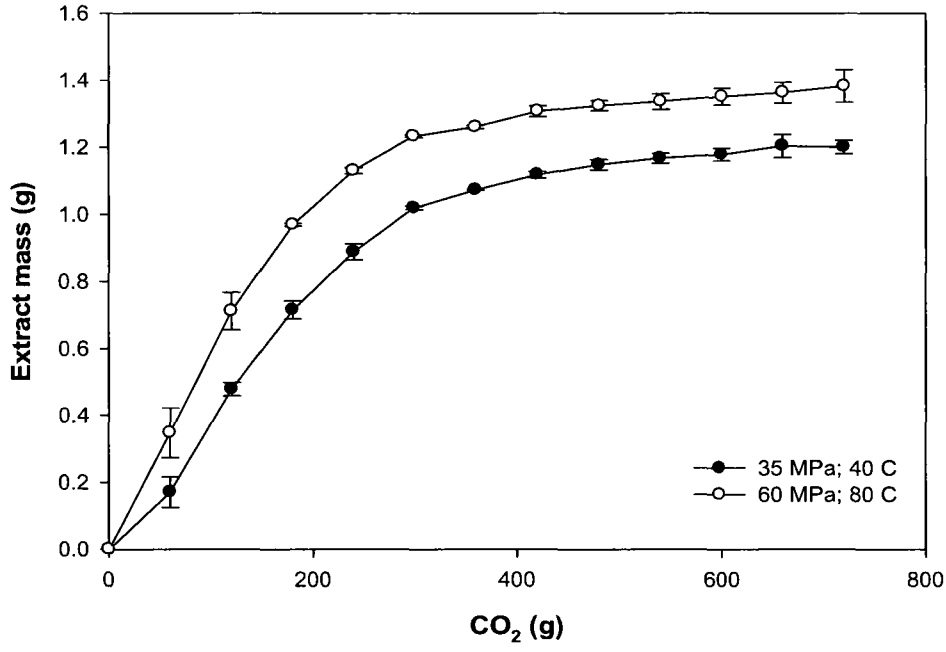

Figure 30: Extraction efficiency: 35 MPa; 40°C (CV<7.6) vs 60 MPa; 80 °C (CV<3.6).

The effect of flow rate on extraction efficiency (Figure 31) used increasing flow rates of 3, 6 and 9 L/min. System constraints made the incorporation of lower or higher flow rates in this study challenging. Results indicate that extraction efficiency is highest at the highest flow rate tested (9 L/min), followed by 6 L/min and 3 L/min respectively. This result is indicative that at 9 L/min the residence time of CO_2 (solvent) with the biomass was near optimal for the rates tested. While the lower flow rates achieved final yields which were not significantly different from the highest flow rate, they took longer. At 9 L/min it took 20 min to achieve 90% yield compared to 35 min and 50 min to achieve 90% yields for 6 and 3 L/min respectively. While flow rates too high may result in wasteful solvent use and increased energy requirements; a result of too short a duration (residence time) of CO_2 in the extractor, too low flow rates may result in saturation of the

CO_2 solvent in oil, which also wastes time and energy; the result of too long a duration (residence time) of CO_2 in the extractor. While flow rate is a key factor to consider for optimal extraction, another factor to consider is flow direction of CO_2, which was not investigated in this study.

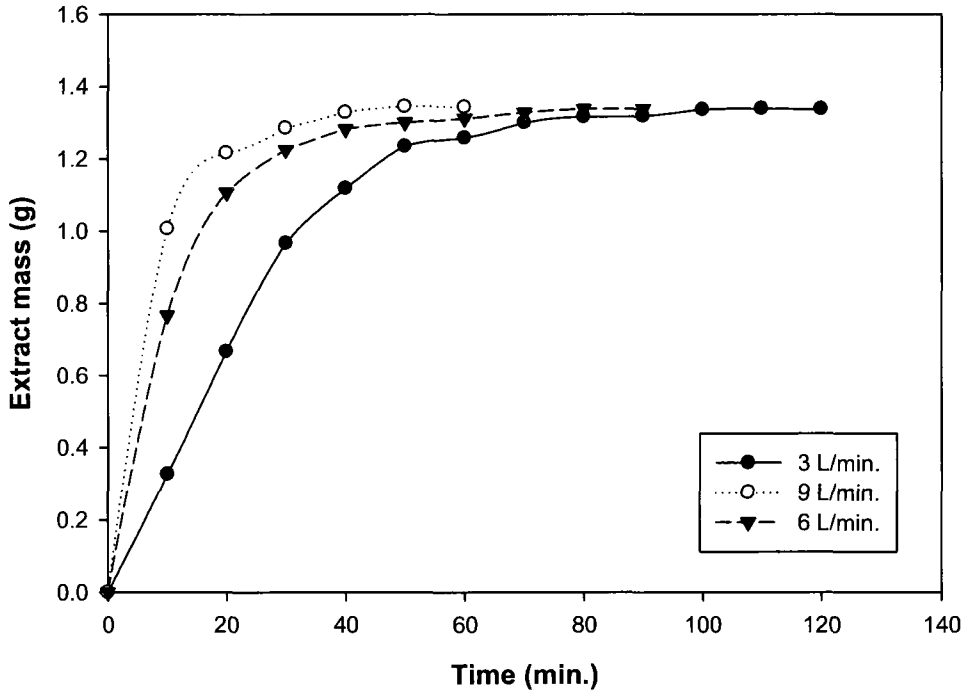

Figure 31: Effect of flow rate on extraction efficiency

Pilot scale (4L) extraction at 60 MPa and 80°C resulted in an average extraction yield of 10.83% vs the 13.83% achieved at the research scale. The PUFA profile (EPA and DHA) was 8.03 ± 0.04 and 5.59 ±0.08 respectively and were not significantly different from the laboratory scale. Table 12 shows the complete fatty acid profile of the two experimental (100 mL extraction vessel) conditions, 35 MPa; 40°C, and 65 MPa; 80°C, as well as the pilot (4L extraction vessel) extraction at 65 MPa; 80°C.

Table 12: Fatty acid profiles of Northern Shrimp residue SFE extracts

	Fatty acid	35 MPa; 40°C	60 MPa; 40°C	60 MPa; 40°C (Pilot)
1	Myristic C14:0	4.32±0.03	4.01±0.02	3.93±0.07
2	Palmitic C16:0	7.87±0.06	7.16±0.22	8.06±0.10
3	Palmitoleic C16:1	9.41±0.11	8.87±0.38	12.19±0.10
4	Stearic C18:0	1.41±0.01	1.39±0.13	1.69±0.02
5	Oleic C18:1n9c	10.35±0.08	10.06±0.50	9.32±0.06
6	Vaccenic C18:1	2.39±0.02	2.52±0.47	3.73±0.04
7	Linoleic C18:2n6c	0.83±0.00	0.92±0.21	1.28±0.01
8	Gamma-linolenic C18:3n6	0.34±0.01	0.23±0.12	0.26±0.01
9	Linolenic C18:3n3	0.51±0.02	0.46±0.01	0.44±0.01
10	Eicosenoic C20:1	8.55±0.22	8.23±0.31	6.61±0.07
11	Eicosadienoic C20:2	0.26±0.01	0.34±0.14	0.54±0.01
12	Arachidonic C20:4n6	0.21±0.03	0.27±0.04	0.42±0.03
13	**EPA C20:5n3**	**7.80±0.06**	**6.08±1.81**	**6.30±0.06**
14	Cetoleic C22:1n11	10.61±0.37	10.74±0.85	8.03±0.04
15	Erucic C22:1n9	5.69±0.17	4.15±1.73	3.88±0.04
16	DPA C22:5	0.65±0.01	0.76±0.20	0.80±0.02
17	**DHA C22:6n3**	**7.97±0.07**	**6.43±1.60**	**5.59±0.08**
18	Nervonic C24:1	0.30±0.01	0.32±0.07	0.25±0.01
	Total	**79.48±0.77**	**72.95±2.23**	**73.31±0.41**

Comparable sources of omega-3 oils include cold water fish such as salmon and mackerel. A recent source which has been identified with exceptionally high levels of EPA and DHA is the marine crustacean krill (*Euphausia superba*); 17.4% and 12.4% respectively (Tou et al., 2007). SFE of krill has been reported in the literature and research is underway for commercial development of this biomass (Harland, 2006) . Our study however, is focused on the utilization of a formally discarded industry by-product as the source of PUFA's. Commercial exploitation of Northern shrimp by-products using SFE represents an opportunity for Northern communities to generate a value-added rich source of PUFA's using a technology which is rapidly garnering support. We

recommend moving toward full scale production and completing clinical trials for a variety of outcomes (ie. eczyma, reduced cancer risk etc..).

