University of Alberta

Isolation and Fast Analysis of Phytochemical Constituents in *Echinacea* species and *Rhodiola rosea* L. using High-Speed Counter-Current Chromatography and Ultra Fast Liquid Chromatography-Mass Spectrometry

by

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Abstract

High-speed counter-current chromatography was used for the purification of phytochemical components from the roots of *Echinacea angustifolia* (DC.) Hell and *Rhodiola rosea* L. Five alkylamides were purified from *Echinacea angustifolia* roots using two solvent systems and seven phenylalkanoid and monoterpene glycosides were isolated from *Rhodiola rosea* roots using one solvent system and semi-preparative HPLC. Fast analytical methods were developed for the identification of alkylamides in *Echinacea* roots and commercial products available in the Canadian marketplace. 24 alkylamides were identified in 15 minutes using ultra-fast liquid chromatography with diode array and mass spectrometric detection. The three major alkylamides obtained by HSCCC were used as quantitative standards to determine alkylamide contents in different products. Also, a 22 minute method using UFLC-DAD-MS was developed for the characterization of 27 components in *Rhodiola rosea* roots. These techniques can be used in quality and authenticity control of natural health products containing *Echinacea* and *Rhodiola rosea*.

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List of Abbreviations

m/z	mass to charge ratio
ADAPT	dietary supplement containing mixture of adaptogenic herbs
amu/s	atomic mass units per second
AOAC Int	Association of Official Analytical Chemists International
APCI	atmospheric pressure chemical ionization
BRM	biomass reference material
C18	reversed-phase column packing (octa decyl silane)
CB2	cannabinoid receptor
CCC	counter-current chromatography
COX	cyclooxygenase
CRM	certified reference material
Da	daltons
DAD	diode array detection
EMS	enhanced mass spectrometry scan
EPI	enhanced product ion scan
ESI	electrospray ionization
GS1	gas 1
GS2	gas 2
HEMWat	hexane-ethyl acetate-methanol-water
HPLC	high-performance liquid chromatography
HSCCC	high-speed counter-current chromatography
ICH	International Conference on Harmonization
IDA	information-dependent acquisition
IL-1β	interleukin-1β
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantitation
LPS	lipopolysaccharide
MPLC	medium-pressure liquid chromatography
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NHP	natural health product
NFκB	nuclear factor κB
NMR	nuclear magnetic resonance
NOS	nitric oxide synthase
PGE2	prostaglandin E2
prep-HPLC	preparative high-performance liquid chromatography
PS I	polysaccharide I
PS II	polysaccharide II
PTFE	Polytetrafluoroethylene or teflon
qNMR	quantitative NMR
QTRAP	triple quadrupole linear ion trap mass spectrometer

RRLC	rapid resolution liquid chromatography
RSD	relative standard deviation
SHR-5	*Rhodiola rosea* supplement produced by Swedish Herbal Institute
TBARS	Thiobarbituric Acid Reactive Substances
TLC	thin layer chromatography
TNF-α	tumor necrosis fractor-alpha
UFLC	ultra fast liquid chromatography
UPLC	ultra performance liquid chromatography
UV	ultraviolet spectrum

Chapter 1. Literature review

1.1 Introduction

Herbal medicinal plants have traditionally been used to treat a variety of ailments, diseases and infections and are still used as the primary treatment in several regions of the World. Although traditionally the intact plant materials were used, they are now commonly available as extracts in the form of capsules or tinctures in Europe, North America and Australia. While they are referred to as natural health products (NHPs) in Canada, they are also called phytomedicines in Europe and dietary supplements in the United States. Consumer interest in these products continues to grow throughout these regions due to several factors including a lack of trust in conventional medicines, growing interest in self medication and a perception of lower side effects compared to many pharmaceuticals in the marketplace.

As herbal products are produced from plant materials, which are very chemically diverse, there is a wide variety of components which can be present in NHPs. This is contrary to pharmaceutical drugs, which typically contain a single component. The multiple components present in these products can cause a large variability in product quality, efficacy and safety. Consumers perceive that since these products are natural, then they are also safe. This is not the case as these components can potentially interact with prescribed drugs, resulting in harmful side effects. Other issues with NHPs include contamination with heavy metals, pesticides, toxins or other plant materials, or deliberate addition of pharmaceutical drugs (*1*). These unknown contaminants can lead to serious safety issues with these products.

NHP quality is dependent on several factors including growing conditions, extraction parameters and final product formulation. The variability in these factors can significantly impact the phytochemical composition of the components including the ratio of different phytochemicals and the presence/absence of components. The quality of these products is further complicated by the addition of different plant species, the amount of plant materials used, and the use of other plant species from the same genus; ie. *Echinacea angustifolia* and/or *Echinacea*

purpurea are used in different products while these products are all referred to as *Echinacea* (*1*). Since the active components are typically unknown or may only be effective when other synergistic components are present, standardization similar to pharmaceuticals may impact the overall product quality. Therefore, it has been proposed that fingerprint chromatograms of phytochemicals present in plant materials are developed in order to evaluate product quality and for authenticity control of the plant materials (*2*).

The development of fingerprint chromatograms for the monitoring of phytochemical compositions of natural health products requires the use of isolated reference standards for the components of interest, which should be available at high purity and their structure has been confirmed using several analytical techniques including MS and NMR. Traditional fingerprinting has been performed using HPLC chromatographic systems, which have long run times, which reduce productivity in manufacturing processes; therefore development of fast methods for phytochemical fingerprinting and quality determinations is necessary (*2*). As many phytochemical reference standards are not available commercially, the development of methods to isolate these components is also essential, with interest growing in the development of faster, more environmentally friendly methods. These isolated standards can then be used both in fingerprint analysis and quantitative analysis of phytochemical components in natural health products.

The objective of this thesis is to develop quality and potential authenticity control methods for two medicinal herbs: *Echinacea* and *Rhodiola rosea*. Due to the limited availability of reference standards, isolation techniques using high-speed counter-current chromatography were developed. Methods for quality control of these phytochemicals using fast LC methods were coupled with mass spectrometry for compound identification. The isolated reference standards were also used to quantify components in a variety of product matrices using the fast LC methods. These methods focus on enhancing quality and safety of natural health products, which can by used in the NHP industry by manufacturers, suppliers and regulatory bodies.

1.2 *Echinacea* species

1.2.1 Origin of use

Medicinal herbs in North America were first studied and used by the indigenous people many years ago. *Echinacea* sp. grew in the North American Great Plains areas from the Canadian prairies down through Montana to Texas, mostly growing in rocky regions (*3*). It was one of the most versatile plants as it was used to treat a variety of ailments including inflammation and pain, antidote to venom, reducing bacterial infections and many more (*3,4*). Knowledge of these herbs was transferred to European settlers and after its introduction to the medical profession its popularity increased substantially (*4*).

The genus *Echinacea* is a small group of perennial plants in the family Asteraceae. Original taxonomic classification by McGregor (1968) identified nine species and four varieties using morphological characteristics and chromosome numbers (*3,5*). The three species of *Echinacea* that are used medicinally are *Echinacea pallida* (Nutt.) Nutt, *Echinacea purpurea* (L.) Moench and *Echinacea angustifolia* (DC.) Hell. These classifications have been re-evaluated to confirm that there are two subgenera and four species (*6*). *Echinacea pallida* and *Echinacea angustifolia* are two varieties of *Echinacea pallida* (*Echinacea pallida* var. *pallida* and *Echinacea pallida* var. *angustifolia*), while *Echinacea purpurea* is still one species with the same nomenclature (*6*). Although these taxonomic revisions have been accepted, the traditional naming system has remained in scientific literature and on product packaging, therefore is used for the remainder of this work. Some of the common names of *Echinacea* include purple coneflower, snakeroot, black sampson, Indian head and comb flower (*3,7*).

1.2.2 Description

Conrad Moench, an 18th century botanist, named the genus *Echinacea* using the greek word “echinos” or hedgehog because of the similarities with the spiny and round seed head. The plants stems range from 10-100 cm in height. The stems contain a single flower and blooms in early summer with petals between light pink to purple in colour. There are distinct differences in the leaves of *Echinacea purpurea* and *Echinacea angustifolia*, as *Echinacea purpurea* leaves

are larger and ovate shaped, while *Echinacea angustifolia* leaves are alternate, oblong to lanced shaped. The leaves of *Echinacea pallida* are similar to *Echinacea angustifolia*, but the flowers are narrower in shape and droop (*3*).

The similarities between the *Echinacea* plant species have resulted in inaccurate plant identification. For example, in the mid 1900's *Echinacea pallida* was misidentified as *Echinacea angustifolia*, resulting in issues pertaining to articles published during this time (*4*). Adulteration of *Echinacea purpurea* with *Parthenium integrifolium* has commonly occurred due to the decreased cost in cultivation and larger root yield compared to *Echinacea purpurea* (*3,4*). Although indigenous to North America, *Echinacea* has been successfully cultivated in Europe, Asia and Australia.

1.2.3 Chemical Composition

The three species used in medicinal herbs have considerably different phytochemical compositions. There are four groups of constituents that have shown bioactivity including caffeic acid derivatives, alkylamides, glycoproteins and polysaccharides (*7*). The roots of *Echinacea purpurea*, *Echinacea angustifolia* and *Echinacea pallida*, as well as the aerial parts of *Echinacea purpurea* are used in *Echinacea* preparations. Some products may contain combinations of these plant parts in the final products. Due to the natural variability in the active components between plant species, individual plants of the same species and the location of the plant used, there is a considerable amount of variability in final products of *Echinacea* available commercially (*8*).

1.2.3.1 Caffeic acid derivatives

Caffeic acid is a hydroxycinnamic acid and is conjugated to several components in *Echinacea* roots and aerial parts. Conjugates with sugars, quinic acid and tartaric acid are the most abundant found. 17 caffeic acid derivates have been identified in *Echinacea* species, while only 5 of these are used as marker compounds for quality control methods (*9,10*).

Echinacoside, β-(3,4-dihydroxyphenyl-ethyl-*O*-α-L-rhamnopyranosyl (1→3)-β-D-glycopyranoside (1→6)-4-*O*-caffeoyl-β-D-glucopyranoside, is most abundant in the roots of *Echinacea angustifolia* and *Echinacea pallida* (*7,9*). The

major caffeic acid derivative in *Echinacea purpurea* is cichoric acid, 2,3-dicaffeoyltartaric acid. Chlorogenic acid (5-caffeoylquinic acid), caftartic acid (2-caffeoyltartaric acid) and cynarin (1,3-dicaffeoylquinic acid) are all minor components.

1.2.3.2 Phenolic compounds

Besides the caffeic acid derivatives, many other phenolic compounds have been identified in the aerial parts of both *Echinacea angustifolia* and *Echinacea purpurea*. The flavonoids include luteolin, kaempferol, rutin, apigenin, isorhamnetin and quercetin, and several glycosides of these flavonoids (*9*). The flowers of *Echinacea pallida* and *Echinacea purpurea* also contain anthocyanins (*9*).

1.2.3.3 Polysaccharides

Three polysaccharides were isolated from the aerial parts of *Echinacea purpurea*. PS I, PS II and a xyloglucan (*7*). The PS I is a 4-*O*-methyl-glucuronoarabinoxylan with the main chains composed of β-1,4-D-xylose residues, arabinose residues that occur on the C3 positions and the 4-*O*-methyl-β-glucuroic acid residues which occur on the C5 position. The average molecular weight of PS I is 35 kDa (*11*). The PS II is an acidic rhamnoarabinogalactan with a molecular weight of 45 kDa (*11*). The xyloglucan has a molecular weight of 79.5 kDa (*11*). Another polysaccharide, Echinacin B, was extracted from the roots of *Echinacea angustifolia* and *Echinacea pallida* and is composed of an acidic mucopolysaccharide (*9*).

1.2.3.4 Glycoproteins

Four glycoproteins have been reported in *Echinacea angustifolia* and *Echinacea purpurea* roots. The molecular weights of the four proteins are 17, 21, 30 and 40 kDa (*11*). They contain approximately 3% protein with four major amino acids; aspartate, glycine, glutamate and alanine and the main sugars are arabinose, galactose and glucosamine (*12*).

1.2.3.5 Alkylamides

Alkylamides, or alkamides, are present in the lipophilic fractions of the roots and aerial parts of *Echinacea angustifolia* and *Echinacea purpurea*. They are fatty acid amides with varying degrees of unsaturation in the alkyl chain. They are mainly isobutylamides and 2-methylbutylamides with the alkyl chain lengths varying from eleven to sixteen carbons (*13,14*). Alkylamides are associated with the tingling effect in the mouth, which in Native American medicine indicates a high quality herb (*15*).

The original isolation and structure elucidation of alkylamides was performed in the 1980's where a total of 19 alkylamides were identified (*13,14*). The main alkylamides in the roots of both *Echinacea angustifolia* and *Echinacea purpurea* is the tetraenoic isomeric pair dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamide, with the *E* isomer shown in Figure 1-1. The alkylamides present in *Echinacea angustifolia* differ from those in *Echinacea purpurea*. The acetylenic alkylamides in *Echinacea purpurea* contain mainly 2,4-dienoic structures, while in *Echinacea angustifolia* the acetylenic alkylamides are composed of both 2-monoene and 2,4-dienoic structures (*7*).

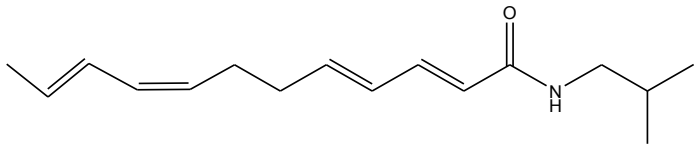

Figure 1-1. Structure of one of the two main alkylamides identified in *Echinacea* roots, dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide.

1.2.3.6 Polyacetylenes and polyenes

The roots of *Echinacea pallida* contain high levels of polyacetylenes and polyenes, primarily ketoalkenes and ketoalkenynes. They been identified as pentadeca-8*Z*-ene-2-one, pentadeca-8*Z*,13*Z*-diene-11-yne-2-one and pentadeca-9*E*,13*Z*-diene- 11-yne-2,8-dione (*7*). Due to their low stability, oxidation occurs to produce 8-hydroxy derivatives, which have been isolated from the roots of *Echinacea pallida* (*16*).

1.2.4. Pharmacology

With the differences in the chemical composition of the three medicinal *Echinacea* species used in commercial preparations, the components responsible for pharmacological effects have not been determined. Current views are that the combination of chemical components is responsible for the pharmacological effects (*7*). *Echinacea* is primarily used in immunomodulation, which includes the treatment of upper respiratory tract infections, colds and flu. Antiviral, antimicrobial, and anti-inflammatory activities have also been observed.

Commercial preparations of *Echinacea* may include hydroalcoholic extracts, which are high in caffeic acid derivatives and alkylamides, or pressed juices, which contain high levels of hydrophilic components such as polysaccharides and glycoproteins. The effect of the final product depends significantly on the extraction procedure and plant material used. To overcome these issues, several pharmacological evaluations have been performed using individual components with comparisons to the original extracts or fractions of the extracts. As the focus of this thesis is on alkylamides, the review will concentrate on alcoholic preparations and purified alkylamides.