Chapter 7

7.0 General Discussion

7.1. Claims to Originality

This thesis documents new and optimized methods for the SFE of bioactive compounds from natural products and demonstrates the wide applicability of the methods to different biosynthetic classes of compounds. In the scope (Chapter 2-5 and Appendix I-V) of this work I have successfully reported the SFE of several distinct (based on biosynthetic approach) classes of bioactive compounds (pentacyclic triterpenoids, irregular monoterpenes, alkaloids, polyunsaturated fatty acids) from different natural matrices (leaf, root, bark, seed, shell, animal tissues) and conducted subsequent qualitative and quantitative analysis (GC-FID, GC-MS, HPLC-UV (DAD), LC-MS) to better understand extraction selectivity. Critical to the biological application of these studies, the characterized extracts were subjected to appropriate bioassays for evaluation of bioactivity.

With respect to the specific chapter studies, this is the first report of the SFE of *Souroubea sympetala* (Chapter 2) and, based on review of the literature, the first report showing the selectivity of SC CO_2 for triterpenoids, and specifically, betulinic acid. When compared with conventional methods of extraction, the SC CO_2 extract contained the highest concentration of BA and demonstrated the highest anxiolytic activity in rodent behavioral assays

In Chapter 3, the crude oleoresin prepared from the *Chrysanthemum cinerariifoliuim,* valued for its potent insecticidal properties, is an impure product, a result of solvent extraction techniques used in developing countries. When the oleoresin

was extracted using SFE the resultant extract contained no detectable hexane. To our knowledge, this is the first study addressing this concern of residual solvent contamination, a key issue in organic agriculture. This report also includes the most comprehensive SFE investigation on the effects of pressure on extraction efficiency and individual pyrethrin profiles, while demonstrating the comparative efficacy of SC CO_2 extracts in insecticidal bioassays.

Chapter 4 builds on insecticidal studies conducted by Ian Scott and Helen Jensen in the Arnason laboratory on solvent extracted *P. nigrum* extracts. A novel *P. nigrum* extract was prepared using SFE and the biological activity confirmed in spray trials using a Potter's Tower on the Colorado Potato Beetle, an agricultural pest that has developed resistance to all major classes of insecticide chemistry. Following an extensive systematic study of SFE parameters and their respective effects on yield and piperamide profile, this thesis reports that the piperine concentration resulting from SFE at 35 MPa/40°C is twice that obtained when using solvent (hexane) extraction.

Chapter 5 and 6 report the SFE of northern shrimp (*Pandalus borealis)* by-products, a promising and highly concentrated source of PUFA's, specifically EPA and DHA. This research provides rationale to replace conventional solvent extraction with SFE and presents for the first time bench-top and semi-commercial pilot plant extraction fatty acid profiles of an optimized parameter set.

7.2. Comparison to the scientific literature

In general the field of SFE has made a significant impact on the science community. To date, there have been 33 articles related to SFE published in the journal *Science*. The field has expanded exponentially in the past twenty-five years, and most notably in the last 7 years with respect to natural product research (Figure 32).

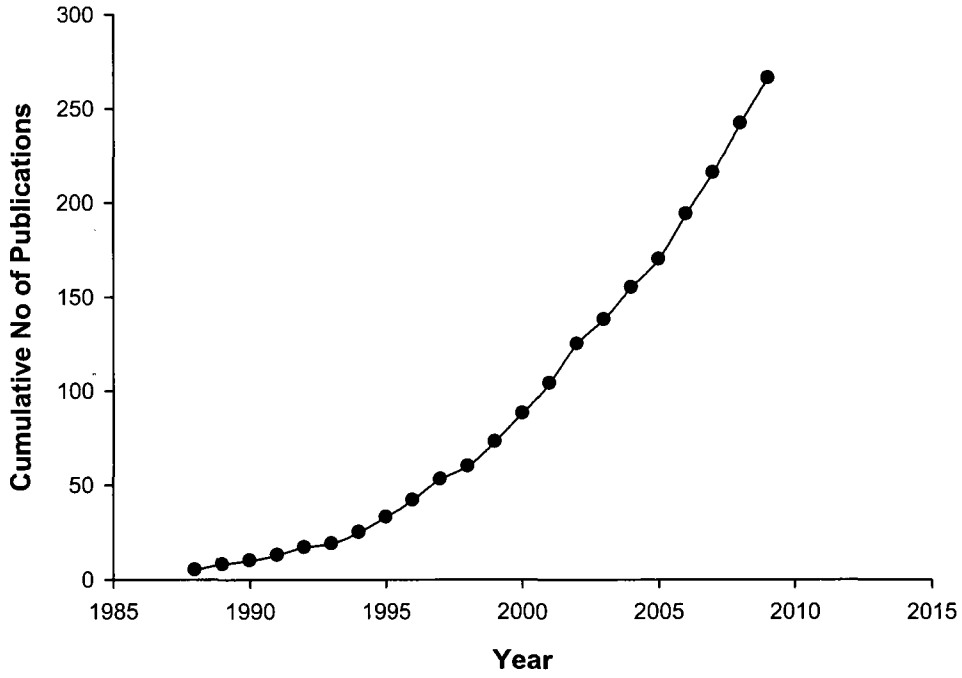

Figure 32: Cumulative number of publications that appeared in the literature according to SCOPUS database search (supercritical and natural products) between 1988 and 2009.

The results which are compiled in this thesis and that have been submitted for publication in the peer reviewed literature have been incorporated among several relevant studies of the SFE of related products (Table 13).

Table 13: Review of the literature associated with the SFE of natural products studied and their respective phytochemicals /target bioactive(s). Results obtained from Chapters 2-5 have been incorporated among other literature reports.

Natural product	Source	Target phytochemical or bioactive	Biosynthetic class of compound	Focus Bioactivity	SFE Conditions	Other	References
Souroubea sympetala Sin Susto **(Chapter 2)**	Leaves	Betulinic acid (BA)	Triterpenoids	*Anxiolytic*	60 MPa; 80°C	***Selective for BA***	(Mullally et al., 2008)* (Kramp et al., (submitted)
Chrysanthemum cinerariifoliuim Pyrethrum **(Chapter 3)**	Flower	Pyrethrin I,II, II Cinerin I, II, III	Irregular monoterpene	*Insecticidal*	7,10, 25 MPa; 20-40°C SCC 30 MPa, 100°C 1200, 2000, 2800, 3600 psi, 100°C 6-10 MPa		(Kiriamiti et al., 2003); (Wenclawiak & Otterbach, 2000); (Wenclawiak et al., 1997); (Pan et al., 1995); (Bunzenberger et al., 1984)
	Oleoresin	Pyrethrin I,II, II Cinerin I, II, III		*Insecticidal*	8MPa, 29°C; 8MPa, 29°C 8, 10, 12, 14 16, 18. 20. 25, 30 and 35 Mpa, 40-60 °C	***No hexane detected***	(Kiriamiti et al., 2003); (Kramp et al., submitted)
Piper nigrum Black pepper **(Chapter 4)**	Fruit	Essential oil	Alkaloid	*Nutraceutical*	90,100,150-300 bar; 30-50 C	***SC CO_2 extracts 2X more piperine than hexane***	(Topal et al., 2008); (Izadifar et al., 2006); (Perakis et al., 2005); (Catchpole et al., 2003); (Ferreira & Meireles, 2002); (Ferreira et al., 1999); (Sovová et al., 1995); (Braumann et al., 1995);
		Piperine		*Nutraceutical*	300 bar, (313 K)		(Catchpole et al., 2003)
		Piperine		*Insecticidal*	10-60 MPa, 40-100°C		(Kramp et al., submitted)
Pandalus borealis Northern Shrimp **(Chapter 5)**	By-products	EPA; DHA	PUFA's	*Nutraceutical*	15-60 MPa; 40-100°C	***Research and pilot scale***	(Treyvaud Amiguet et al.,submitted) (Kramp et al., submitted)

Chapter 2 builds on our laboratory's earlier work on the genus *Souroubea* and provides scientific evidence for the ethnobotanical uses of this plant (J. T. Arnason, 2003; Bourbonnais-Spear et al., 2007). The first thorough phytochemical investigation of *Souroubea* (extraction, isolation, characterization) was presented in the thesis of Eva Puniani (2004). Bioassay guided fractionation revealed anti-anxiety effects for the triterpenoid rich fraction. The bioactivity demonstrated in the triterpene fraction was not surprising. Triterpenoid research in the past decade has resulted in the discovery of a myriad of pharmacological effects for this family of compounds (Jäger et al., 2009). Bioassay guided fractionation of plant extracts consistently report the co-occurence of 3 major pentacyclic triterpene betulinic acid, oleanolic acid and ursolic acid. Evidence has accumulated for use of betulinic acid as a promising candidate for melanoma, HIV, malaria, and most recently anxiety (Galgon, et al., 2005; Cichewicz & Kouzi, 2004; De Sá et al., 2009; Fulda & Kroemer, *in press.*) Our recent publication has provided an advanced analytical method (LC-MS) for the quantification of the main triterpenoid markers (Mullally & Kramp et al., 2008).

SFE has been observed for other well known anxiolytic botanicals; the extraction of flavonoids (20 MPa 40-80 C) from skullcap and THC and CBD from cannabis (Bergeron et al., 2005; Russo, 2003). Chapter 2 represents the first publication in the literature on the SFE of anxiolytic *Souroubea sympetela,* the first demonstration of the selective capacity of SFE for betulinic acid and a clear indication of the anxiolytic effect in the extract in standardized tests for anxiety with positive control diazepam (Table 13). This collaborative body of work presents part of a series of several papers introducing *Sin Susto* for the first time to the scientific literature.

As a result of the extensive use of pyrethrum insecticides for well over a century the accumulation of data has been substantial (Krief, 2009). Since the mid-nineties, there have been a number of studies on the SFE of pyrethrins from *Chrysanthemum cinerariifoliuim* flowers (Table 13). The data presented in Chapter 3 builds on a single preliminary study by Kiriamit et al. which investigated the SFE of pyrethrum oleoresin (Table 13). As a result of the relevance of the oleoresin to the development of pyrethrum formulations, our thorough investigation of oleoresin extraction by sc CO_2, the resulting pyrethrin profiles and subsequent insecticidal activity of the extracts provides data which warrants additional fractionation and larger scale studies. Notable to the organic sector was the reduction of solvent contamination in the oleoresin. While not directly related to the work conducted in this thesis, the increase in prevalence SC CO_2 being used for the extraction of pyrethrins for determination of pyrethrin and pyrethroid residue levels in crops, food, and environmental samples was notable. The rationale for this is likely attributed to the efficiency of the process; no solvent removal step necessary, more focused extracts, increased selectivity, and ability to directly couple SFE to solid phase extraction (SPE) cartridges for clean-up prior to analysis. We have also observed this advantage where we compare the extraction of DDT and its metabolites from ginseng using ASE and SFE-SPE.

The investigation of *P. nigrum* (Chapter 4) was based primarily on work previously conducted by Ian Scott. Scott's research demonstrated the effectiveness of the botanical insecticide (*p.nigrum*) against a wide range of economically relevant insect pests (Scott et al., 2004; Scott et al., 2003; Scott et al., 2005; Scott et al., 2007). Jensen (2006b) reported an exceptional synergism ratio for black pepper combined with

pyrethrum. Their work, combined, has made a considerable contribution to the investigation of botanical alternatives to synthetic insecticides. The pepper extracts used in the aforementioned studies were prepared with solvents such as ethyl acetate, whereas we report the use of sc CO_2. Other botanical insecticides have been investigated using sc CO_2, however they have been primarily focused on the essential oil portion (Hu et al., 2008; Pavela et al., 2008; Pavela et al., 2009). Our comprehensive (10-60 MPa; 40-100°C) systematic inquiry of piperamide extraction by SFE compliments previous reports of the SFE of *P.nigrum*, the vast majority of which were consistently conducted at lower pressures and remained focused on the essential oil fraction. (Table 13) Two stage extractions of *P. nigrum* by SFE have also been observed; the aromatic essential oil which is extracted at low pressures was fractionated from the pungent alkaloid oleoresin which was extracted at high pressures (Table 13). The combined SFE, quantification of piperamides, and evaluation of the insecticidal efficacy of sc CO_2 extracts of *P. nigrum* has not been reported elsewhere in the peer reviewed literature (Table 13). Our results affirm previous literature that has correlated the concentration of piperamides in the extract with insecticidal efficacy (Scott et al., 2005a) and demonstrate the suitability of SFE for piperamide extraction compared to conventional solvent methods.

Omega-3 fatty acids are prominent in the literature, their health benefits have been well-documented (Griffin, 2008; Risérus, Willett, & Hu, 2009; Serini et al., 2009; Siddiqui et al., 2008) and several population studies show evidence supporting the benefits associated with their long-term consumption (Dewailly et al., 2001; Dewailly et al., 2003). Fatty acids represent the natural product most thoroughly studied by SFE and research suggests that SFE is now becoming an industry standard, gradually replacing

conventional solvent methods of lipid extraction (Sahena et al., 2009). Recently the marine crustacean krill (*Euphausia superba*) has made a significant market presence with reported high levels of EPA and DHA and significant biomass with which to draw from (Atkinson et al., in press; Tou et al., 2007; Watkins, 2007). Industrial research is currently exploring the optimization of the SFE process with respect to krill (Harland, 2006; Yamaguchi et al., 1986). The SFE of fish by-products from industry as a source of high-quality, clean and concentrated PUFAs has only recently been reported (Davarnejad et al., 2008; Rubio-Rodríguez et al., 2008; Létisse et al., 2006). Chapters 5 and 6 present two publications which introduce the SFE of North Atlantic shrimp *(Pandalus borealis)* by-products to the literature (Table 13). The fatty acid extracts generated revealed comparable PUFA levels to previously studied sources (Table 13). The direct comparison we used (SFE vs hexane soxhlet) demonstrated that SFE was superior to conventional solvent methods for fatty acid extraction at the research and pilot scale in terms of yield, fatty acid profile, efficiency and quality of extract.

While significant work remains to accurately delineate the experimental conditions for the extraction and selection of target bioactives, this work compliments existing data available in the literature and has addressed several gaps in the SFE of natural products important to the advanced research conducted by our laboratory group and with our collaborators.

7.3. Future directions and concluding remarks

Future directions for the investigation of the traditional medicine (*Souroubea sympetala*) (Chapter 2) include study of its mode of action and human clinical studies of well characterized extracts. The bioactivity which has been demonstrated reinforces the role of ethnobotany in the development of alternative forms of therapy in health. With greater than 80% of the population in several developing counties depending on traditional medicine for primary health care and 70-80% of the population in developed countries having used some form of alternative therapy, the evidence for an increased emphasis on efficacy and safety is clear (WHO, 2009). Ethnobotanical studies are of paramount importance to preserve traditional knowledge and to develop a deeper understanding of uses and preparations; expanding on the significant contributions which have informally been made to science. Concerted efforts of conservation, benefit sharing and sustainable practices are necessary to ensure the growth of existing relationships and to foster new ones based on respect. Our lab is currently investigating several natural product leads based on this approach which may present new collaborations, benefit sharing and possible considerations for natural health product development.

With respect to the insecticidal section of this thesis (Chapter 3, 4), future work on pyrethrum involves completion of an ongoing study which has successfully isolated the six known actives (Pyrethrins I and Pyrethrins II) and four additional compounds from oleoresin using a simple and efficient approach of SFE followed by preparative scale RP-HPLC coupled to an online fraction collector. Structure elucidation of the unknowns and confirmation of the major markers is being accomplished by spectroscopic analysis (NMR, IR, MS). Subsequent insecticidal assays are planned in collaboration with

Agriculture Canada to determine the respective insecticidal activity of individual compounds compared to characterized SFE and solvent extracts respectively. Advanced fractionation (SC chromatography) and insecticidal studies on previously identified promising natural product synergists (Bernard et al., 1989) are planned.

Value added utilization of waste material, specifically shrimp by-products (Chapters 5, 6), represents a trend toward more ecological and economic strategies in industry. By-products of the food industry are often excellent sources of biologically active materials. Lycopene from tomato waste has been promoted as an antioxidant and anticancer natural product (Nobre et al., 2009; Naviglio et al., 2008); resveratrol commonly found in wine making by-products (skins and seeds) is held in high regard due to its cardioprotective and anticancer properties (Casas et al., 2010).) The residue free "clean" extracts generated by SFE are well suited for natural product, food, pharmaceutical, nutraceutical and natural health product applications. As a result of the favourable results achieved in Chapter 6 pilot commercial scale studies are underway in order to generate an omega-3 concentrated extract for clinical work with potential for natural health product development.

Additional ongoing research projects have generated positive preliminary results, and will advanced toward publication. These include work on evening primrose (*Oenothera biennis*), purple coneflower (*Echinacea purpurea*) and Northern prickly ash (*Zanthoxylum americanum*). Preliminary results/chromatograms are presented in Appendix III and IV.

Throughout this thesis I have utilized analytical tools for characterization (qualitative and quantitative) of variable extracts (Chapter 2-6; Appendix I-IV) and have conducted

both *in-vitro* and *in-vivo* studies in order to better understand efficacy (Chapter 2-6). Our focused approach on SFE of natural products fits well within this mandate due to the GRAS status of CO_2, the residue free extracts, and the fractionation capability which is important to gaining a better understanding of the effective mechanisms of action. Future work will use SFE to generate clean, safe extracts to characterize specific bioactive (marker) compounds and to determine efficacy and any possible side-effects. These steps are necessary to ensure the growth and confidence in the burgeoning market.

The natural products presented in this thesis and the literature represents a diverse and biologically active collection of compounds. Even within a particular class of natural products there are wide ranges of molecular weight, varying polarities and vastly different biological effects. The diversity found across and within respective classes of compounds requires development of a selection of methods to effectively extract, analyze and determine biological efficacy. SFE from natural sources remains relatively unexplored. Several theoretical and technical obstacles remain which highlight the substantial work remaining to develop protocols based on formal experiments. A preliminary strategy for SFE of natural products which I developed is outlined in Appendix V. With this knowledge we can expect to gain a more in-depth understanding of the often complex mechanisms involved and more effectively incorporate SFE into advanced natural product investigations. The great potential of natural products derived from plant, marine or microbial sources remains largerly unexplored. With increases in understanding and recent advances in strategies for extraction, analysis and determination of biological activity we can expect rapid progress to be made in the near future.