1.2.4.1 Immunomodulatory activity

Immunomodulation describes medicines that modify immune function including both immunostimulative and immunosuppressive actions. Some immune responses can be harmful; therefore immunosuppressive effects can also be beneficial in immunity. Early research describes *Echinacea* as an immunostimulant, but recent agreement is that immunomodulation is a more appropriate term (*17*).

An immune response occurs when a foreign substance enters the body, which it wants to fight off. *Echinacea* is thought to enhance both innate and adaptive immune function, although the majority of the research focuses on innate immune function (*18*). The molecules of the foreign substance, such as a bacterial lipopolysaccharide (LPS), bind to receptors on the surface of macrophages and engulf the foreign substance (*19*). Toll receptors are activated, which initiate the expression of NFκB, a gene transcription enzyme regulating the expression of

cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), cyclooxygenase-2 (COX-2) and nitric oxide synthase 2 (NOS2) (*19,20*). These can increase nitric oxide production and prostaglandin E2 (PGE2).

Recent studies concentrate on a product containing both *Echinacea angustifolia* and *Echinacea purpurea*, allowing for the full spectrum of alkylamides to be studied simultaneously. Several individual components and fractions were evaluated including: pure cichoric acid, 2 pure alkylamides (one acetylenic alkylamide and the main tetraenoic alkylamide) and the alkylamide fraction of the extract. Using macrophages, there was a significant decrease in NFκB expression and TNF-α when the cells are LPS stimulated (*21*). The decrease in NFκB was observed with all components and the TNF-α was significant with the alkylamide mixture and the cichoric acid (*21*). In T-cells, the NFκB expression was evaluated after LPS-induced inhibition. The inhibition was significantly reversed by the *Echinacea* extract, the alkylamide fraction, the tetraenoic alkylamide, and cichoric acid (*22*). The alcoholic extracts of all three medicinal *Echinacea* species also reduced nitric oxide production in LPS-stimulated macrophages (*23*).

A potential molecular mode of action of alkylamides related to TNF-α was proposed. Alkylamides from *Echinacea* increased the expression of TNF-α mRNA and increased multiple signal transduction pathways while the levels of TNF-α were inhibited (*24*). It was proposed that this occurred because alkylamides acted as potential ligands for the cannabinoid receptor CB2 (*24*). Twelve different alkylamides were found to have selective affinity for the CB2 receptor (*25*). The alkylamides dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*-dienoic acid isobutylamide, have higher binding affinities compared to the endogenous cannabinoid anandamide (*26*). This tetraenoic alkylamide has the highest binding affinity compared to three other isomeric configurations (*27*). By binding to the CB2 receptors, primarily found in macrophages and T-cells, they function as agonists with CB2 receptors and modulate the immune system (*25*).

Several animal models have been used to assess the *in vivo* immune responses of *Echinacea*. Rats administered an *Echinacea purpurea* extract standardized for polysaccharides, cichoric acid and alkylamides showed an increase in phagocytic activity of alveolar macrophages (*28*). The *in vitro* LPS stimulation of these macrophages produced an increase in TNF-α and nitric oxide (*28*). The extract was fractionated into three fractions, one fraction containing cichoric acid, one containing the polysaccharides and the third containing the alkylamide mixture. The alkylamide fraction increased the phagocytic activity of the alveolar macrophages, while the other fractions did not produce an immune response (*29*).

Several clinical trials have assessed *Echinacea* in the treatment and prevention of cold symptoms. Due to the variability in the composition of the *Echinacea* preparations these results are inconclusive. Several meta-analyses have reported clinical trials as either effective or ineffective, indicating that efficacy may be associated with product variability and quality (*30-32*). It was concluded that standardization of the multiple components is an important factor for product comparison, as the effects may be caused by the synergistic effects of the components in the extracts (*32*).

1.2.4.2 Anti-inflammatory activity

Echinacea has been found to be responsible for the inhibition of COX enxymes, which are involved in the production of prostaglandins resulting in inflammation. Initial discovery of the inhibition of COX enzymes was performed by Muller-Jakic et al (*33*). Several acetylenic alkylamides were assessed and found to have both COX-1 and COX-2 inhibition (*34*). The inhibition of COX-2 enzymes was monitored by the production of PGE2. *Echinacea* extracts from all three medicinal species were compared, where *Echinacea angustifolia* and *Echinacea pallida* were most active, while there was no reduction in PGE2 by *Echinacea purpurea* extract (*35*). The reduction in PGE2 occurred due to the presence of dodeca-2*E*,4*E*-dienoic acid isobutylamide and the ketone pentadeca-8*Z*-ene-11,13-dyn-2-one (*36*). Enrichment with these two components in *Echinacea* extracts confirmed their activity (*37*). The anti-inflammatory activity

may reduce the symptoms caused by colds, flus and upper respiratory tract infections such as sore throat when *Echinacea* products are administered.

1.2.4.3 Antiviral activity

Recent interest has emerged in the antiviral activity of *Echinacea* products as treatment for flu viruses and as topical prevention of the herpes virus. All species of *Echinacea* including roots and aerial parts of the plants were studied against the herpes virus. The hexane portion of *Echinacea purpurea* had the highest inhibition, which contains alkylamide and ketoalkenes (*38*). An *Echinacea purpurea* polysaccharide fraction and *Echinacea pallida* hydroalcoholic extract and pressed juices have also shown anti-herpetic activities (*39,40*). It was proposed that the extracts interfere with the viral attachment to the cells, therefore inhibiting replication (*40.41*). A clinical trial evaluating *Echinacea* as a topical treatment for herpes resulted in no significant differences observed (*42*).

Influenza viruses have been widely publicized recently, triggering research into antiviral remedies. Although *Echinacea* products are used in the treatment of colds and flu research on the antiviral effects on influenza are not extensive. The extract Echinaforce, produced by A.Vogel, has antiviral activity against rhinovirus, adenovirus, influenza virus, respiratory syncytial virus, herpes simplex virus 1, human H1N1-type IV, highly pathogenic avian IV of H5 and H7 types and swine origin IV (*41,43*). Inhibition occurred when direct contact with the extract occurred before infection, inhibiting the receptor binding activity of the virus into the cells and inhibited viral replication (*41,43*). Also, the secretion of pro-inflammatory cytokines was significantly lower and potentially alleviates the symptoms of the infections (*43*).

1.2.4.4 Antimicrobial activity

A lipophilic extract of *Echinacea purpurea* roots has a strong antifungal activity against *Candida albicans* and *Saccharomyces cerevisiae* (*44*). An isolated ketoalkyne was confirmed to inhibit the growth of *S. cerevisiae*, but not *C. albicans* (*44*). The antibacterial activity of *Echinacea* extracts was evaluated against 15 strains of pathogenic bacteria and two pathogenic fugi, including *Candida albicans* (*45*). There was a 3 log10 reduction in growth of the five

bacterial strains, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Legionella pneumophila*, *Clostridium difficile*, and *Propionibacterium acnes*, while there was insignificant activity on the other bacteria (*45*). *Echinacea* has antimicrobial activity against several clinical strains of *Propionibacterium acnes* and also reduced the secretion of cytokines, which could also reduce inflammation caused by acne on the skin (*46*).

1.2.4.5 Bioavailability, pharmacokinetics and metabolism

The *in vitro* evaluations on the bioactivity of these chemical constituents assume that these components are bioavailable. A preliminary investigation using one subject, confirmed that the main tetraenoic alkylamide was absorbed after oral administration (*47*). A scale up study with 11 subjects confirmed the bioavailability of six alkylamides (*48*). The olefinic alkylamide dodeca-2*E*,4*E*-dienoic acid isobutylamie was not detected in the blood, indicating that the structure affects absorption (*48*). Using caco-2 cell monolayer to imitate absorption, 12 alkylamides and the five main caffeic acid derivatives were evaluated. The caffeic acid derivatives did not cross the monolayer, while all alkylamides passed through the layer, although the apparent permeability varied by structure (*49*).

The oral administration of alkylamides revealed two absorption rates. There are “fast absorbers”, where the maximum concentration in the blood is reached within 10 minutes and “slow absorbers”, where the maximum concentration is reached by 40 minutes (*48*). Based on this quick absorption in the blood, it has been proposed that the absorption sites are the mucous membranes in the mouth and esophagus (*48*).

The metabolism of alkylamides has been evaluated *in vitro* using liver microsomes. NADPH was required, suggesting that metabolism occurs with cytochrome P450 enzymes (*50*). The main metabolites include hydroxylated alkylamides, epoxides and carboxylic acid metabolites (*51*). Pure acetylenic alkylamides were metabolized to hydroxylated alkylamides and no carboxylic acid metabolites were observed (*51*). The olefinic alkylamides were rapidly

metabolized, while the 2-monoene acetylenic alkylamides were only minimally metabolized, indicating that structure influences the degree of metabolism (*50*).

1.2.4.6 Safety of *Echinacea* products

Side effects are rare after oral administration of *Echinacea* products. Potential risks arise from allergies to plants from the Asteraceae family, where consumption of the aerial parts should be avoided (*7*). The doses recommended for these products do not produce toxic effects, and acute toxicity testing in animals required significant amounts of the extracts and/or plant materials to be administered before toxic effects were observed (*7*).

1.2.5 Outlook on *Echinacea* research

There are several factors contributing to the variability in final *Echinacea* products including: plant material used, part of the plant used, manufacturing procedure and the final product form (*52*). Therefore, it is difficult to determine the specific dose of these products. Further research on the pharmacological effects of the individual components is required. Some researchers agree that alkylamides are most likely to be effective in the immunomodulation due to their bioavailability, while other components should still be considered (*15*). Based on these results, it is important to include information on the composition of the extracts in scientific reports and on product labels, to ensure high quality products are being evaluated and provided to the public.

1.3 *Rhodiola rosea*

1.3.1 Origin of use

Rhodiola grows in high altitudes in the arctic and mountainous regions of Asia, Europe and North America. Although these regions are dry, sandy or rocky, they provide ideal growing conditions for this plant. *Rhodiola rosea* been used in traditional medicines in Russia and Scandinavia, while interest in western regions has only occurred in the past century. Traditional uses of *Rhodiola* have included treatment of fatigue, headaches, pain, colds and flu, tuberculosis, cancer, depression, anemia, impotence, increase in physical endurance and work

productivity, etc (*53*). *Rhodiola rosea* is currently being used as an adaptogen for non-specific resistance to stress and fatigue (*54*).

The availability of the past research on the efficacy of *Rhodiola rosea* as an adaptogen is limiting since it was performed by Russian researchers and has either been published in Slavic and Scandinavian languages or has not been published at all (*53*). Review articles summarizing these works are available and several studies have been done to confirm their results.

1.3.2 Description

Rhodiola rosea is a perennial plant belonging to the family Crassulaceae. It is further categorized in a sub-family sedoideae (*53*). Over 20 species of *Rhodiola* have been identified, while *Rhodiola rosea* is separated from these other species due to its distinct phytochemical composition.

Rhodiola rosea is a flowering plant that can grow up to 30 inches tall. Multiple leafy stems grow from the same root system and flowers at the top of the stems can be singular or in clusters. The flowers of *Rhodiola rosea* are yellow and can range in size from 5 to 15 mm in diameter (*53*). The roots and rhizomes are thick with a large system of small roots and when cut produce a strong rose-like fragrance. Some of the common names used for *Rhodiola rosea* include: roseroot, arctic root, and golden root.

1.3.3 Chemical composition

The roots and rhizomes are extracted using hydroalcoholic solvents for commercial preparations. There are six main groups of chemical constituents that have been identified in these extracts with over 30 individual compounds identified (*55*). The two main groups that are used for quality evaluation are phenylethanol derivatives and phenylpropanoids. The four other groups of compounds are flavonoids, monoterpenes, triterpenes and phenolic acids.

1.3.3.1 Phenylethanol derivatives

The two main phenylethanol derivatives are *p*-tyrosol and salidroside. Salidroside is a glycoside derivative of tyrosol as shown in Figure 1-2. Originally, *Rhodiola* products were standardized to a minimum of 0.8% salidroside (*53*).

Since salidroside is present in all *Rhodiola* species and several other plants in different genera, other *Rhodiola* materials were being substituted to reduce costs (*53*). Therefore, quality and authenticity control required compounds exclusive to *Rhodiola rosea*.

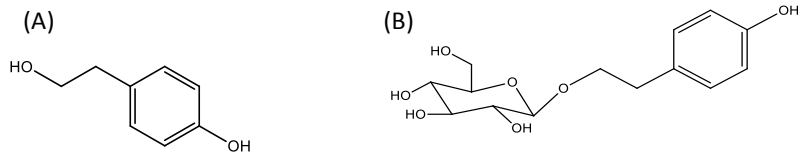

Figure 1-2. Structures of the two main phenylethanol derivatives in *Rhodiola rosea* roots: (A) *p*-tyrosol and (B) salidroside.

1.3.3.2 Phenylpropanoid derivatives

Three phenylpropanoid derivatives composed of cinnamyl alcohol glycosides were identified in *Rhodiola rosea* roots in 1982 (*56*). Since these compounds are specific to *Rhodiola rosea*, they are considered biomarkers for plant identification and quality. The structures of the three compounds, rosavin (cinnamyl *O*-(6'-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside)), rosin (cinnamyl *O*-β-D-glucopyranoside) and rosarin (cinnamyl *O*-(6'-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside)), can be found in Figure 1-3. This group of compounds is referred to as rosavins, with rosavin in the highest abundance in *Rhodiola rosea* roots (*53*). Standardization of *Rhodiola rosea* preparations now includes a minimum 3% rosavins with the 0.8% salidroside, which mimics the naturally occurring ratio of 3:1 (*51*).

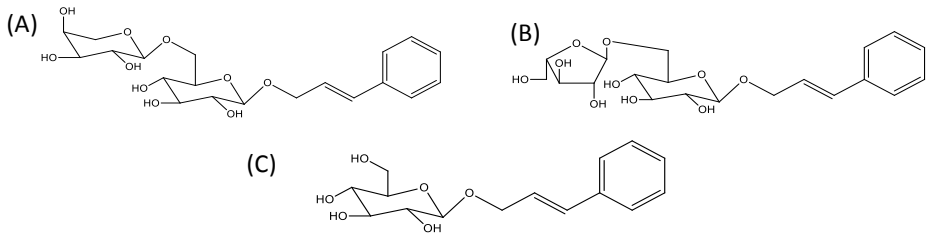

Figure 1-3. Main phenylpropanoid glycosides in *Rhodiola rosea* roots: (A) rosavin, (B) rosarin and (C) rosin.

There are several other phenylmethanoids, phenylethanoids and phenylpropanoids that have been isolated in *Rhodiola rosea* roots (*55*). These are minor compounds that are not generally used as biomarkers and structures vary depending on the sugar groups, and the structure of the phenyl groups.