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Appendix I: Supercritical Fluid Extraction (SFE)

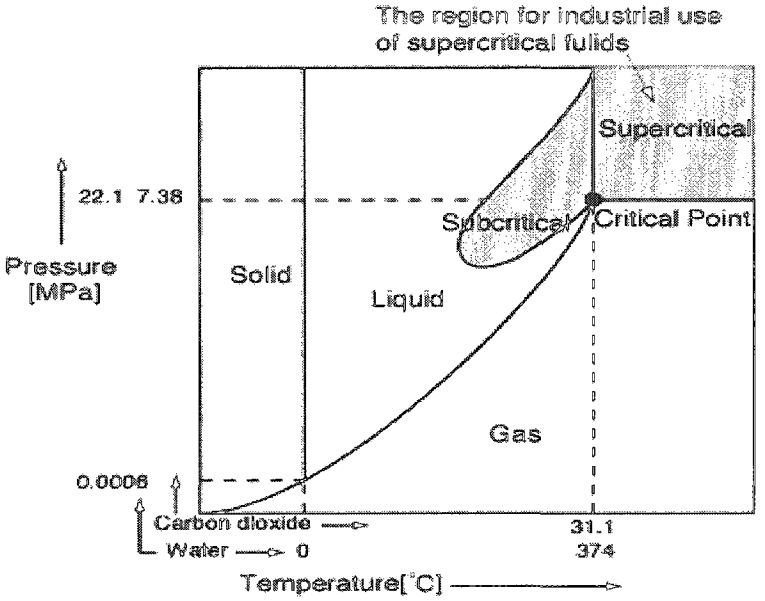


Figure A1: Phase diagram for CO_2

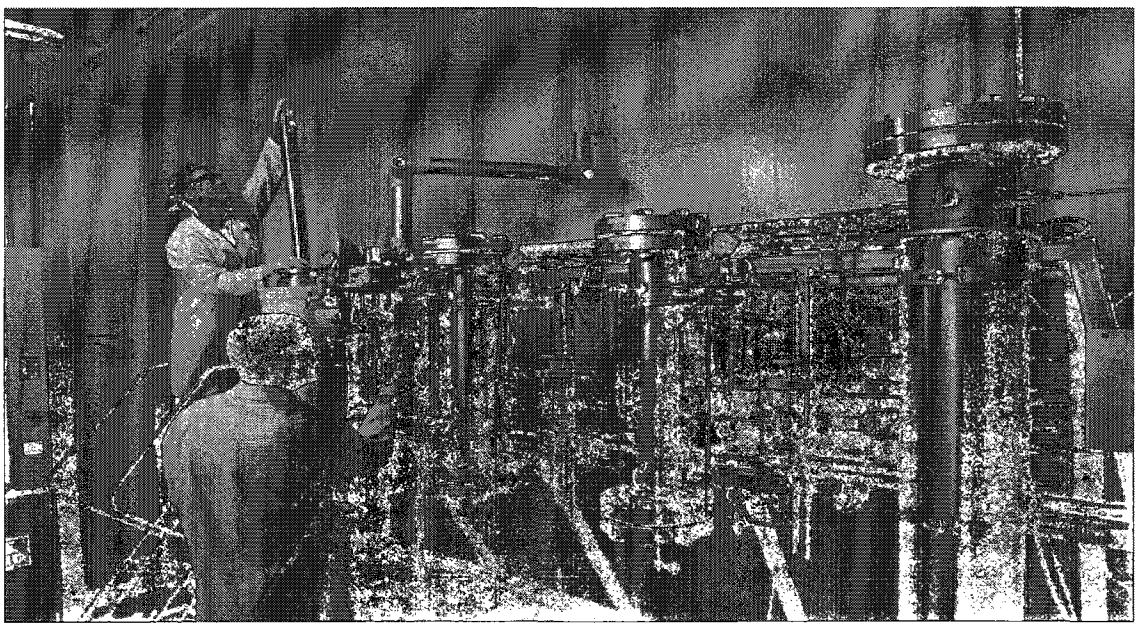
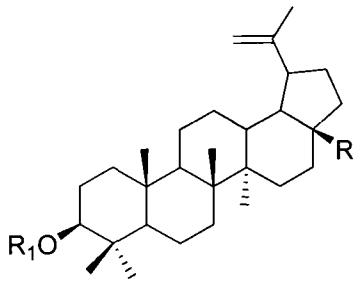
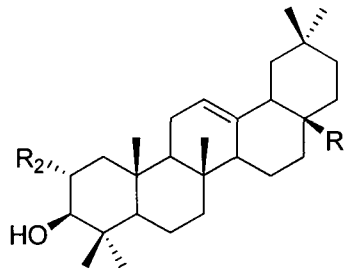


Figure A2: Pilot (4 L) scale SFE

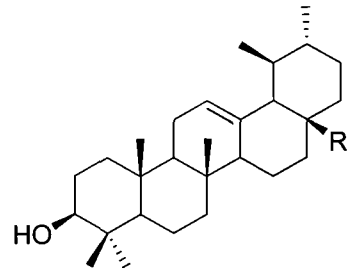
Appendix II: Sin susto



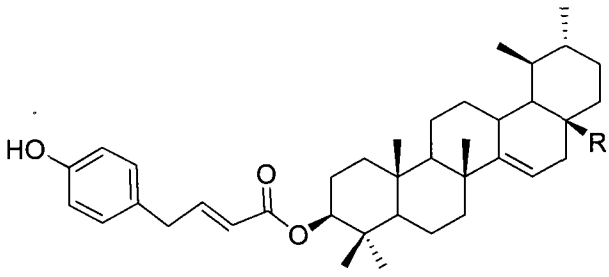
1. R = CO_2H; R_1 = H
2. R = CO_2CH_3; R_1 = H
4. R = CO_2H; R_1=$COCH_2C(CH_3)_2CO_2H$



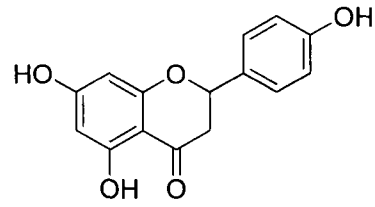
3. R = CO_2H
6. R = CH_3
12. R = CO_2CH_3; R_2 = OH



5. R = CH_3
9. R = CO_2CH_3; R_2 = H
11. R = CO_2CH_3; R_2 = OH



7



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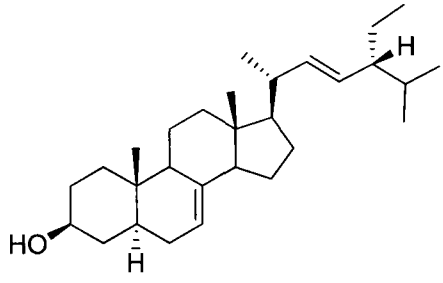

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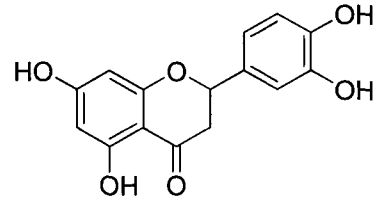

10

Figure A3: EtOAc soluble phytochemical fraction of *Souroubea sympetala* leaves 1. Betulinic acid, 2. Methyl betulinate, 3. Oleanolic acid, 4. 2,2-dimethyl succinic anhydride, 5. α-amyrin, 6. β-amyrin, 7. Taraxenyl trans-4-hydroxy-cinnamate, 8. Naringinin, 9. Methyl ursolate, 10. Eriodictyol, 11. Methyl-2- α-hydroxyursolate, 12. Methyl-2- α-hydroxymaslinate
*numbering does not reflect thesis (Puniani et al., manuscript in press)

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Characterization and Quantification of Triterpenes in the Neotropical Medicinal Plant *Souroubea sympetala* (Marcgraviaceae) by HPLC-APCI-MS

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A rapid, two-solvent, HPLC-APCI-MS method was developed to identify and quantify four pentacyclic triterpenes (betulinic acid, ursolic acid, α-amyrin and β-amyrin) in extracts of the neotropical medicinal plant *Souroubea sympetala*. Analysis of various plant organs, wood, bark, leaves, immature fruit and flowers, indicated that the phytochemical distribution and quantity of marker triterpenes varies across the plant, with betulinic acid and ursolic acid the major constituents in the bark, wood, fruit and flowers and the amyrins the major constituents in the leaves.

Keywords: *Souroubea sympetala*, Marcgraviaceae, pentacyclic triterpenes, anxiolysis.

The Marcgraviaceae is a neotropical plant family, indigenous to tropical America consisting of 5 genera and 125 species [1a] for which the phytochemistry is not well described. The genus *Souroubea* (Marcgraviaceae) was identified during a natural product discovery study in Costa Rica (C.R.) as an anxiolytic. *S. guianensis* may also be used to treat *susto* (fright) [1b]. *Susto* is a condition of folk etiology known throughout Latin America and understood to occur following a sudden frightening event that leads to the loss of "soul" or essence. The physiological characteristics of *susto* include diarrhea, loss of appetite, and restlessness [1c]. For diagnostic purposes, *susto* is considered a "culture-bound syndrome" linked to both anxiety and depression [1d,1e]. Preliminary *in vivo* evidence indicates that *Souroubea* sp. significantly reduces anxiety in a rodent behavioural assay of anxiety. Treatment of rats with 1 mg/kg of an ethanolic extract of *Souroubea* sp. exerted significant anxiolysis in the elevated plus maze (EPM), a standard behavioural assay of anxiety [f]. Further, the ethanolic extract of *S. gilgii* inhibits rat gamma amino butyric acid-transaminase (GABA-T) activity, (IC_{50} = 0.6 mg/mL) [2a], a major pharmacological target in the treatment of epilepsy and anxiety [2 b,c].

Currently there are no methods available for the phytochemical analysis of Marcgraviaceae. From *S. sympetala* we have isolated a variety of known triterpenes and flavonoids [1f]. The anxiolytic activity is associated with a terpene fraction containing four pentacyclic triterpenes, betulinic acid (BA), ursolic acid (UA), α-amyrin (α-A) and β-amyrin (β-A), and a method for their analysis in extracts of this plant is described here.

HPLC method development

Preliminary HPLC study of BA in *S. sympetala* extracts (Figure 1A) was initiated using diode array detection (DAD) (UV, 205 nm). DAD sensitivity was low due to poor light absorbance. Detection was enhanced with the use of MS detection versus DAD (Figure 1B). The detection method was optimized for all four compounds by selected ion mode (SIM). The gradient was optimized to increase separation. Separation was complicated by the fact that BA and UA have the same molar mass and α-A and β-A are structural isomers. Initial isocractic conditions used caused co-elution of BA and UA. A 10 min gradient followed by 8 min isocratic at 100% acetonitrile eliminated co-elution and resulted in two distinct peaks.

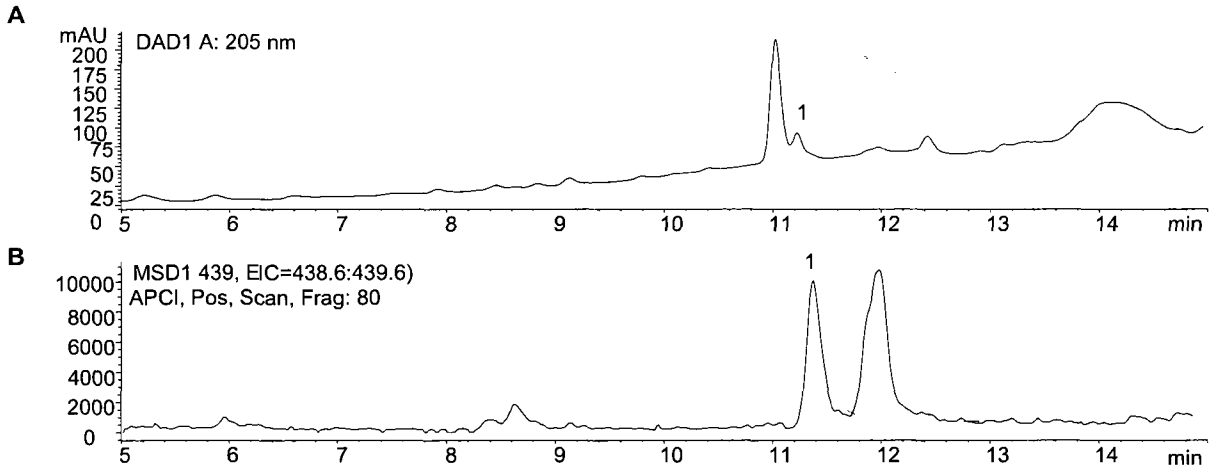

Figure 1: HPLC chromatogram of betulinic acid (1) detected via diode array detection (UV = 205 nm) in an ethanolic extract of *S. sympetala* (A), versus the same extract detected via mass spectrometry detection (B).

Chromatographic profiles of *S. sympetala* extracts and compound identification: The chromatograms (Figure 2) show the presence of the four triterpenes in all plant parts, with BA and UA detected at highest levels in the bark and the wood and the amyrins detected at highest levels in the leaves. There is a differential distribution of amyrins in the leaves with α-A most prevalent in the old leaves and β-A the major amyrin detected in the young leaves. Lower levels of triterpenoids were detected in the fruit and flowers, in both the major peak detected was BA.

Quantification of triterpenoids in *S. sympetala* extracts: Extraction yields from the ASE extracts (Table 1) were highest in the flowers and lowest in the immature fruit. Quantification of the phytochemicals across the plant parts (Figure 3) showed more BA in the bark,

Figure 2 presents the quantification of the phytochemicals across the plant parts, calculated as μg/mg extract. Although not statistically significant, there is a trend of considerably more BA in the bark, with a mean value of 85.7 ± 30.3 μg/mg extract, than in the wood, 46.6 ± 11.7 μg/mg. There is significantly more BA in the bark versus the old leaves, young leaves, flowers and fruit ($p < 0.05$). There are higher levels of amyrins in the leaves than in the other plant organs. α-A is the major triterpene present in the old leaves, with a mean value of 11.5 ± 7.7 μg/mg, 3.3 times that greater than that measured in the young leaves, 3.4 ± 3.1 μg/mg. There is more α-A in the old leaves than in the wood, bark, flowers and fruit, although a significant difference exists only between the α-A content of the old leaves, 11.5 ± 7.7 μg/mg, and α-A levels of the fruit, 0.04 ± 0.01 μg/mg ($p < 0.05$). Finally, β-A is the major triterpene in the young leaves, with a mean value of 12.2 ± 6.4 μg/mg versus 6.1 ± 3.0 μg/mg in the old leaves. There is significantly more β-A present in the young leaves than in the wood, flowers and fruit ($p < 0.05$). β-A levels in the old leaves are lower than in the young leaves, but significantly greater than in the flowers or fruit ($p < 0.05$).

Table 1: Percent yield for ASE extraction of each of the *S. sympetala* plant organs investigated.

Plant Organ	% Yield
Wood	3.4
Bark	5.0
Old Leaves	16.2
Young Leaves	15.5
Flowers	26.5
Fruit	0.3

This report provides the first method for identification of *S. sympetala* plant organs by HPLC-APCI-MS. The method is straightforward and allows for detection and quantification of the four marker triterpenes of *S. sympetala* from a biologically active fraction. The extraction method is also rapid and simple, with the added value that ASE extraction methods consume less solvent and are less labor intensive than conventional extraction approaches [2d]. The HPLC-APCI-MS method is similarly rapid (28 mins) and employs a two solvent system that effectively accomplishes the challenging separation of a pair of molecules with identical molecular mass (BA and UA) and a pair of isomers (α-A and β-A).

While other methods to separate triterpenes exist [3a,3b], our method is, to the best of our knowledge, the first to separate this particular combination of molecules. Further,

we have characterized the phytochemical profile of *S. sympetala* across the plant organs. From this characterization it is clear that the phytochemistry varies across the plant, with more BA and UA present in the wood and the bark versus the leaves, whereas the leaves contain more amyrins. The anxiety reducing properties identified in the traditionally used material, the leaves, may be due to the presence of the amyrins, which have been shown to exert anxiolysis [3c]. The phytochemical characterization described here will facilitate phytochemical identification of *S. sympetala* and on-going identification of plant organs that represent best candidates for medicinal application. The present study has addressed new strategic priorities in the characterization of *S. sympetala*. Future work will investigate the variability and phytochemical diversity of this species and compare it with other members of the same genus, particularly *S. gilgii*, a species also found in Costa Rica. Finally, due to the lipophilic nature of these triterpenes, emerging extraction technologies (for example, super critical CO_2) are under development to optimize complete extraction.

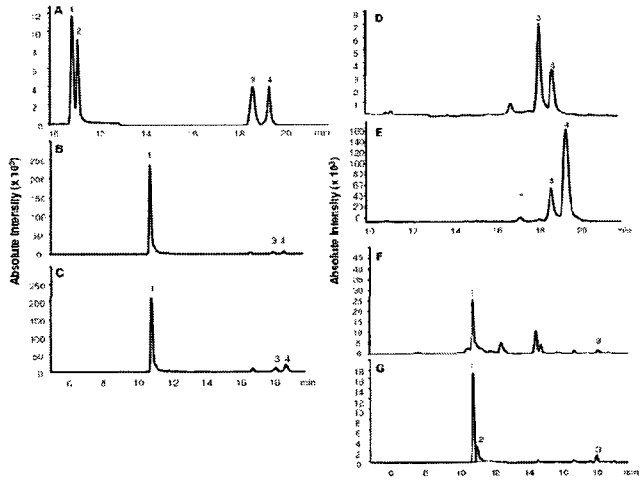

Figure 2: HPLC-APCI-MS profiles of *S. sympetala* extracts and standard mix of triterpenes. (A) standard mix, (B) wood, (C) bark, (D) young leaves, (E) old leaves and (F) flowers and (G) immature fruit. Marker triterpenes detected: **1**: betulinic acid, **2**: ursolic acid, **3**: β-amyrin, and **4**: α-amyrin. For each sample, 1 µL of a 20 mg/mL extract was injected into the autosampler

Experimental

Materials: Analytical grade HPLC solvents were purchased from J.T. Baker (USA). Standards of BA, UA, α-A and β-A were obtained from Sigma (St. Louis, MO).