1.3.3.3 Flavonoids

The flavonoid glycosides identified in the roots of *Rhodiola rosea* contain kaempferol or herbacetin (*57*). Tricin, 4',5,7-trihydroxy-3',5'-dimethylflavone, and two glycoside derivatives, 5- or 7-glycopyranoside, were also identified (*56*). Five of the herbacetin flavonoid glycosides identified are rhodionin, rhodiosin, rhodiose, rhodalgin and acetylrhodalgin (*55,59*). The roots also contain procyanidins and catechins with a variety of structures (*55*).

1.3.3.4 Monoterpenes

The monoterpenes sachalinol A and rosiridol have been detected in *Rhodiola rosea* roots. Six monoterpene glycosides identified as rhodiolosides A-F have also been identified (*60,61*). Another monoterpene glycoside, rosiridin, is a glycoside of rhosiridol, while rhodioloside D and E are glycosides of sachalinol A. Two cyanogenic glucosides, rhodiocyanoside A and lotaustralin, have also been detected in *Rhodiola* (*55,62*).

1.3.3.5 Triterpenes

Two phytosterols, daucosterol and β-sitosterol were identified in *Rhodiola rosea* (*63*). Daucosterol is a glycoside of β-sitosterol with a structure of β-sitosterol-β-D-glucoside.

1.3.3.6 Phenolic acids

The three main phenolic acids in *Rhodiola rosea* include chlorogenic acid, *p*-coumeric acid and gallic acid (*64,65*). The gallic acid derivative methyl gallate has also been isolated (*57*).

1.3.4 Pharmacology

The chemical diversity of *Rhodiola rosea* is very complex and several different components may be responsible for the pharmacological effects. Although salidroside and rosavins are used in quality and standardization, there is

no consensus on whether these components are responsible for the pharmacological effects.

1.3.4.1 Adaptogenic activity

The adaptogenic properties of *Rhodiola* relate to a decrease in stress related fatigue and improvements in physical performance. Simple organism models have been used to confirm adaptogenic properties. *Caenorhabditis elegans* and snail embryos were exposed to environmental stressors including heat, UV light, heavy metals, and oxidative stress (*66,67*). Lifespans were evaluated where the longer the lifespan of the organism indicates there is adaption to the stressor. Although the individual responses of the organisms varied depending on the stressor, the *Rhodiola rosea* treated organisms survived longer after exposure compared to untreated organisms (*66,67*). When the *C. elegans* were treated with *Rhodiola rosea* there was a translocation of the transcription factor DAF-16 into the nucleus, therefore, one proposed mechanism of action is due to changes in gene expression (*67*).

Animal studies on *Rhodiola rosea* use exhaustive swimming tests to evaluate increases in physical endurance. Rodents were placed in water tanks with weights attached to them, making them reach exhaustion quickly. Two studies report that administration of *Rhodiola rosea* extracts significantly prolonged the time to exhaustion (*68,69*). The muscle mitochondria increased in the treatment group compared to the control (*69*). Rosavins are thought to be responsible for the adaptogenic properties of *Rhodiola rosea*, therefore an extract which contained only salidroside was compared (*Rhodiola crenulata*). *R. crenulata* did not produce a significant effect compared to the control, therefore confirming this hypothesis (*69*).

Recent clinical trials focused on the proprietary *Rhodiola* extract SHR-5 produced by the Swedish Herbal Institute. There is no report on the chemical composition of this product. *Rhodiola* was evaluated in acute physical performance where an increase in physical endurance was observed (*70*). Several biomarkers of physical exhaustion were evaluated including blood lactate concentration. The lactate levels for the treated group after the exhaustive exercise

were comparable to athletes who train their bodies after a significant amount of training (*70*).

Administration of 100-576 mg of SHR-5 *Rhodiola rosea* extract per day was used for the evaluation of the reduction of stress induced fatigue (*71-74*). The subjects evaluated varied from young soldiers to physicians and stressed students, demonstrating that *Rhodiola* is able to function regardless of the type of stress that is being applied. All studies confirmed the effectiveness of the extract SHR-5 as an adaptogen for non-specific stress induced fatigue.

Another mechanism of action for the adaptogenic properties of *Rhodiola rosea* has only been proposed based on the evaluation of a proprietary blend of adaptogens, ADAPT-232 also produced by the Swedish Herbal Institute. It was proposed that the adaptogens modulate stress response by acting as a stress mimetic (*75*). The cells produce stress related compounds including nitric oxide and cortisol, which induces the production of a heat shock protein that repairs proteins which would become damaged under stress, therefore if any stress occurs, these proteins will be in sufficient supply to maintain proper function (*75*).

1.3.4.2 Cardioprotective effects

The cardioprotective effects of *Rhodiola rosea* relate to the stress-induced damages that occur to the heart (*53*). In mice *Rhodiola rosea* reduced the buildup of 99mTc-pyrophosphate in the blood, which is a marker for cardiac damage (*76*). *Rhodiola rosea* extracts have also reduced arrhythmias, which are associated with the opioid receptors (*77,78*). The mechanism of action for the cardioprotective effects has not yet been demonstrated.

1.3.4.3 Antioxidant activity

There are several compounds present in *Rhodiola rosea* that may produce antioxidant activity including the phenolics, flavonoids and the phenyl derivatives. The *in vitro* antioxidant activity methods used to evaluate to *Rhodiola rosea* root extracts include DPPH radical scavenging activity, xanthine oxidase, β-carotene bleaching, TBARS, deoxyribose model, and antioxidant activity in keratinocytes (*79-81*). The extracts were effective in suppressing the generation of free radicals and scavenging free radicals, although efficacy was variable

compared to other synthetic antioxidants (*79-81*). When keratinocytes were preincubated with the extract, there was significant antioxidant activity based on several antioxidant activity tests (*79*).

1.3.4.4 Central nervous system stimulation

Rhodiola improves cognitive functions including learning, attention, thinking and memory (*53,82*). In a mouse model, *Rhodiola rosea* enhanced the function of the catecholamine neurotransmitters serotonin, dopamine and norepinephrine, while the permeability of serotonin and dopamine through the blood brain barrier also increased (*53*). The mechanism for improved cognitive function is proposed to be that these increases in neurotransmitters activate the cerebral cortex and the limbic systems in the brain (*53*). Therefore improvements in cognitive functions including thinking, planning, learning, memory and calculating can be observed.

Rhodiola was administered to students before the final exam periods, and several physical and cognitive tests were performed. Students treated with *Rhodiola* showed significant increases in some of the cognitive tests and treated groups showed improvements in grades (*74*). A similar study performed on physicians working night duty also showed significant improvements in cognitive tests (*71*). The association with improved cognitive function is thought to be caused by the decrease in stress related fatigue when *Rhodiola* is administered.

1.3.4.5 Antidepressant activity

Depression was evaluated in a mouse model where, according to the behavioural despair theory, depressed rats swim less compared to normal rats. A significant increase in swimming time was observed compared to non-treated rats and was more effective compared to known antidepressants. When the individual components in the extract were evaluated, the most effective were salidroside and tyrosol. A synergistic effect was also observed when salidroside and rosavin were combined (*83*). *Rhodiola* extracts restored serotonin levels in rat brains and low dosages also repaired cells in the hippocampus restoring them to normal levels (*84*).

A clinical trial using 90 patients in a double-blind placebo-controlled study on the use of *Rhodiola rosea* for the treatment of mild to moderate depression was performed. The proprietary *Rhodiola* extract SHR-5 was used in this trial at two separate dosage levels of 340 and 680 mg/day. A decrease in depression, emotional stability, somatization and insomnia was observed in both treatment groups, while there was no effect in the control group (*85*).

1.3.4.6 Anti-carcinogenic activity

Due to the ethical practices of using *Rhodiola* extracts on human cancer patients, evaluations use animal models. Animals with Lewis lung carcinoma and Ehrlich ascites tumors were treated with cyclophosphamide, *Rhodiola* extract or both. They were both found to suppress tumor growth (*86-88*). Cyclophosphamide and other cancer treatment drugs have been shown to decrease normal bone marrow cells, while *Rhodiola* maintained normal levels when used in combination with the cancer drugs (*87*). Also, *Rhodiola* enhanced the effectiveness of the drug while reducing the side effects (*89*).

The mechanism of anti-carcinogenic activity was assessed using an *in vitro* model using promyelotic leukemia cells on HL-60 line. *Rhodiola rosea* inhibited the division of the cells leading to the induction of apoptosis and necrosis, therefore, decreasing the survival of the cells (*90*). *Rhodiola rosea* did not affect the chromosomes, indicating that the antiproliferative action is mild on the cells, but anti-carcinogenic effects do occur (*90*).

1.3.4.7 Bioavailability and pharmacokinetics

Information on the bioavailability, pharmacokinetics and metabolism of active components in *Rhodiola rosea* extracts is limited. Animal models for bioavailability and pharmacokinetics are available, while no studies on metabolism have been performed. Salidroside was shown to be 32% bioavailable in rats after oral administration, but no data is available for the rosavins (*91*). The pharmacokinetics of rosavin and salidroside was evaluated in rats, where rosavin is absorbed and eliminated faster than salidroside (*83*).

1.3.4.8 Safety of *Rhodiola rosea* products

Clinical trials using *Rhodiola* have shown very few side effects of the drug. Subjects generally feel better after administration. Observations in one clinical trial found that effectiveness may decrease over time due to adaptation to the herb, therefore it is recommended only for short periods of time (*71*). Some individuals have felt anxious, agitated, nervous or experienced lack of sleep or vivid dreams, so taking these supplements early in the day and gradually increasing the dose is recommended (*53*). Since *Rhodiola* acts as an antidepressant, it is recommended to limit use for subjects with bipolar disorders who may potentially become manic due to the medicinal herb (*53*).

1.3.5 Outlook on *Rhodiola* research

Although standardization parameters have been implemented for *Rhodiola rosea* extracts in several countries, it does not confirm that producers are complying with these standardizations. It is essential to ensure proper labeling and quality assessments of *Rhodiola* products are performed, which will ensure these levels of components are maintained. Since the chemical composition of *Rhodiola rosea* extracts is complex, it is essential to continue evaluating the components which are responsible for the bioactivity. There are only a limited number of clinical trials on *Rhodiola* which are restricted to the proprietary extract SHR-5. Other commercial preparations of *Rhodiola rosea* should be assessed for pharmacological activity to confirm the efficacy of other *Rhodiola rosea* products.

1.4 Instrumental methods of analysis

1.4.1 High-speed counter-current chromatography (HSCCC)

High-speed counter-current chromatography (HSCCC) is a preparative technique to purify components using liquid-liquid separation. This technique was originally developed by Ito & Bowman in 1970 (*91*). Although the newer designs of HSCCC instruments have reduced the separation time, the term high-speed is relative to the original developments of counter-current chromatography, which ranged from overnight up to three days (*93*).

Separation times have been reduced significantly due to the numerous advancements in the instrument set-up and technology of the systems which are

commercially available today. Original HSCCC instruments contained a rotary seal and one coiled column with a counter weight, which has been replaced with the columns set up in three multilayer coils in series, removing the need for the counter weight and rotary seal (*94,95*). The three coils maintain the balance within the instrument, which allows speeds of 800-1250 revolutions per minute (rpm) (*96*). The column holders are arranged in a type-J coil planet centrifuge. As the instrument spins, the column holders rotate around their own axis and the axis of the centrifuge (*93*). By using this arrangement, the tubing does not get tangled or twisted, which could cause leaking or contamination problems (*93*).

Since HSCCC separation is performed using a liquid stationary phase, the systems require an immiscible two-phase solvent system. There are two different modes in which the solvent systems can be used in the HSCCC. The more common mode uses the lighter phase (upper phase) as the stationary phase and the heavier phase (lower phase) as the mobile phase. The other mode uses the lower phase as the stationary phase and the upper phase as the mobile phase. An important aspect of solvent phase introduction into the column is that when a closed column is filled with two immiscible solvents, the lighter phase will migrate to the head of the column and the heavier phase will move to the tail of the column, as shown in Figure 1-4. During HSCCC separation with the upper phase as the stationary phase, the lower phase must enter through the head of the column, which allows equilibrium of the two phases to occur. If the lower phase entered the tail of the column, the upper phase would be pushed out of the column and separation would not be possible. This separation mode is called head-to-tail elution, while using the lower phase as the stationary phase would be called tail-to-head elution, as the upper phase is introduced into the tail of the column (*93*).

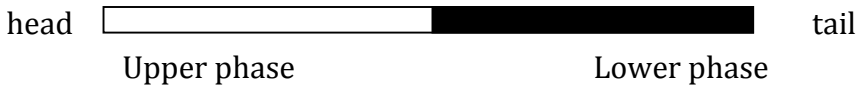

Figure 1-4. Distribution of upper and lower phases of two-phase solvent system when introduced to closed column. Adapted from Ito, Y. (*93*).

As the column is rotating, the two phases distribute within the column and equilibrate. The amount of the stationary phase that remains depends on several factors including revolution speed, flow rate and the composition of the solvent system (*93,97*). The distribution of the two phases in the column undergoes type-J planetary motion, which results in two distinct zones within the column: the mixing and settling zones (*93*). As the coil spins, the mixing occurs near the center of the centrifuge, while the settling occurs away from the center of the centrifuge, allowing the same spot in the column to undergo mixing and settling during one rotation, as shown in Figure 1-5. This is similar to liquid-liquid partitioning using a separatory funnel, with the exception that there is a continuous flow allowing the components to move from others based on their preference to be in the upper or lower phase.

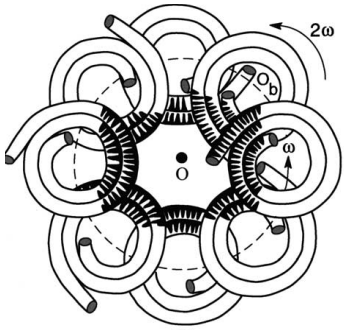

Figure 1-5. The distribution of the two phases during an HSCCC run. The mixing zone occurs at the center of the axis, while settling is observed away from the center of the coils. Source: Ito, Y. (*93*)

The development of the two-phase solvent system is the most important aspect to consider when separating components using HSCCC. There are several factors that must be considered when developing the solvent system. The first is the partition coefficient of the compound of interest. The partition coefficient is the ratio of the solute equilibrated between equal amounts of the upper phase and lower phases shown in Equation 1-1, which can be measured using one of several techniques including TLC, HPLC, UV spectroscopy and MS (*93,98*). The optimal partition coefficient for the compound of interest should be between $0.5 < K_{U/L} < 1.0$. The second important factor is the settling time of the solvent system. The solvents should easily separate from one another in order to reduce the

chance of an emulsion forming in the column, a settling time of less than 20 seconds is recommended. The third important factor is the ratio of the upper phase to the lower phase. Generally, a ratio of 1:1 is optimal in order to reduce solvent waste (*93*).

$$K_{U/L} = \left(\frac{C_{UP}}{C_{LP}} \right)$$

Equation 1-1. Equation for determining the partition coefficients for components to be separated using HSCCC. C_{UP} is the concentration of analyte in the upper phase and C_{LP} is the concentration of the analyte in the lower phase.

There are several benefits of HSCCC over traditional separation techniques. Traditional methods require a solid stationary support, which can lead to peak tailing and loss of sample due to irreversible adsorption of the components. Due to the absence of the solid support, this does not occur in HSCCC separations, allowing for complete recovery of the sample. Semi-preparative HSCCC systems have been able to separate 100 to 1000 mg of extract in a few hours with lower solvent consumption compared to other techniques. HSCCC is a more versatile system compared to preparative-HPLC as the composition of the stationary phase can vary to select for a particular component, increasing the selectivity of the separation and reducing the cost of new preparative columns in HPLC (*93*).

1.4.2 High-performance liquid chromatography (HPLC)

HPLC is a chromatographic technique used in the separation of complex mixtures into individual components. HPLC columns contain a stationary support in which a liquid mobile phase is pumped through. The separation of the components occurs based on their interactions between the stationary and mobile phases (*99*). The compounds that elute later from the column interact more with the stationary phase compared to compounds that elute earlier. HPLC has been widely used in the analysis of pharmaceuticals, natural products, environmental samples, etc. and the purpose of the analyses can range from qualitative identification of metabolites, impurities, active ingredients to quantitative analysis of these compounds (*100*). Other uses of HPLC have included pharmacodynamic

and pharmacokinetic studies, determination of degradation products, or monitoring manufacturing cleanliness (*101,102*).

HPLC systems require several components in order to work efficiently. Present day instruments contain a solvent degasser to remove any gas from the mobile phases. The degasser is connected to the binary solvent pump. The pump is connected to the autosampler and injection valve, the autosampler allows for overnight runs and better reproducibility compared with manual injections (*103*). The column, which may be contained in a temperature controlled oven, is where the separation of the components occurs. The column is connected to a detector, which can be a diode-array detector (DAD), electrochemical cell, fluorescence detector, evaporative light scattering detector or a mass spectrometer (*104*). This will depend on the type of analysis that is performed and the components which are being separated. The instrument is controlled by a computer containing acquisition software.

The separation of the components in liquid chromatography is dependent on the column and the type of components. Normal-phase HPLC was first developed using silica beads as the stationary phase and a non-polar organic mobile phase (*99,102*). Reversed-phase HPLC contains a non-polar bonded phase, which can contain alkyl groups with carbon chains ranging from C4-C30. Other bonded phases include phenyl groups, polar endcapped C18 and high load C18 columns and other separation modes are possible including ion exchange chromatography and size exclusion chromatography, which are not commonly employed in natural product analysis. In natural product separations, gradient elution is typically employed using reversed-phase columns in order to separate the variety of components in an extract.

Traditional HPLC methods run times range from 20 minutes to greater than one hour (*105*). The particle size of the stationary phase ranges from 3 to 5 μm in diameter (*102*). Faster analysis times became desirable and run times were reduced when particles were decreased from 5 to 3 μm. When developing fast HPLC methods, compromises were made which reduced resolution to increase speed (*106*). The next logical step for decreasing analysis time and maintain

resolution was to use smaller particle sizes, less than 2 μm in diameter, but the back pressure caused by pumping the mobile phase through the smaller particles exceeded the pressure limitations of traditional HPLC systems (*107*). These limitations led to the development of ultra performance liquid chromatography (UPLC) instruments.

1.4.3 Ultra performance liquid chromatography (UPLC)

UPLC is a branch of liquid chromatography where instruments can reach higher pressures than traditional HPLC. The first commercially available UPLC was released by Waters called Aquity UPLC system in 2004, which led to the development of other high pressure systems by other liquid chromatograph producers (*107*). Waters trademarked the name UPLC, therefore other companies have released instruments with similar names and capabilities including Agilent's rapid resolution liquid chromatography (RRLC) and Shimadzu's ultra fast liquid chromatography (UFLC) and Ultrahigh performance liquid chromatography (UHPLC).

Several modifications have been made in the development of UPLC columns in order to provide optimal separations. Development of porous particles with 1.7 μm and bridged methyl groups in the silica matrix had higher mechanical strength and improved separation (*105,106*). This technology was developed by Waters, therefore different technologies are used in other UPLC columns (*107*). The smaller particles increase the frictional heating so column diameters have been reduced from 3.0-4.6 mm to 1.0-2.1 mm and the interior surface of the columns were smoothed (*105,107*). Traditional HPLC injection volumes would overload these smaller columns, therefore injection volumes must be reduced (*107*). The instruments were adapted by increasing sampling speed of detectors and improvements in injectors in order to perform UPLC separations efficiently (*105-107*).

The main advantage which has been stated in the development of UPLC systems and separations is the decreased analysis time with no loss in resolution. This occurs because with smaller particle sizes, the change in plate height is very low regardless of the flow rate, as described by the Van Deemter equation (*106*).

There are several other advantages of these methods over traditional HPLC which need to be considered. In some cases, the resolution is actually increased in UPLC systems and high sensitivity can be observed in several detectors (*105*). For example, in mass spectrometers the faster peaks are more concentrated compared to eluting compounds in HPLC and there is a reduction in the volume of the mobile phase, since the flow rates are usually lower. This increases the sensitivity of MS detectors by increasing the ionization efficiency (*105*). An environmental advantage is the lower consumption of solvents for the same number of samples. The shorter analysis time also allows for faster decisions associated with the results, for example, decisions on process monitoring, release of final products in manufacturing or decisions associated with research directions (*106*).

1.4.4 Mass spectrometry (MS)

Mass spectrometry is a powerful analytical tool used for structure elucidation, compound identification and quantification. Mass spectrometers detect gaseous ions according to their mass to charge ratio (*m/z*). The information obtained from MS can be used for both qualitative and quantitative analysis.

Originally mass spectrometers were coupled with GC instruments with the components already in a gaseous state, but with the development of soft ionization techniques, mass spectrometers are now commonly coupled with LC instruments. The most widely used techniques are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) (*108*). In electrospray ionization, which is used in this thesis, the mobile phase contains a volatile acid such as formic or acetic acid. The liquid travels through a charged capillary forming charged droplets producing a spray that travels toward the mass spectrometer (*109*). The liquid in the droplets evaporates causing the charges to become more concentrated. The charges repel each other and the droplets separate into smaller droplets until singly charged ions are formed (*110*). In ESI, the ions are generally formed by the addition or loss of a proton or by the formation of adducts such as sodium or acetate in positive and negative ionization respectively (*109*).

The ions generated are separated by mass in a mass analyzer and detected. The instrument used in this work is a triple quadrupole linear ion trap (QTRAP) hybrid instrument. The quadrupole mass analyzers contains four rods, two have a direct current voltage applied while the other two have a radio frequency applied, which can select for different ions to pass to the detector (*108*). This instrument has three mass analyzers in series, where the final quadrupole has the capability of acting as an ion trap, which can perform multiple fragmentations in space. In this instrument the second quadrupole is used to perform fragmentation, where it contains a collision gas, which fragments ions before entering the final quadrupole for detection.

There are several acquisition modes in mass spectrometry that can be used for structure elucidation, compound identification and to increase selectivity for quantitation. As quantitation was not performed using mass spectrometry in this work, only full scan and product ion scans were performed. In product ion scans the molecular ions are fragmented in the second quadrupole and all fragments are detected.

1.4.5 Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance (NMR) spectroscopy is a technique used to elucidate structures of organic molecules. NMR signals are measured from the relaxation of unpaired nuclei in the nucleus of an atom in magnetic fields (*110*). The two most common nuclei measured in NMR are proton, ^{1}H, and carbon 13, ^{13}C, which both contain spin numbers of ½ due to the odd number of protons and neutrons within the nucleus. The NMR signals for protons or carbon 13 produce chemical shifts based on the environment of the nuclei in the organic molecule, which provide information on functional groups within the structure (*110*). NMR can provide information on the number of protons or carbons, how these carbons and hydrogens are connected and the configuration of the molecules, including double bond (*E*/*Z*) configurations, which cannot be determined with MS. NMR, is a non-destructive technique, which requires anything from 1-20 mg of sample.

1.5 Method validation

Analytical methods for evaluating product compliance, presence or absence of components or quantitation of those components are validated to prove that the method is suitable for its intended purpose. The level of validation applied to a method is determined by the future use of the method. Partial validation is used for methods used within a specific laboratory, such as research methods that confirm components in an extract (*111*). Methods used for compliance to regulations, either by the regulatory bodies or the manufacturers, should be fully validated by a single laboratory validation or a collaborative study (*111,112*). There are several international organizations with guidelines for validation with varying parameters to evaluate. Below are some of the most commonly validated parameters.

1.5.1 Specificity

The selectivity of a method for a particular analyte is determined by its ability to quantify the specific analyte without interferences, such as co-eluting compounds or matrix effects (*112*). Typically, matrix interferences are present with botanicals that may decrease the analyte signal impacting the quantitative results. With efficient solvent extraction and chromatographic separations, the matrix effects can be eliminated. If this is unsuccessful, clean-up procedures including liquid-liquid separations or solid-phase extraction methods are required. Selectivity in chromatography is assessed by high peak resolution of at least 1.5 and a minimum of 1.0 between the analyte and other eluting compounds (*112*).

1.5.2 Calibration/linearity

Quantitative methods require calibration to quantify the amount of analyte in an unknown sample. The signal of the unknown concentration is compared to a reference standard with known concentration. A series of reference standard solutions covering the concentration range above and below the concentration of the analyte in the sample are used to construct a calibration equation fitting the response versus concentration curve. Linearity is defined as a linear response between the two variables (*112,113*).

1.5.3 Accuracy

The accuracy or trueness of a quantitative method is the closeness of the calculated result to the true value (*114*). Due to the uncertainty in all measured values, accuracy can only be estimated. There are several methods to determine accuracy. In some cases, certified reference materials (CRMs) are available for the analytes present in a plant material. These reference materials do not contain the plant materials, but the analytes in a solution with the reported concentration. Results obtained with the new method are compared to the reported concentration (*112*). If CRMs are not available, analyte free matrix materials may be used where the analyte is spiked at a known concentration. In some cases, analyte-free matrix materials are unavailable and standard addition is performed. The two later methods are also used in recovery determinations (*112*).

1.5.4 Precision

The variability of duplicate or replicate results is considered the precision of a method. Precision is determined by the standard deviation of the multiple measurements. Repeatability precision is the precision of results from simultaneously acquired data (*113*). This precision has low variability because the samples are prepared using the same pipettes, calibration curve, instruments, analysts, etc. The intermediate precision is calculated when results are obtained on different days, with different instruments, calibration curves and analysts (*112*).

1.5.5 Limit of detection/limit of quantitation

The limit of detection (LOD) is the lowest concentration of an analyte that can be detected with a reasonable amount of reliability from the signal noise (*114*). The limit of quantitation is the lowest concentration of an analyte that can be quantified with reasonable accuracy and precision (*113*). Equations based on the signal to noise ratios of a blank sample have been used to determine LOD and LOQ. The LOD is defined as the blank plus three times the standard deviation of the noise ($x_{BL}+3s_{BL}$) and the LOQ is defined as the blank plus ten times the standard deviation of the noise ($x_{BL}+10s_{BL}$) (*113*). These equations depend on the signal of the blank, which is difficult to measure and highly variable (*115*). Other methods that have been proposed for LOQ and LOD are described in the ICH

validation guidelines. These include visual evaluation, signal to noise ratio calculations, and calculating based on the standard deviation of the response compared to the slope of the calibration curve (*113*).

1.5.6 Stability

Reference materials and samples are susceptible to degradation during storage, and it is essential to determine their shelf life. Analysis must be performed before losses are observed, which would affect the quantitative results of the components. This measurement is performed using normal and/or accelerated storage conditions in which the analytes of interest are measured periodically to determine if degradation has occurred (*112*).

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Chapter 2. HSCCC isolation of alkylamides from *Echinacea angustifolia* roots[*]

2.1 Introduction

Phytochemical reference standards are used in the development of analytical methods and in the determination of biological activity of individual components. Several limiting factors are associated with commercially produced standards as they can be expensive, available at low purity or their availability is not consistent from the producers (*1*). During the investigations of this study, it became evident that alkylamides from *Echinacea* are unavailable commercially; therefore isolation was necessary for further analytical method developments.

Alkylamides are one of the major groups of proposed active components in *Echinacea* species (*2*). These fatty acid amides can have carbon chain lengths from 11 to 16 carbons with several degrees of unsaturation. Over 20 alkylamides have been identified in *Echinacea angustifolia* and *Echinacea purpurea*, which typically contain either isobutylamide or 2-methylbutylamide groups (*3,4*). The main alkylamide in the roots of these two species is an isobutylamide with four double bonds, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide.

The initial characterization of the alkylamides in *Echinacea* was carried out by Bauer et al. (*3,4*). The alkylamides were isolated from *Echinacea angustifolia* and *Echinacea purpurea* roots using multiple steps, including fractionation by silica gel column chromatography and purification using preparative HPLC or MPLC with reversed-phase columns (*3,4*). Poor separation and peak tailing occur using silica gel separation of basic amides, therefore purification using preparative thin layer chromatography or column chromatography is limited. The further purification using reversed-phase columns is time consuming and requires the use of large amounts of organic solvents.

High-speed counter-current chromatography (HSCCC) is an emerging technique used in the isolation of phytochemical standards with several benefits over traditional separation techniques as described in Chapter 1. With the development of suitable solvent systems, complex mixtures can be separated into

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individual components based on their partition between the two immiscible solvents (*5*). Alkylamides from *Echinacea* have not previously been separated using this technique, although alkylamides from other plant materials have undergone HSCCC purification. One alkylamide from *Mallotus lianus* Croiz with a chain length of 18 carbons was isolated using a one step separation with the solvent system 1:5:1:5 (hexane-ethyl acetate-methanol-water) (*6*). Three alkylamides from *Piper longum* L. were purified in two steps, where prefractionation was performed using HSCCC and the fractions were purified using semi-preparative HPLC (*7*).

The objective of this study was to isolate alkylamides from the roots of *Echinacea angustifolia* using HSCCC. As the polarities of the alkylamides in *Echinacea angustifolia* vary significantly, two HSCCC runs were developed to isolate different groups of alkylamides. The alkylamides were isolated as reference standards to be used in analytical method development and alkylamide quantitation.

2.2 Experimental

2.2.1 Solvents and reagents

The organic solvents hexane, ethyl acetate and methanol were analytical grade and purchased from Fischer Scientific (Ottawa, ON). The water, acetonitrile and formic acid (>98%) were HPLC grade and purchased from Fisher Scientific.

2.2.2 Plant materials

Two sources of *Echinacea angustifolia* roots were obtained for the HSCCC separation of alkylamides. The first source was obtained as a powdered material from Three Feather Farms (Sherwood Park, AB). These roots were harvested in fall 2009. The second source was also obtained as a powdered material and was provided by Naturex (South Hackensack, NJ). This species was harvested in 2008 under the supervision of Dr. Wendy Applequist and deposited with the Missouri Botanical Garden Herbarium, voucher number 217. All materials were stored at -20°C.