Sample preparation and extraction: Fresh samples of wild *S. gilgii* wood, bark, early and late foliage (young & old leaves), flowers and fruits were collected in Tortuguero, C.R. and stored in 95% ethanol. Storage ethanol was removed and filtered. Plant material was dried, weighed, coarsely ground via manual blender and extracted via pressurized liquid extraction with an Accelerated Solvent Extraction (ASE) 200 Extractor (Dionex, Sunnydale, USA). The extraction was conducted with 80% ethanol at a temperature of 110°C, pressure of 120 bar, for two 5 mins static cycles, parameters previously demonstrated to optimize triterpenoid extraction [10]. The ASE extract was combined with the original 95% ethanol extract and dried down via speed vacuum at 40°C and lyophilized. All extracts were stored in opaque glass vials at 4°C.

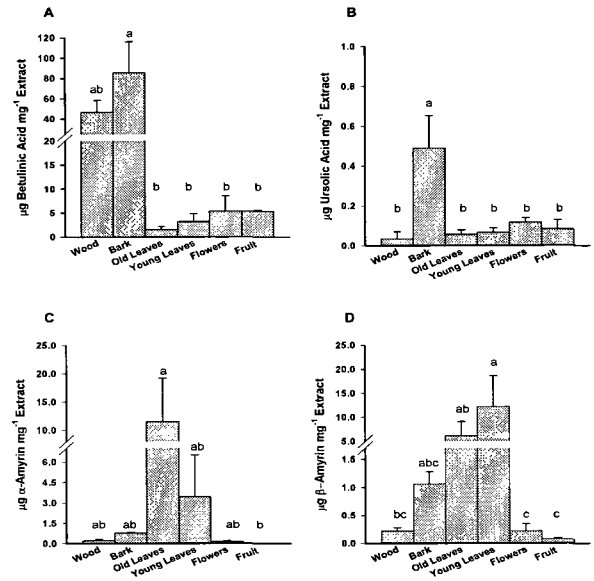

Figure 3: Quantitative comparisons of the triterpenes, betulinic acid (A), ursolic acid (B), α-amyrin (C) and β-amyrin (D) in wood, bark, old leaf, young leaf, flower and immature fruit extracts of *S. sympetala*. Letters indicate significant differences ($p < 0.05$) as determined by a Tukey multiple comparison of means.

HPLC-APCI-MS analyses: HPLC-APCI-MS analyses were conducted on wood, bark, young leaf, old leaf, flower and immature fruit extracts. Analyses were performed with a 1100 LC MSD VL APCI system consisting of an autosampler, quaternary pump, photodiode array detector (DAD) and an online APCI-MS with a mass range of 50 – 1500 a.m.u. (Agilent, Palo Alto, CA, USA). A Waters YMC ODS-AM column (100 x 2 mm I.D.; 3 µm particle size, 120 Å), maintained at 45°C was used at a flow rate of 0.4 mL/min. The elution conditions were optimized with a mobile phase of water (solvent A) and acetonitrile (solvent B) as follows: initial conditions: 70% A: 30% B, linear gradient to 100% B in 10 min, maintained at 100% B for 8 min and returned to 70% A: 30% B in 7 min, post-time 3 min, for a total run time of 28 min. One microlitre of each extract was injected through the autosampler for each run and the elution profiles monitored via MS.

Detection and quantification of triterpenes was conducted via MS. The mass spectrometer was tuned in positive ion mode at the beginning of all experiments. The optimized spray chamber conditions were: drying gas flow rate of 5.0 L/min; nebulizer pressure of 60 psi; drying gas temperature of 200°C; vaporizer temperature of 325°C; capillary voltage of 3200 V; and corona current of 5.0 μA. The MS was operated in SIM and tuned to detect ions with a mass/charge (m/z) ratio of 439.1 (BA), 439.2 (UA) and 409.2 (α-A and β-A) which correspond to the molecular mass of each marker triterpene following the loss of a hydroxyl group and hydrogen atom during fragmentation.

Calibration standards: Individual stock solutions of the four standards were dissolved in methanol at a concentration of 2 mg/mL. The stock solutions were diluted through the addition of the appropriate volume of methanol to a range of 1 μg/mL - 1 mg/mL to yield the solutions used to generate the calibration curve. The identities of the triterpenes in the extracts were determined by comparing the retention times and mass data with those of the calibration standards.

Statistical analysis: All statistical analyses were performed with S-PLUS software version 7.0 (Insightful Corp., Seattle, USA). Tukey multiple mean comparison tests were conducted on log-transformed raw data to compare phytochemical distribution across the plant.

Acknowledgments - Special thanks to L. Kimpe for technical assistance with the ASE extractions. Financial support to MM is provided by Natural Sciences and Engineering Research Council of Canada.

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Figure A4: Characterization and Quantification of Triterpenes in the Neotropical Medicinal Plant *Souroubea sympetala* (Marcgraviaceae) by HPLC-APCI-MS

Appendix III: Evening primrose oil (EPO)

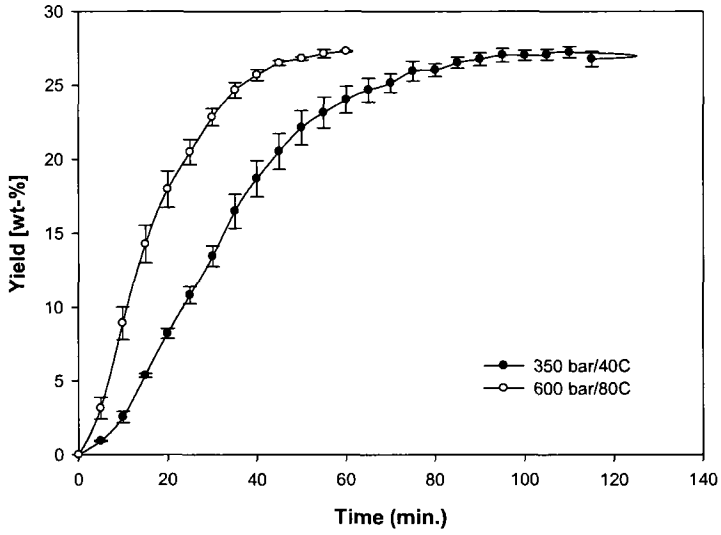


Figure A5: SFE extraction efficiency (EPO)

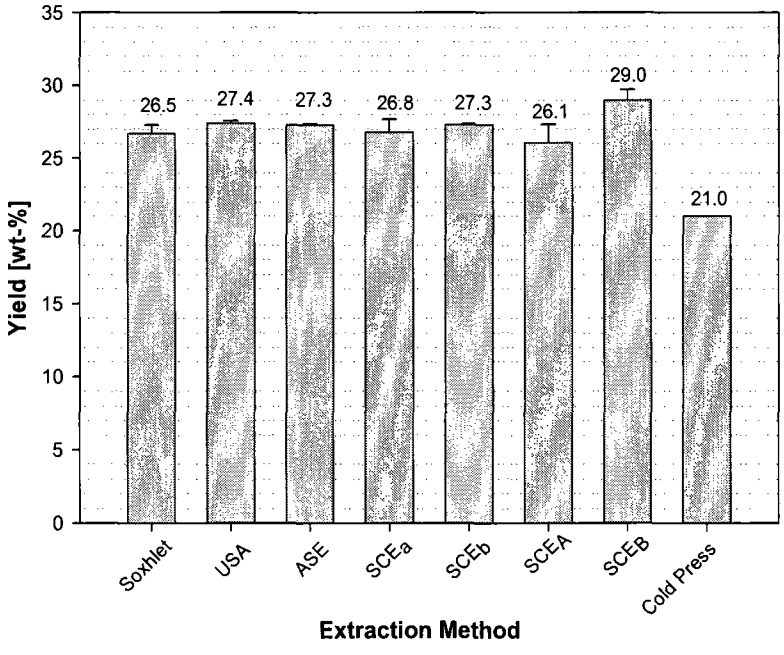


Figure A6: Extraction method comparison (EPO)