2.2.3 Extraction of alkylamides from *Echinacea angustifolia* roots

The alkylamides were extracted from *Echinacea angustifolia* roots using Soxhlet extraction with hexane for six hours. 55 grams of plant material was extracted with 400 mL of hexanes. The resulting extract was dried under vacuum to obtain a yield of 1.21% (w/w) from the root of the plants grown in Alberta and a yield of 0.53% (w/w) from the roots obtained from Naturex.

2.2.4 Solvent system selection for alkylamides

Solvent systems were screened based on partition coefficients of six main alkylamides, while a partition coefficient ranging from 0.5-1.0 for the main alkylamide dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraeneoic acid isobuytlamide was preferred. The other properties that were screened were the settling time and phase ratio of the upper to lower phase. Solvent systems were evaluated using the hexane-ethyl acetate-methanol-water systems using the schematic search according to Ito, Y. (*5*). The ratios of the solvents were varied to find the optimal solvent system. The settling time and solvent phase ratios were evaluated by preparing 10 mL of each solvent system. In a test tube, 2 mg of the crude hexane extract from Alberta was combined with 2 mL of the upper and lower phases of the solvent systems and mixed thoroughly using a vortex mixer. When the systems settled into two distinct phases, aliquots of the upper and lower phases were analyzed using the HPLC-DAD method described in section 2.2.7. The partition coefficient was determined using the ratio of the peak area of the six main alkylamides in the upper and lower phases.

2.2.5 Instrumentation

A model TBE-300B high-speed counter-current chromatograph (Tauto Biotech, Shanghai, China) was used in the HSCCC separations. This instrument is equipped with a type-J planet coil centrifuge with three multilayer coils connected in series. The total volume of the column was 300 mL, containing PTFE tubes with 1.68 mm inner diameter. The rotation speed was adjustable from 0-1000 rpm. The column contains a manual injection port with 20 mL sample loop. The column was connected to an isocratic preparative pump, model 501 PrimeLine (Analytical Scientific Instruments, El Sorbante, CA), a UV-vis detector, model

VUV-24 Visacon (Reflect Scientific Inc., Orem, UT) and a fraction collector, model CHF 122SC (Avantec Toyo Kaisha Ltd., Tokyo, Japan). The column was temperature controlled using a Neslab RTE 7 refrigerated cooling bath (Thermo Scientific, Rockford, IL).

2.2.6 Separation of alkylamides by HSCCC

2.2.6.1 Separation of olefinic alkylamides

The solvent system consisting of hexane, ethyl acetate, methanol and water with the ratios of 4:1:2:1, respectively, was mixed and equilibrated at room temperature overnight in a separatory funnel. The phases were separated and degassed shortly before use. The head-to-tail elution mode was used, therefore the upper phase was used as the stationary phase and the lower phase was the mobile phase.

The sample solution was prepared by mixing 250 mg of the crude hexane extract with 5 mL of both phases of the solvent system. After the coil was filled with the stationary phase, the column was rotated at 1000 rpm. The column temperature was maintained at 20°C. The lower phase was introduced to the column at 3 mL/min until the mobile phase eluted from the column, indicating that hydrodynamic equilibrium was achieved. The sample was injected into the system and the fractions were collected at a rate of 3 min/tube (9 mL total per tube). After two hours, the mobile phase was switched to the upper phase and ran for another two hours. The 80 fractions collected were analyzed using HPLC-DAD as described in section 2.2.7. This separation was performed on the plant materials from Three Feather Farms and Naturex.

2.2.6.2 Separation of acetylenic alkylamides

The acetylenic alkylamides are minor components in *Echinacea angustifolia*. Therefore, in order to isolate larger quantities the fractions containing the two acetylenic alkylamides, with retention times of 15.2 and 17.5 minutes in the HPLC, were pooled from 10 runs using the HSCCC separation described in section 2.2.6.1. These samples were stored at -20°C and dried under vacuum immediately before use.

The solvent system consisting of hexane, ethyl acetate, methanol and water with the ratio of 3:1:2:2, respectively, was mixed and equilibrated at room temperature overnight in a separatory funnel. The phases were separated and degassed shortly before use. The head-to-tail elution mode was used in this separation.

A total of 20.3 mg of sample was obtained from the ten HSCCC runs. This sample was dissolved in 5 mL of both phases of the solvent system. After the coil was filled with the stationary phase, the column was rotated at 1000 rpm and the lower phase was introduced into the column at 3 mL/min. The column temperature was maintained at 20°C. When the lower phase emerged from the column, the sample was injected and the fractions were collected at a rate of 3 min/tube (9mL total per tube). After 160 minutes, the mobile phase was switched to the upper phase and the separation was terminated when the second alkylamide eluted from the column. The total run time was 245 minutes. The fractions were analyzed for purity using the HPLC-DAD method described in section 2.2.7.

2.2.7 HPLC-DAD analysis

The alkylamide profile of the crude extracts was evaluated by HPLC using a series 1200 Agilent liquid chromatograph (Agilent Technologies, Mississauga, ON) equipped with a solvent degasser, a binary gradient pump, a thermo-autosampler, a column oven and a diode array detector. The data collection was performed using ChemStation software. 1 mg of the crude extract was dissolved in 1 mL of methanol and filtered using a 0.45 nylon filter into an HPLC vial. This HPLC method was adapted from Luo et al. (*8*). The separation was performed using a Waters Nova-Pak C_{18} column with 150 x 3.9 mm, 4 µm particle size (Waters, Wexford, Ireland). The mobile phase consisted of A: 0.1 % formic acid in water and B: acetonitrile with gradient elution of 0-9 min (10-18.5% B), 9-9.5 min (18.5-45% B), 9.5-39.5 min (45-80% B), 39.5-42 min (80-100% B), and 42-45 min (100-10% B). The injection volume was 10 µL and the alkylamides were monitored at 254 nm. The column oven was maintained at 30°C. This HPLC method was used to assess the fractions from the HSCCC separation for purity.

2.2.8 Structure elucidation of alkylamides

The fractions that contained high purity of the same alkylamides were combined and dried under vacuum. The resulting purity and structures were assessed with both HPLC-MS/MS and NMR spectroscopy. The HPLC-MS/MS analysis was performed using the HPLC separation described above, which was connected to a 4000 QTRAP system (ABSciex, Streetsville, ON). The mass spectrometer was equipped with electrospray ionization (ESI) operating in positive mode. The main alkylamide dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobuytlamide, isolated using HSCCC, was used to optimize the mass spectrometer for signal intensity of the molecular ion and fragmentation using full scan and product ion scans, respectively. Nitrogen gas (>99%) was used as nebulizing (Gas 1, GS1) and collision gas (Gas 2, GS2). The values for optimum spray voltage, source temperature, GS1, GS2 and curtain gases were +3.5 kV, 400°C, 50, 30 and 15 psi, respectively. The declustering potential was 66.0 V, the collision energy was 23.0 eV, the entrance potential was 10 V and the collision exit potential was 10 V. The spectra were obtained over a mass range of *m/z* 50 to 500. MS/MS analyses were performed using the information-dependent acquisition (IDA) method where the mass spectrometer alternates between the enhanced MS (EMS) scan for the full scan and the enhanced product ion (EPI) scan and generates MS/MS data on the eight most intense peaks. The scan rate for EMS was 1000 amu/s and for EPI was 4000 amu/s. The data acquisition was performed using Analyst 1.5 software (ABSciex).

^{1}H and ^{13}C NMR spectra were collected using a Varian VNMRS 500 NMR spectrometer with $^{13}C/^{1}H$ dual cold probe (Agilent Technologies, Santa Clara, USA) at 499.82 and 125.69 MHz, respectively. The solvent chloroform ($CDCl_3$) was used with tetramethylsilane (TMS) as the internal standard.

2.3 Results and discussion

2.3.1 Solvent system selection for alkylamides

The HEMWat solvent systems were used to determine a starting point for optimizing the solvent system suitable for the alkylamides. Once the region was selected, small adjustments were performed to obtain a system which fulfilled all

of the requirements for a good solvent system. Several of the solvent systems are summarized in Table 2-1.

Table 2-1. Partition coefficients for alkylamides in *Echinacea angustifolia* using several two phase solvent systems for HSCCC solvent system development composed of hexane-ethyl acetate-methanol-water.

	partition coefficient						percentage	
solvent ratio	15.1* min	17.5* min	20.1* min	22.9* min	27.1* min	29.9* min	UP	LP
9:1:2:1	0.09	0.49	0.86	1.68	3.05	7.01	76	24
6:1:2:1	0.19	0.64	1.34	2.49	3.76	8.10	67	33
4:1:2:1	0.15	0.54	1.18	2.06	3.41	7.29	57	43
3:1:2:2	1.48	6.10	11.20	24.11	40.01	100	46	54
3:1:1:1	1.14	5.34	8.00	20.74	31.54	100	62	38
1:2:1.75:1	0.92	2.12	2.57	3.86	4.10	6.67	18	82
4:1:4:1	0.04	0.13	0.28	0.42	0.66	0.95	42	58

* Retention time in HPLC

The six main alkylamides selected for partition coefficient determination are identified by HPLC retention times. The acetylenic alkylamides had retention times of 15.1 and 17.5 minutes, while the olefinic alkylamides had later eluting retention times. A partition coefficient in the range of 0.5-1.0 for the tetraenoic alkylamide, retention time of 20.1 minutes, was used for determining the solvent system for the olefinic alkylamides. From Table 2-1, there are several solvent systems which are close to this range, but due to the more reasonable ratio of the upper to lower phases, the solvent system 4:1:2:1 (hexane:ethyl acetate: methanol:water) was selected. The solvent system with a partition coefficient of approximately 1.0 for the alkylamide eluting at 15.1 minutes was used to select the solvent system for the acetylenic alkylamides. Based on the solvent systems evaluated, the solvent system 3:1:2:2 (hexane: ethyl acetate: methanol: water) was selected.

2.3.2 HSCCC separation of olefinic alkylamides

The alkylamide profiles of the hexane extracts of *Echinacea angustifolia* roots from separate sources are compared using the HPLC-DAD separation in Figure 2-1. The relative abundance of the main alkylamide, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobuytlamide is much higher in the Alberta source hexane extract compared with the roots provided by Naturex. Also, a co-

eluting isomer of the alkylamide eluting at 22.9 minutes is present in the Alberta source and not the Naturex source.

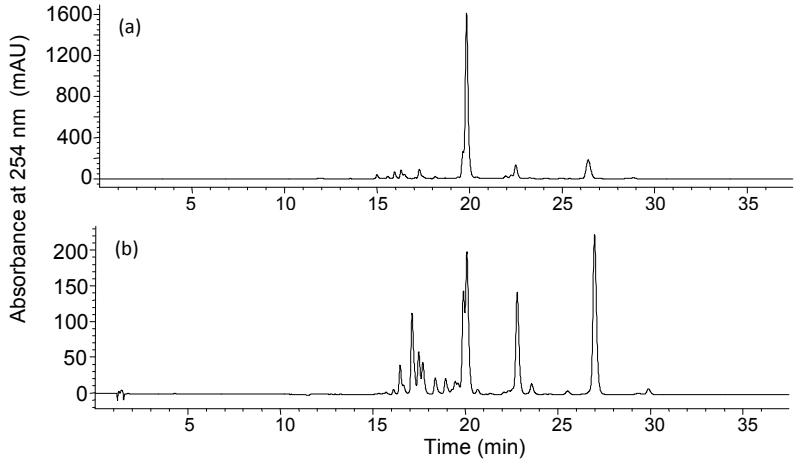

Figure 2-1. HPLC profiles of alkylamides in the hexane extracts of *Echinacea angustifolia* from two different sources monitored at 254 nm (a) Three Feather Farms, Alberta and (b) Naturex, Missouri.

The solvent system 4:1:2:1 was selected for the separation of the olefinic alkylamides and with the conditions used in this separation the stationary phase was satisfactory with 73% retention. The HSCCC profile obtained from injecting 250 mg of the Alberta extract is displayed in Figure 2-2. The resolution of the four final peaks is higher in comparison to the components eluting in the first 75 minutes of this separation. The HPLC-DAD analysis confirmed the separation of the six main alkylamides, which are summarized in Table 2-2. The structure elucidation was performed on the alkylamides with greater than 90% purity. The MS data have been summarized in Table 2-3 and the ^{1}H and ^{13}C NMR signals have been summarized in Table 2-4.

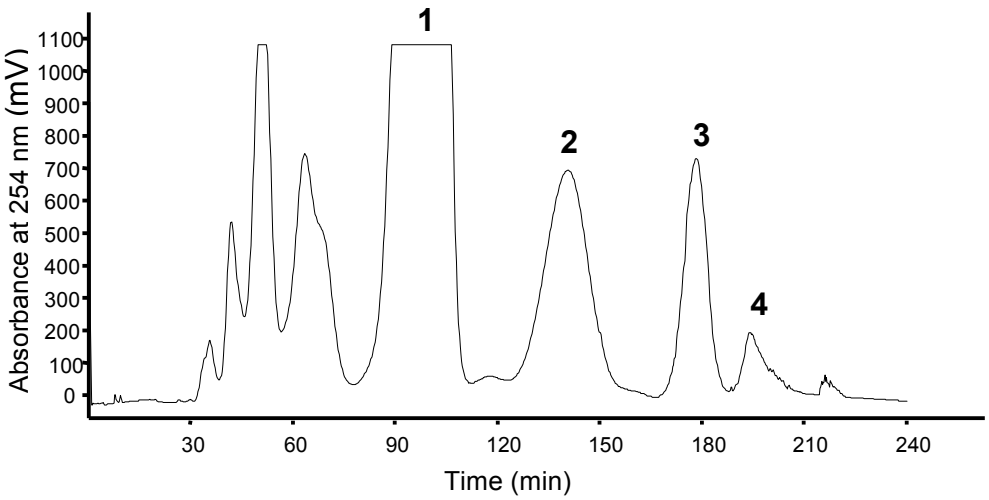

Figure 2-2. HSCCC profile of the alkylamide separation using the solvent system 4:1:2:1 (hexane:ethyl acetate: methanol: water) in the head-to-tail elution mode. Peaks are labelled according to alkylamide number in Table 2-2.

Table 2-2. Alkylamides isolated by using HSCCC with the solvent system 4:1:2:1 in head-to-tail elution mode.

Alkylamide No.	Fraction	Retention time (min)	Peak area (%)	Sample weight (mg)	Compound
	22	15.1	74.1	9.4	Undeca-2*E*-ene-8,10-diynoic acid isobutylamide
	28-29	17.5	74.9	10.1	Dodeca-2,4,10-triene-8-ynoic acid isobutylamide
1	34-37	20.1	96.8	38.9	Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide
2	43-46	22.9	77.3	2.5	Dodeca-2*E*,4*E*,8*Z*-trienoic acid
	40*	22.9	92.1	4.4	isobutylamide
3	58-62	27.1	99.4	3.2	Dodeca-2*E*,4*E*-dienoic acid isobutylamide
4	65	29.9	91.7	0.3	Dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide

* Pure fraction obtained from *E. angustifolia* from Naturex source.