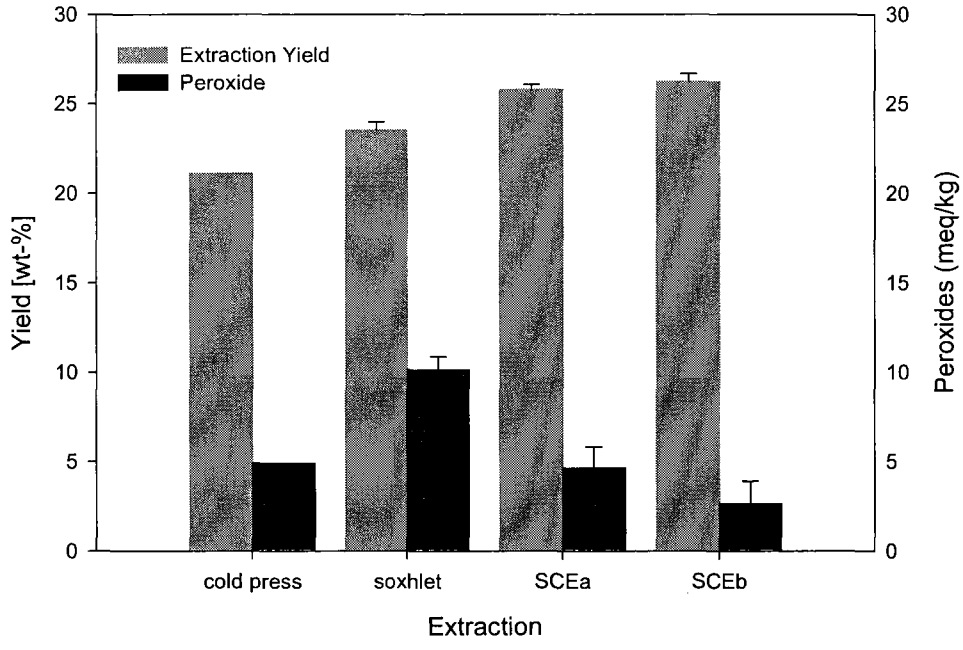


Figure A7: Yield and peroxides comparison (EPO)

Table A1: Fatty acid profiles dependent on extraction method (EPO)

	Fatty acids [wt-%]				
	Palmitic	Stearic	Oleic	Linoleic	GLA
ASE	6.11 ± 0.09	2.55 ± 0.03	9.51 ± 0.02	69.40 ± 0.17	11.29 ± 0.04
Soxhlet	6.17± 0.12	2.35 ± 0.16	9.47 ± 0.12	69.99 ± 0.32	11.11 ± 0.07
Ultrasonic	6.20 ± 0.06	2.51 ± 0.08	9.49 ± 0.03	69.32 ± 0.17	11.20 ± 0.07
Cold Press	6.12 ± 0.11	2.50 ± 0.02	8.92 ± 0.03	69.91 ± 0.15	11.24 ± 0.04
Pilot Scale Low Pressure	6.15 ± 0.16	2.43 ± 0.06	9.04 ± 0.08	69.91 ± 0.46	11.26 ± 0.10
Pilot Scale High Pressure	6.13 ± 0.13	2.53 ± 0.02	9.30 ± 0.05	69.52 ± 0.32	11.16 ± 0.05
Benchtop Low Pressure	6.00 ± 0.04	2.47 ± 0.04	8.98 ± 0.03	70.10 ± 0.17	11.18 ± 0.02
Benchtop High Pressure	6.12 ± 0.07	2.50 ± 0.02	9.03 ± 0.01	69.73 ± 0.17	11.19 ± 0.02

Mean values of three measurements ± standard deviation

Table A2: Pilot extraction industrial tests (EPO)

Extraction Conditions	Description	Peroxides	Free Fatty Acids	Total Plate Count	Total Yeast and Combined Moulds	Salmonella	E.Coli
(MPa/°C)		(meq/kg)	(mL/g)	(cfu/g)	(cfu/g)	(Presence/Absence)	(Presence/Absence)
35/40	Golden, viscous liquid with white ppt. which goes into sol'n when mixed.	3.25	17.57	<10	<10	Absence	Absence
60/80	Golden, viscous liquid with white ppt. which goes into sol'n when mixed.	2.37	17.45	<10	<10	Absence	Absence