Table 2-3. MS fragmentation data obtained for the four olefinic alkylamides isolated by HSCCC.

Compound	Retention time (min)	UV λ_{max} (nm)	Molecular ion (*m/z*) $[M+H]^+$	Fragments (*m/z*)
Alkylamide 1	20.1	236, 260	248	192, 175, 166, 149, 147, 142, 133, 121, 107, 105, 93
Alkylamide 2	22.9	260	250	194, 177, 167, 152, 149, 135, 121, 109, 95
Alkylamide 3	27.1	260	252	196, 179, 161, 137, 119, 105, 96
Alkylamide 4	29.9	260	266	196, 179, 161, 133, 119, 109, 95

Table 2-4. ^{1}H and ^{13}C NMR assignments obtained for the four olefinic alkylamides isolated by HSCCC.

	Alkylamide 1		Alkylamide 2		Alkylamide 3		Alkylamide 4	
	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)
1		166.3		166.4		166.6		166.6
2	5.76 *d* (15)	122.1	5.75 *d* (15)	122.0	5.76 *d* (16)	121.9	5.76 *d* (16)	121.4
3	7.19 *dd* (15,13)	141.2	7.18 *dd* (15,10)	141.3	7.20 *dd* (16,11)	141.6	7.21 *dd* (16,11)	141.8
4	6.16 *dd* (15,13)	128.7	6.14 *dd* (15,10)	128.6	6.14 *dd* (15,11)	128.4	6.12 *dd* (15,11)	128.4
5	6.08 *dt* (15,6)	142.1	6.08 *dt* (15.6)	142.2	6.06 *dt* (15,6)	143.5	6.07 *dt* (15,6)	143.6
6	2.25 *m*	33.1	2.18 *m* (2H)	33.1	2.14 *m*	33.2	2.16 *m*	33.0
7	2.30 *m*	27.0		26.6	1.28 *m* (5H)	32.0	1.28 *m* (5H)	31.8
8	5.25 *dt* (10,6)	128.0	5.36 *m* (2H)	128.5		29.4		29.8
9	5.97 *t* (10)	129.4		130.8		29.3		29.2
10	6.30 *dd* (15,13)	126.8	2.00 *dt* (7,7)	29.4		29.0		29.0
11	5.69 *m*	129.9	1.37 *m*	22.8		22.8		22.7
12	1.78 *dd* (6,1)	18.3	0.90 *t* (7)	13.8	0.88 *t* (6)	14.3	0.88 *t* (6)	14.1
N-H	5.46 br *s*		5.46 br *s*		5.49 br *s*		5.48 br *s*	
1’	3.17 *dd* (6,6)	47.0	3.17 *dd* (7)	47.0	3.17 *t* (6)	47.2	3.18 *m*	45.4
2’	1.80 *m*	28.7	1.80 *m*	28.7	1.81 *m*	28.9	1.56 *m*	35.1
3’	0.93 *d* (6)	20.2	0.93 *d* (7)	20.2	0.93 *d* (6)	20.3	1.41/1.15 *m*	27.1
4’	0.93 *d* (6)	20.2	0.93 *d* (7)	20.2	0.93 *d* (6)	20.3	0.91 *t (6)*	11.3
5’							0.91 *d* (6)	17.2

Measured in $CDCl_3$

The purity of the fractions containing the acetylenic alkylamides (22, 28-29) were not high enough for structure elucidation by NMR. Purity measurements were taken as the peak area percentage of the alkylamides at 254 nm after HPLC separation. These fractions were combined for further HSCCC separation described in section 2.3.3.

The structures of the alkylamides isolated using this HSCCC solvent system are shown in Figure 2-3. Fractions 34-37 were combined and contained the main alkylamide dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide with a purity of 96% (alkylamide 1). This alkylamide was obtained in highest abundance with 38.9 mg isolated. The molecular ion detected at *m/z* 248 and fragmentation pattern was consistent with previously published data (*9,10*). Based on the NMR data, the ^{13}C shift for carbon 12 of 18.3 ppm is consistent with the *E* isomer, the more abundant alkylamide in this isomeric pair is the *E* isomer (*4,11*). These two alkylamides have only been separated previously using preparative argentation

reversed-phase HPLC (*12*). Traditionally, they are isolated together and used as a combined standard when used in quantitation analysis.

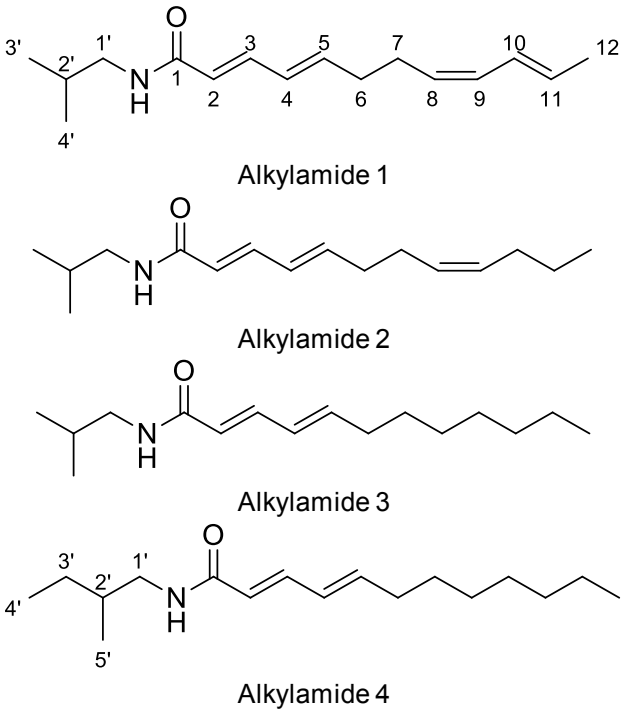

Figure 2-3. Structures of the four alkylamides isolated from *Echinacea angustifolia* roots by HSCCC.

The HSCCC separation of the Alberta hexane extract was unable to separate the alkylamide dodeca-2*E*,4*E*,8*Z*-trienoic acid isobuytlamide from the co-eluting component in fractions 43-46. These fractions were only able to yield 2.5 mg of material at 77.3 % purity. The Naturex hexane extract does not contain this isomer, therefore 250 mg of this extract was separated using the HSCCC method, where 4.4 mg of this alkylamide was obtained at 92% purity (alkylamide 2). The MS and ^{1}H NMR were consistent with previously published data, while there was no ^{13}C NMR available (*3,9,10*). The ^{13}C NMR data was compared with the NMR data of the tetraenoic and dienoic alkylamides for proper identification.

The alkylamide dodeca-2*E*,4*E*-dienoic acid isobutylamide was isolated in fractions 58-62 at 99% purity (alkylamide 3). The MS and NMR data were all consistent with published data confirming the structure of this alkylamide (*3,9,10*).

Alkylamide 4 was isolated using HSCCC was obtained in fraction 65 with a purity of 91.7%. This alkylamide is a minor component and only 0.3 mg were collected in one HSCCC run. This alkylamide was identified as dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide, which has not previously been identified in *Echinacea angustifolia* roots. The MS data confirmed a molecular ion of *m/z* 266, therefore a molecular weight of 265. This is consistent with a molecular formula of $C_{17}H_{31}NO$. The fragmentation confirmed the 2-methylbutylamide group with the fragments *m/z* 196 and *m/z* 179 which correspond to the loss of the methylbutyl group (-70 Da) and the methylbutyl amine (-87 Da). The ^{1}H and ^{13}C NMR for the structure of the alkyl chain were compared with the signals from the alkylamide dodeca-2*E*,4*E*-dienoic acid isobutylamide, while the ^{1}H and ^{13}C NMR for the structure of the 2-methylbutylamide chain was confirmed by comparing the signals to the compound dodeca-2*E*-ene-8,10-dynoic acid 2-methylbutylamide and dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide (*3,4,13*). These comparisons allowed for the confirmation of this new compound.

2.3.3 HSCCC separation of acetylenic alkylamides

Initial attempts to separate the acetylenic alkylamides using the solvent system 4:1:2:1 were unsuccessful, therefore new solvent systems were explored, which would be more selective to these more polar alkylamides. The solvent system 3:1:2:2, which is selective to the polar alkylamides was also tested using 250 mg of the hexane extract to isolate these two alkylamides, but was unsuccessful due to low yields and co-elution. Therefore, the HSCCC solvent system 4:1:2:1 was used as a pre-fractionation step, where the fractions with high purity of the alkylamides with HPLC retention times of 15.1 and 17.5 minutes were collected and used as the sample for this separation.

Ten runs of the olefinic alkylamide HSCCC resulted in a total of 20.3 milligrams of the two components injected into the HSCCC. Due to the storage of these components, decomposition of the alkylamides may have occurred. The solvent system 3:1:2:2 had a satisfactory retention of the stationary phase of 70%. The profile of the HSCCC separation is shown in Figure 2-4. The initial peak

eluting is the alkylamide with the retention time of 15.1 minutes in the HPLC. There is good resolution between these two alkylamides with this solvent system.

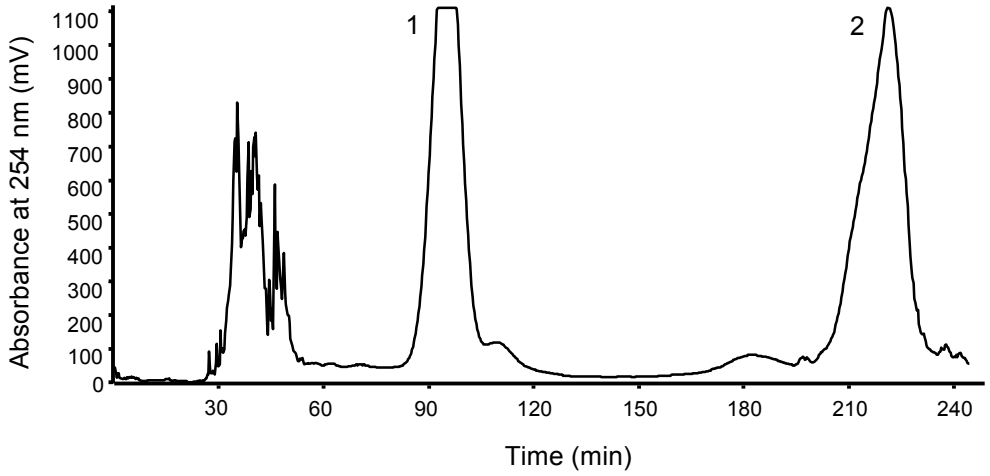

Figure 2-4. HSCCC profile of the aceytlenic alkylamides using the solvent system 3:1:2:2.

The fractions 32-37 contained the 2.6 mg of the alkylamide undeca-2*E*,4*E*-diene-8,10-dynoic acid isobuytlamide at 94% purity. The MS data confirmed the molecular ion as *m/z* 230, therefore a molecular weight of 229. This alkylamide has not previously been identified in *Echinacea angustifolia* roots, but has been identified in *Echinacea atrorubens* roots (*14*). The ^{1}H and ^{13}C NMR signals were compared with those previously published, which confirmed the structure of this alkylamide (*14*). The structure of this alkylamide is shown in Figure 2-5. The NMR and MS data are summarized in Tables 2-5 and 2-6, respectively.

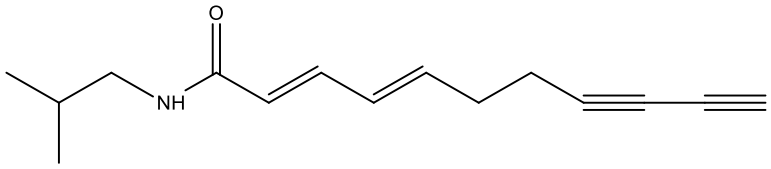

Figure 2-5. Structure of the acetylenic alkylamide undeca-2*E*,4*E*-diene-8,10-dynoic acid isobutylamide isolated with HSCCC using the solvent system 3:1:2:2.

Table 2-5. ^{1}H and ^{13}C NMR assignments for the acetylenic alkylamide isolated using a two-step HSCCC separation.

	Undeca-2*E*,4*E*-diene-8,10-dynoic acid isobutylamide	
	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)
1		166.1
2	5.80 *d* (15)	123.2
3	7.18 *dd* (15,11)	140.4
4	6.20 *dd* (15,11)	129.8
5	6.05 *dt* (15,7)	139.1
6	2.39 *m*	31.3
7	2.39 *m*	18.9
8		nd*
9		68.3
10		65.5
11	1.98 *s*	65.1
N-H	5.48 br *s*	
1’	3.17 *dd* (7,7)	47.0
2’	1.80 *m*	28.6
3’	0.90 *d* (7)	20.2
4’	0.90 *d* (7)	20.2

Measured in $CDCl_3$
*not detected

Table 2-6. MS fragmentation data obtained for the acetylenic alkylamide undeca-2*E*,4*E*-diene-8,10-dynoic acid isobutylamide using a two-step HSCCC separation.

Compound	Retention time (min)	UV λ_{max} (nm)	Molecular ion (*m/z*) $[M+H]^+$	Fragments (*m/z*)
Undeca-2*E*,4*E*-diene-8,10-diynoic acid isobutylamide	15.1	260	230	174, 167, 157, 129, 128, 116, 91

The second peak contained the alkylamide eluting at 17.5 minutes in the HPLC, with a purity of 95%. With the development of the UFLC-DAD method in section 3, it was confirmed that this peak contains two co-eluting components that could be separated with this new method. Therefore, the structure of this compound was not analyzed with NMR. The tentative structures of the two alkylamides were identified by MS as dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide and dodeca-2*E*,4*Z*,10*Z*-triene-8-ynoic acid isobuytlamide.

2.4 Conclusions

HSCCC is a successful separation method for alkylamides from *Echinacea angustifolia* roots due to its selectivity for different components based on the partition coefficients. Five alkylamides were isolated and elucidated using two

different solvent systems. One single HSCCC run was capable of isolating four alkylamides with greater than 90% purity. The second HSCCC run required a pre-fractionation step prior to isolation of a single alkylamide. Two alkylamides which have not previously been identified in *Echinacea angustifolia* roots were isolated, one of which is a new compound. HSCCC was not capable of separating alkylamides from co-eluting isomers, therefore it is recommended to evaluate the alkylamide profiles using liquid chromatography prior to HSCCC separation to determine if co-eluting alkylamides may be present.

2.5 References

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Chapter 3. UFLC-DAD-MS/MS identification of alkylamides in *Echinacea* plant materials and dietary supplements[*]

3.1 Introduction

Although five alkylamides were isolated using HSCCC, these alkylamides do not cover the range of all of these components in *Echinacea* roots and commercial products. There have been over 20 alkylamides identified in *Echinacea angustifolia* and *Echinacea purpurea* roots, while the HSCCC isolation focused on the most abundant alkylamides in *Echinacea angustifolia* roots. The use of liquid chromatography mass spectrometry (LC-MS) allows for the identification and phytochemical profiling of both major and minor alkylamides present in *Echinacea*.

Echinacea products have been among the top selling herbal dietary supplements in the United States for several years (*1-3*). In Canada these products are referred to as natural health products (NHPs) and go by a variety of different names throughout the world. In Europe and Australia they are referred to as phytomedicines, in the US as dietary supplements and have also been reported as herbal medicinal products. Interest in these products has increased in the past few decades due to interest in natural medicines, alternatives to conventional medicine and the aging population's interest in self-medication. Contrary to the development of pharmaceuticals, these products are assumed to be safe without rigorous clinical and composition testing (*4,5*). There are several limitations to these assumptions as these products contain pharmacologically active components, which may interact with prescribed drugs or cause toxic effects (*4*). Other issues with NHPs include contamination, adulteration with misidentified species or deliberate addition of cheaper species and/or active pharmaceuticals, leaving consumers unaware of the dangers of these products (*4*). For example, *Echinacea* commercial products have commonly been adulterated with *Parthenium integrifolium* L., therefore diluting or eliminating the pharmacological effects of these products (*6-8*). Therefore, quality control

[*] A version of this chapter has been published. Mudge et al. *J Agric Food Chem*. **2011**, *59*, 8086-8094.

methods are required for product authenticity and detection of product adulteration to ensure the public receives safe and effective NHPs.

Several meta-analyses have confirmed that the clinical efficacy of *Echinacea* products is inconclusive in the treatment and prevention of common colds and flu (*9-11*). NHPs containing *Echinacea* are highly variable due to the use of three different species and the blending of the roots and aerial parts of these plants. As well, NHP quality depends significantly on the quality of the phytochemical composition of the initial plant materials, and other factors including extraction method, manufacturing process, storage and handling of initial and final products (*12,13*). This leads to a wide variety of phytochemical compositions in products assumed to be similar on the marketplace. Most clinical trial and biological assay reports do not contain information on the phytochemical composition of the extracts or plant materials used, limiting the ability to determine the reasons for such inconclusive results of *Echinacea* product efficacy (*11*). Therefore, the development of methods to monitor each of the four types of active components is necessary when reporting data in the future for these types of studies.

Traditionally, alkylamides are analyzed using HPLC with ultraviolet or mass spectrometric detection for structural identification (*6,14-21*). The majority of these methods focus on the identification of alkylamides in roots, extracts and achenes of *Echinacea purpurea*, while *Echinacea angustifolia* and *Echinacea pallida* roots have not been characterized by LC-MS. Generally, the HPLC methods use reversed phase C18 columns with mobile phase compositions of an acidic aqueous solution and acetonitrile with gradient elution (*6,14-21*). Run times for these methods range from 30-45 minutes, reducing the sample throughput and increasing the consumption of solvents. Recently, there is a lot of interest in the development of fast methods for the analysis of phytochemicals in NHPs. Instrument improvements such as higher pressure limits have allowed for the use of sub 2 micron particle size columns. The benefits of these fast LC methods include increased resolution, sensitivity and sample throughput, while the

consumption of solvents is reduced (*22*). Fast LC when coupled with mass spectrometric detectors enhances the detector sensitivity (*23*).

The alkylamide profiles of *Echinacea* roots and commercial products were evaluated using a UFLC-DAD-ESI-MS/MS method. Due to the complexity of the structures of the alkylamides, and the presence of isomeric pairs, there is a possibility for co-elution to occur that may not be observed in ultraviolet detection alone. In contrast, the use of mass spectrometry allows for the identification of these minor co-eluting compounds. This versatile method allows for the identification of alkylamides in a variety of *Echinacea* products and presents the most extensive characterization of alkylamides in *Echinacea angustifolia* roots using LC-MS so far.

3.2 Experimental

3.2.1 Solvents and reagents

The solvents water, acetonitrile and methanol were HPLC grade and purchased from Fisher Scientific (Ottawa, ON). The solvents used in the mobile phase were filtered using a millipore solvent filtration system with 0.2 μm nylon filters. Formic acid (99%) was analytical grade and purchased from Fisher Scientific.

3.2.2 Plant materials and dietary supplements

The roots of *Echinacea angustifolia* and *Echinacea purpurea* were supplied from Three Feather Farms (Sherwood Park, AB). These roots were harvested in 2009 and provided as a powdered material. The roots of *Echinacea angustifolia*, *Echinacea purpurea* and *Echinacea pallida* were supplied by Naturex (South Hackensack, NJ). These were harvested in 2008 under the supervision of Dr. Wendy Applequist and the herbarium species were deposited with the Missouri Botanical Garden Herbarium with voucher numbers 217, 218 and 216 respectively. Dried *Echinacea angustifolia* and *Echinacea purpurea* root extracts were supplied from the Natural Products Research Group at the British Columbia Institute of Technology (BCIT).

Several commercial dietary supplements were purchased from local supermarkets and natural health food stores throughout Edmonton, AB. These included capsules, softgels, and tinctures with varying *Echinacea* compositions.

3.2.3 Extraction of alkylamides

3.2.3.1 Extraction of plant materials

The dried plant materials, 200 mg, were extracted with 8 mL of 70% methanol. The samples were mixed using a vortex mixer for 1 minute and extracted using a sonicating bath at room temperature for 30 minutes. Samples were allowed time to settle before 1 mL of the extract was filtered with a 0.2 μm nylon filter into an HPLC vial for analysis.

3.2.3.2 Extraction and clean-up of dried *Echinacea* extracts

The dried extracts, 200 mg, were extracted with 8 mL of 70% methanol. The samples were mixed using a vortex mixer for 1 minute and extracted using a sonicating bath at room temperature for 30 minutes. The samples were allowed to sit for 30 minutes. The samples were then cleaned up using a solid-phase extraction method described below. The cartridge, Waters Sep-pak 6CC 1.0 g C_{18}, was conditioned sequentially with ethyl acetate, methanol and water. 2 mL of the extract was mixed with 333 μL of water, to change the solvent composition to 60% methanol, which retains the alkylamides. The 2.333 μL extract was added to the Sep-pak and the eluate was discarded. The cartridge was washed with two column volumes (4 mL) of 60 % methanol and the eluate was sent to the waste. The alkylamides were then collected into a 2 mL volumetric flask using 3 mL of ethyl acetate. The extracts were dried under nitrogen to the 2 mL mark on the volumetric flask and then filtered with a 0.2 μm filter into an HPLC vial for analysis.

3.2.3.3 Extraction of dietary supplements: capsules

The contents of 10 capsules were combined and mixed thoroughly before extraction. 300 mg of the capsule contents were extracted with 8 mL of 70% methanol. The samples were mixed using a vortex mixer for 1 minute and then extracted using a sonicating bath for 30 minutes. The samples were allowed to sit

for 30 minutes before filtering with a 0.2 µm nylon filter into an HPLC vial for analysis.

3.2.3.4 Extraction of dietary supplements: tinctures

Echinacea tinctures were mixed thoroughly before extraction and prepared by dilution of the tincture using 70% methanol. 1 mL of the herbal tincture was mixed with 1 mL of 70% methanol. The solution was mixed using a vortex mixture for 1 minute. 1 mL of the solution was filtered using a 0.2 µm nylon filter into an HPLC vial for analysis.

3.2.3.5 Extraction of dietary supplements: soft-gels

The contents of 10 softgels were combined by breaking the softgel cores and removing the contents into a scintillation vial where they were mixed thoroughly before extraction. 300 mg of soft gel contents were extracted using 8 mL of 70% methanol. The samples were mixed using a vortex mixer for 1 minute and then extracted using an orbital shaker at 300 rpm for 30 minutes. The extract was filtered using a 0.2 µm nylon filter into an HPLC vial for analysis.

3.2.4 UFLC-DAD-MS/MS analysis of *Echinacea* plant materials and dietary supplements

The UFLC separation was performed on a Shimadzu (Toyko, Japan) Prominence UFLCXR liquid chromatograph system equipped with a CBM-20A communication bus module, two LC-20AD XR pumps, a DGU-20A3 vacuum degasser, a SIL-20AC XR autosampler, a CTO-20AC column oven and a SPD-M20A diode array detector. The UFLC was coupled to a 4000 QTRAP MS/MS system (ABSciex). The chromatographic separation was performed using a VisionHT C18 HL with 50 x 2.0 mm, 1.5 µm particle size (Mandel Scientific, Guelph, ON). The mobile phase was composed of A: 0.1 % formic acid in water and B: acetonitrile using a gradient elution as follows: 0-4 min (39-40% B), 4-8 min (40-50% B), 8-10.5 min (50-70 % B), 10.5-11.5 min (70% B), 11.5-12 min (70-39% B), 12-15 min (39% B). The mobile phase flow rate was 0.5 mL/min and the column oven was maintained at 20 °C. The injection volume was 2 µL. The UV spectra were collected from 200-400 nm and the alkylamides were monitored at 254 nm. The mass spectrometer was equipped with electrospray ionization

operating in positive mode. Nitrogen gas (>99%) was used as nebulizing gas (Gas 1, GS1) and collision gas (Gas 2, GS2). The sensitivity of the mass spectrometer was optimized for signal intensity of the molecular ion and fragmentation using full scan and product ion scans, respectively with the alkylamide standard dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, previously isolated using HSCCC as described in chapter 2. The values for optimum spray voltage, source temperature, GS1, GS2 and curtain gases were +4.5 kV, 600 °C, 50, 50 and 30 psi, respectively. The declustering potential was 66.0 V, the collision energy was 23.0 eV, the entrance potential was 10 V and the collision exit potential was 10 V. The spectra were obtained over a mass range of 50 to 500 amu. MS/MS analyses were performed using the information-dependent acquisition (IDA) method where the mass spectrometer alternates between the enhanced MS (EMS) scan for the full scan and the enhanced product ion (EPI) scan and generates MS/MS data on the eight most intense peaks. This allows for the identification of both the major and minor components eluting from the column. In this method, extracted ion chromatograms and collision-induced dissociation are simultaneously acquired and high selectivity is obtained. The scan rate for EMS was 1000 amu/s and for EPI was 4000 amu/s. The data acquisition was performed using Analyst 1.5 software (ABSciex).

3.3 Results and Discussion

3.3.1 Extraction optimization of alkylamides from *Echinacea* roots and dietary supplements

Extraction protocols of alkylamides from *Echinacea* root materials are highly variable between publications. Several focus on the separation of caffeic acid derivatives, therefore optimization of alkylamides is not considered. The optimal extraction solvent and the ratio of solvent to material are essential to ensure complete extraction is performed. Since extraction can be time consuming when performed in multiple steps, complete extraction in one step is desirable.

The percentage of methanol used as extraction solvent was assessed from 20-100% when extracting 300 mg of plant material with 15 mL of solvent. The peak area of the tetraenoic alkylamide was plotted versus the percentage of

methanol as shown in Figure 3-1. The optimal methanol concentrations ranged from 70-100%, therefore to be consistent with previous publications 70% methanol was chosen as the extraction solvent.

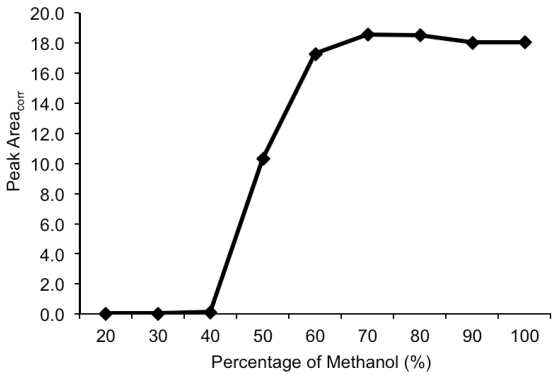

Figure 3-1. Amount of the tetraenoic alkylamide extracted with different percentages of methanol.

In order to evaluate the ratio of plant material to extraction solvent, two extraction solvent levels were chosen (8 and 10 mL). The amount of plant material evaluated ranged from 100-500 mg. The extraction efficiency was determined by removing all of the solvent and adding fresh solvent to the extracted *Echinacea* roots. The results of the extraction efficiency are shown in Table 3-1. Both 100 and 200 mg resulted in over 95% extraction of the alkylamides from *Echinacea angustifolia* roots with 8 mL of solvent, which contains the most abundant levels of alkylamides, therefore was used for selecting the amount of plant material. Due to the increased peak intensity with 200 mg versus 100 mg of plant material, the optimal extraction was 200 mg of *Echinacea angustifolia* roots using 8 mL of 70% methanol.

Table 3-1. Percentage of the tetraenoic alkylamide extracted in the first step evaluated using different ratios of plant material and extraction solvent.

Mass of plant material (mg)	Volume of Extraction Solvent (mL)	Extraction Efficiency of *E. angustifolia* (%)	Extraction Efficiency of *E. purpurea* (%)
100	10	95.1	95.0
200	10	93.7	91.5
300	10	92.8	88.8
400	10	89.5	60.2
500	10	68.7	68.2
100	8	90.3	95.5
200	8	95.2	92.9
300	8	87.8	86.6
400	8	85.5	86
500	8	77.5	81.2

3.3.2 Separation of alkylamides by UFLC

The separation of alkylamides by UFLC was optimized by changing several conditions including the mobile phase composition, the gradient, the column and oven temperature. The separation was reduced from tradition HPLC methods at 30-45 minutes in length to 15 minutes. A high load C18 column was selected to improve the separation of the isomer pairs of alkylamides present in *Echinacea*. Based on the optimal separation conditions described in section 3.2.4, there was an improvement in the separation of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide compared to previously published methods (*15,17,18,21*). Based on the DAD chromatograms at 254 nm, in *Echinacea purpurea* 15 peaks were observed and in *Echinacea angustifolia* 17 peaks were observed as shown in Figures 3-2b and 3-3b respectively. The large peak eluting at the beginning of the chromatogram is the co-extracted phenolic compounds which were not considered in this analysis.

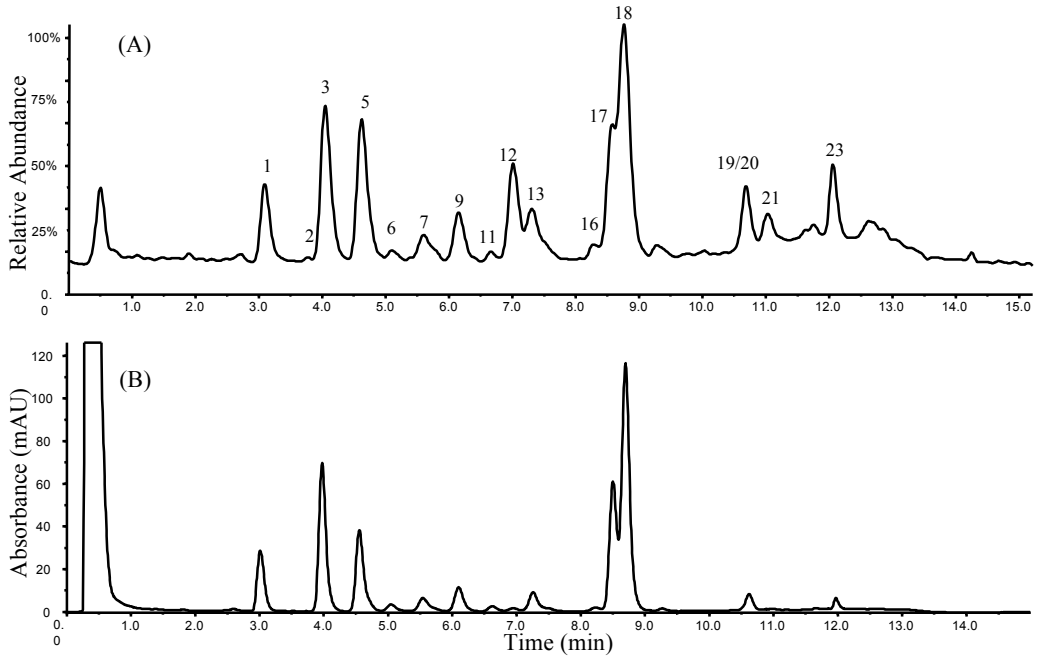

Figure 3-2. Separation of alkylamides from the roots of *Echinacea purpurea* grown in Alberta by UFLC; TIC(A) and UV (B) at 254 nm.