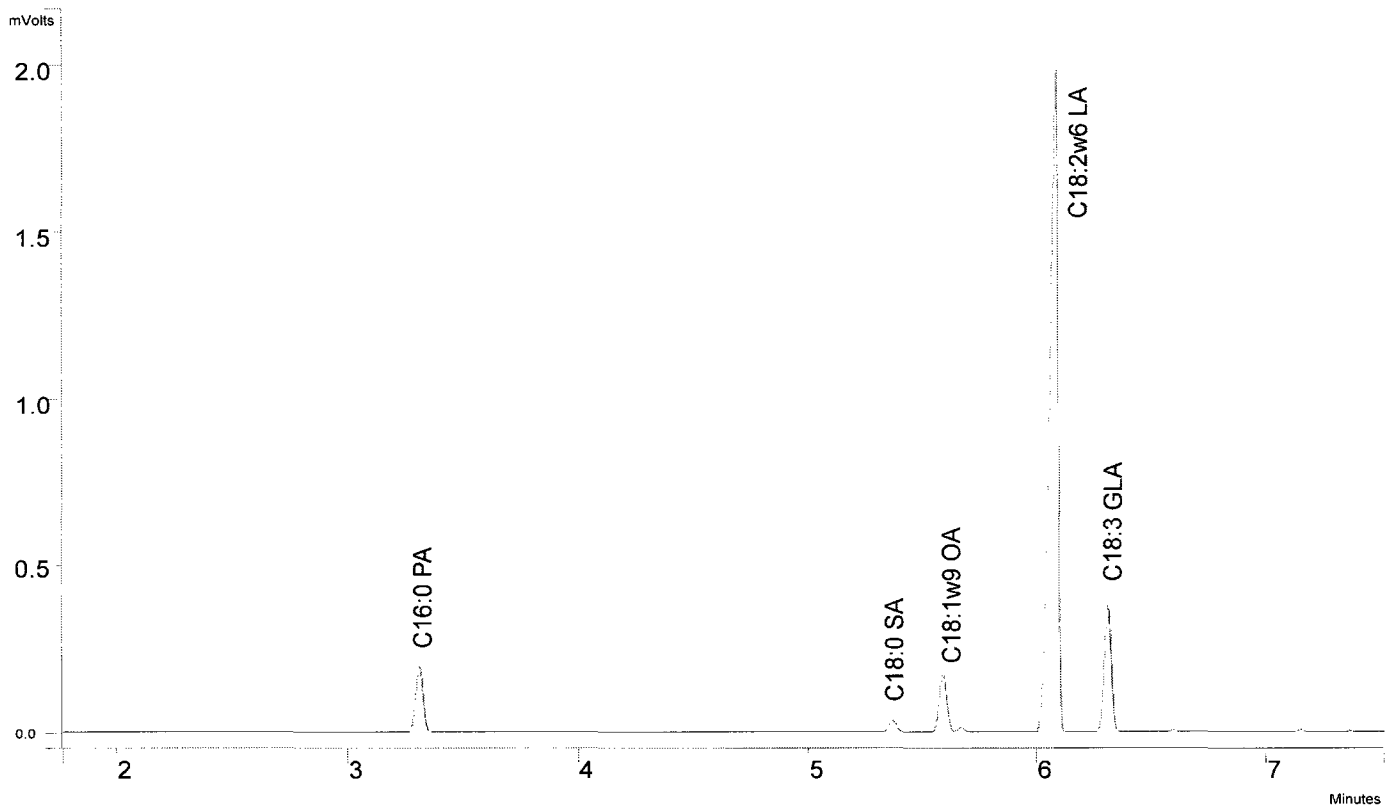


Figure A8: EPO GC-FID Chromatogram

Appendix IV: Echinacea and Northern Prickly Ash

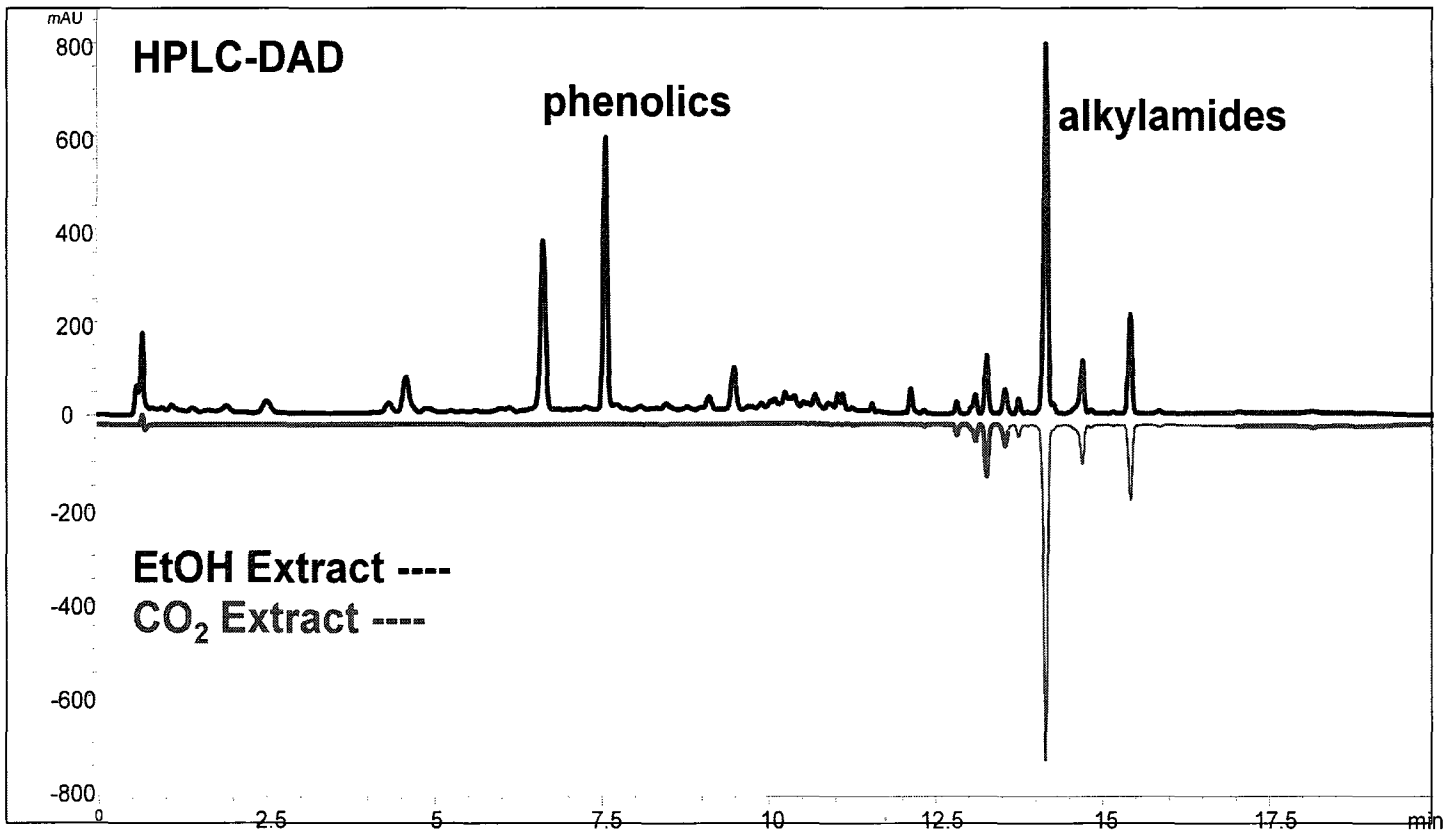


Figure A9: Comparative HPLC chromatogram of solvent (EtOH) extraction vs SFE (CO_2) extraction (Echinacea)

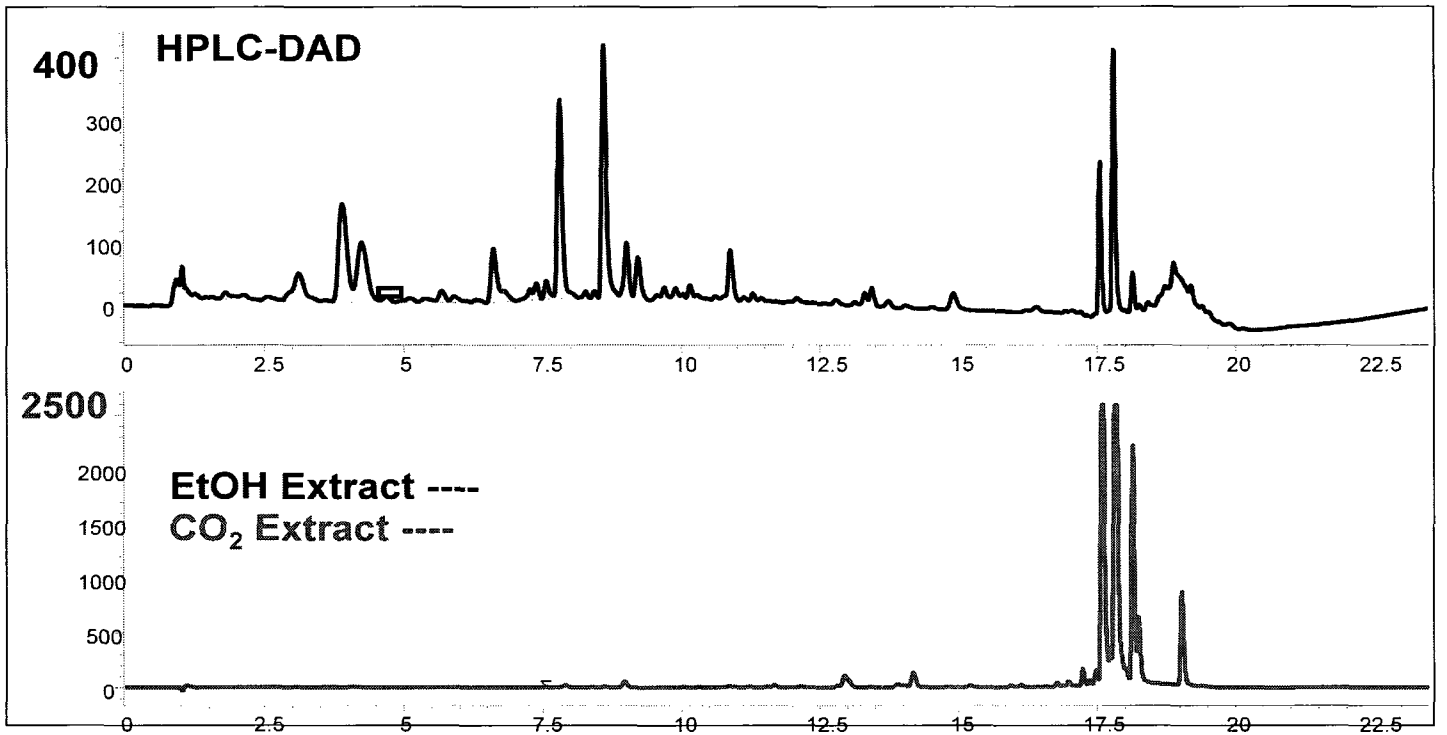
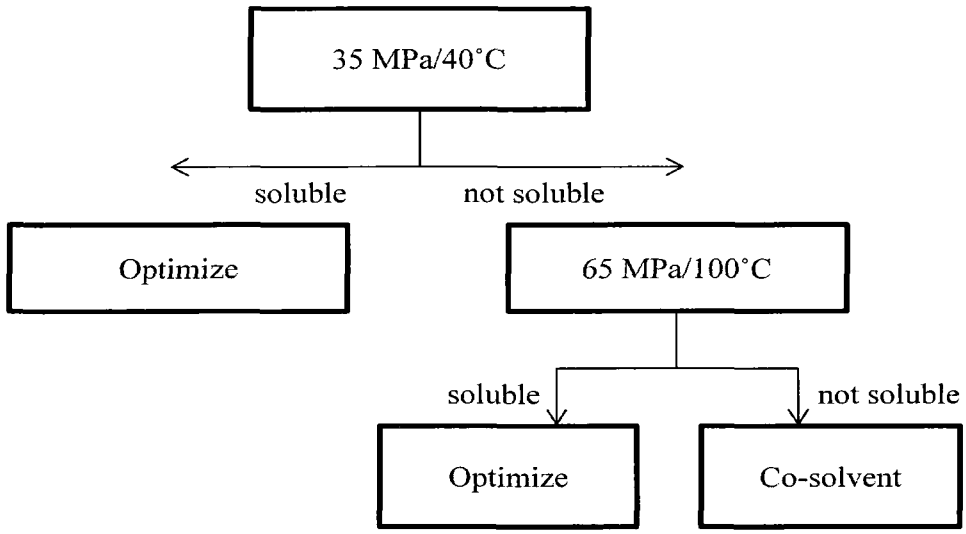


Figure A10: Comparative HPLC chromatogram of solvent (EtOH) extraction vs SFE (CO_2) extraction (Echinacea)

Appendix V: SFE Strategy Flow Chart



SFE Strategy

Start extraction at approximately 35 MPa and 40°C: density is high (0.97 $g.cm^{-3}$). If extraction is successful modify parameters (temperature and pressure) for optimization. The greatest selectivity (highest concentration) is achieved when the density of SC CO_2 only slightly exceeds what is necessary to dissolve target compounds. Reducing extraction time is often achieved by increasing pressure/temperature parameters. *Temperature considerations are necessary for thermally labile compounds.* Reducing particle size and modifying particle shape, reducing moisture, and optimizing flow rate are necessary adjustments for optimal CO_2 usage (solvent:feed). If extraction is not successful at 35 MPa and 40°C, re-extract at 65 MPa and 100°C. If extraction is successful optimize parameters as previously discussed. If extraction is not successful, investigate co-solvent addition and re-extract at high pressure/high temperature parameters.
Non-polar and slightly polar low molecular weight (M.W.) <250 organics are very soluble in liquid and SC CO_2. Higher M.W. (>400) low to moderately polar organics are sparingly soluble. High M.W. highly polar organics are almost insoluble in SC CO_2 without the addition of a polar co-solvent. The co-solvent should present specific interactions with the solute. Fractionation of a mixture of compounds is possible with multiple separators if the differences in mass, vapour pressure or polarity of the constituents in the mixture are significant. The low critical temperature (Tc) of CO_2 allows for easy separation of the extract from the solvent.

Figure A11: SFE Stategy