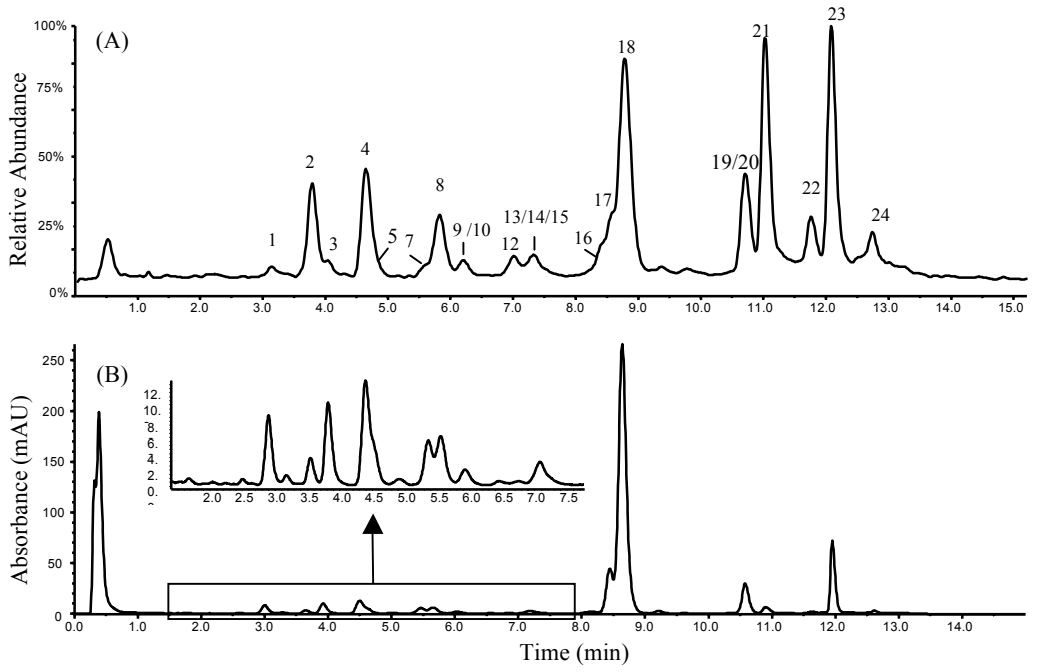

Figure 3-3. Separation of alkylamides from the roots of *Echinacea angustifolia* from plants grown in Alberta by UFLC; TIC (A) and UV (B) at 254 nm.

3.3.3 Identification of alkylamides in *Echinacea* roots

Alkylamides were identified in *Echinacea purpurea* roots from two sources, *Echinacea angustifolia* roots from two sources and *Echinacea pallida* from one source. Based on these results, 17 alkylamides were identified in *Echinacea purpurea*, 22 alkylamides in *Echinacea angustifolia* and no alkylamides were identified in this source of *Echinacea pallida*. The TIC chromatograms for *Echinacea purpurea* roots and *Echinacea angustifolia* roots are shown in Figure 3-2a and 3-3a. Due to the presence of minor alkylamides, which were not observed in the DAD chromatograms, the sensitivity of the mass spectrometer allows for the separation and identification of these minor alkylamides that co-elute with other major alkylamides. All alkylamides identified in *Echinacea angustifolia* and *Echinacea purpurea* roots are summarized in Table 3-2. The corresponding structures are provided in Figure 3-4.

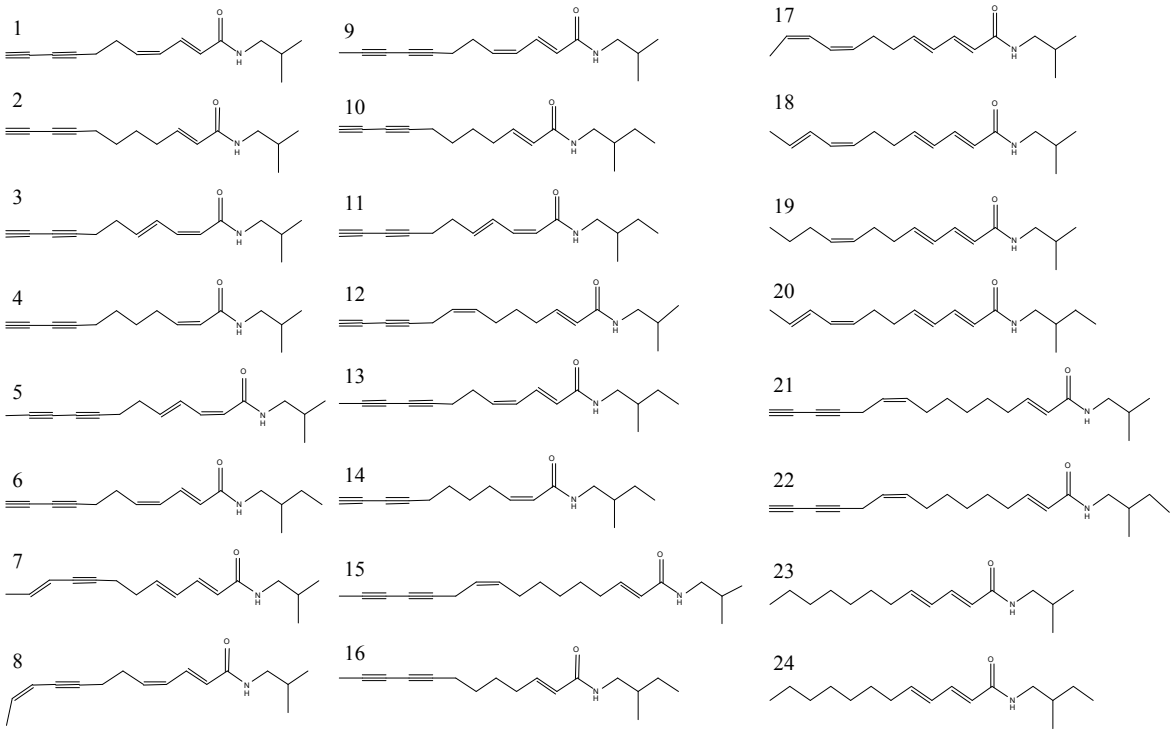

Figure 3-4. Structures of alkylamides found in *Echinacea angustifolia* and *Echinacea purpurea* roots. The compound numbering is based on the elution order using the UFLC method.

Table 3-2. Assignment of alkylamides in *Echinacea angustifolia* and *Echinacea purpurea* roots.

Peak No.	t_R (min)	Precursor $[M+H]^+$ (*m/z*)	Product Ions (*m/z*)	Compound	Present in *E. purpurea*	Present in *E. angustifolia*
1	3.0	230	174, 167, 157, 129, 128, 116, 91	Undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	X	X
2	3.6	232	176, 159, 131, 105, 91, 79	Undeca-2*E*-ene-8,10-diynoic acid isobutylamide	X	X
3	4.0	230	188, 174, 166, 157, 146, 129, 116, 105, 91	Undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	X	X
4	4.5	232	176, 159, 131, 105, 91, 79	Undeca-2*Z*-ene-8,10-diynoic acid isobutylamide		X
5	4.6	244	188, 171, 167, 145, 117, 105,	Dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	X	X
6	5.0	244	174, 157, 131, 129, 116, 91	Undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide	X	
7	5.5	246	190, 173, 145, 119, 105, 91, 79	Dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide	X	X
8	5.7	246	190, 173, 147, 143, 119, 105, 91, 79	Dodeca-2*E*,4*Z*,10*Z*-triene-8-ynoic acid isobutylamide		X
9	6.1	244	188, 171, 148, 128, 117, 105	Dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	X	X
10	6.1	246	176, 148, 133, 131, 105, 91, 77	Undeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide		X
11	6.6	244	180, 174, 157, 146, 131, 129, 117, 91	Undeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide	X	
12	6.9	258	202, 157, 131, 117, 91	Trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide	X	X
13	7.2	258	188, 171, 160, 143, 129, 128, 117, 105	Dodeca-2*E*,4*Z*-diene-8,10-diynioc acid 2-methylbutylamide	X	X
14	7.2	246	218, 176, 159, 131,105, 91	Undeca-2*Z*-ene-8,10-diynoic acid 2-methylbutylamide		X
15	7.2	300	244, 227, 199, 166, 153, 91, 77	Hexadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide		X
16	8.2	260	190, 173, 147, 145, 105, 91	Dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide	X	X
17	8.5	248	192, 175, 167, 166, 152, 149, 107, 79	Dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide	X	X
18	8.7	248	192, 175, 167, 166, 152, 107, 79	Dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide	X	X
19	10.6	250	194, 177, 167, 152, 109, 95	Dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide	X	X
20	10.65	262	180, 166, 145, 107, 79	Dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide	X	X
21	10.9	286	230, 213, 171, 145, 143, 105, 91	Pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide	X*	X
22	11.6	300	230, 213,185, 171, 159, 145, 105, 91	Pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid 2-methylbutylamide		X
23	11.9	252	196, 179, 161, 95	Dodeca-2*E*,4*E*-dienoic acid isobutylamide	X	X
24	12.6	266	210, 196, 179, 133, 109, 95	Dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide		X

* Detected only in *Echinacea purpurea* roots grown in Alberta

The three standards isolated from the HSCCC were used to identify compounds eluting at the same retention time in *Echinacea angustifolia* and *Echinacea purpurea* roots, where their structures were confirmed by the molecular ion and MS/MS fragmentation patterns. They were identified as dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide with a retention time of 8.5-8.7 min , dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide with a retention time

of 10.6 min, and dodeca-2*E*,4*E*-dienoic acid isobutylamide with a retention time of 11.9 min. Since the HSCCC separation was capable of separating the two isomers of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, the *E* isomer was isolated with 97% purity and the structure was confirmed by NMR. Using this standard, it was confirmed that the elution order of the isomeric pair is the *Z* isomer (8.5 min) followed by the *E* isomer (8.7 min). This is consistent with previously published work (*19*).

It has been reported that 17 alkylamides are present in *Echinacea purpurea*, although only 15 reported in other publications could be identified in the *Echinacea purpurea* roots evaluated in this study (*15,18,21*). Undeca-2*E*-ene-8,10-diynoic acid isobutylamide was observed, but dodeca-2*E*-ene-8,10-diynoic acid isobutylamide was not. This is contradictory to the most recently published data and is possibly due to different genotypes or growing conditions (*21*). There are two additional alkylamides, which have been identified in these *Echinacea purpurea* roots. Dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide was identified in both samples while pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide was found only in the roots from the Alberta source. Both alkylamides have not previously been identified in *Echinacea purpurea* roots. All other alkylamides in *Echinacea purpurea* were identified based on molecular ions and fragmentation data available in previously published works (*15,21*).

There is more variation in the structures of the alkylamides in *Echinacea angustifolia* roots when compared with *Echinacea purpurea* roots (*14,24*), which has been confirmed in this study. Based on the LC-MS data, 22 alkylamides were identified in *Echinacea angustifolia* roots, several of which have not previously been characterized using LC-MS. Co-elution does occur with minor alkylamides which would not be observed using DAD analysis only and would most likely not separate using longer separation times, therefore sufficient separation was achieved with this method.

Alkylamides not previously characterized using LC-MS were identified by comparing their fragmentation patterns to those in published methods and comparing molecular ions with alkylamides previously identified using NMR by

Bauer et al. (*24,25*). Molecular formula were assigned for these unknown alkylamides based on the molecular ion minus one proton and the assumptions that these components are alkylamides, contain one nitrogen, one oxygen and the other typical structural patterns of alkylamides. Accurate mass determinations were not performed with this analysis.

The alkylamide eluting at 4.5 minutes had a molecular ion of *m/z* 232 $[M+H]^+$ and the fragmentation pattern was identical to undeca-2*E*-ene-8,10-ynoic acid isobutylamide (peak 2), present in *Echinacea purpurea* roots. Therefore, this alkylamide was identified as undeca-2*Z*-ene-8,10-ynoic acid isobutylamide. UV spectra were assessed to determine if there are variations between the *cis* and *trans* bonds, similar to carotenoids (*26*), but due to the co-elution of the two alkylamides at 4.5 and 4.6 minutes, this could not be verified.

Four alkylamides were detected with molecular ions of *m/z* 246 $[M+H]^+$ in *Echinacea angustifolia*. Two of these alkylamides (peaks 7 and 8) had very close retention times and could not be completely separated. Their fragmentation patterns, shown in Figure 3-5a-b, are also identical. The fragment *m/z* 190 corresponds to the loss of the isobutyl group (-56 Da) and the fragment 173 corresponds to the loss of the isobutyl amine (-73 Da), indicating that these two alkylamides are isobutylamides. The molecular formula, $C_{16}H_{23}NO$, requires a degree of unsaturation of six, therefore the alkyl chain must contain twelve carbons with either one triple bond and three double bonds or two triple bonds and one double bond. It has been proposed that MS/MS fragmentation can be used to differentiate between diene or monoene alkylamides. The presence of two fragments separated by two mass units would represent the fragmentation that occurs between carbons 1 and 2 for diene alkylamides (*21*). According to the fragmentation assignments by Spelman et al. (2009), the fragments observed are the alkyl chain (group ii), and the alkyl chain with 1 bond saturating and the second double bond transitioning to the 3 position (group iii) (*21,27*). MS/MS analysis confirmed the presence of these two fragments (*m/z* 145 and 147) and is labeled in Figure 3-4a-b, confirming these alkylamides as dienes, which contain three double bonds and one triple bond. The alkylamide eluting at 5.5 minutes

was present in *Echinacea purpurea* roots, while the alkylamides eluting at 5.7 minutes was only present in *Echinacea angustifolia*. The configuration of the double bonds cannot be confirmed by MS, but previously published data confirmed that dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide is present in *Echinacea purpurea*, therefore this is the tentative assignment of the alkylamide eluting at 5.5 minutes (*25*). In *Echinacea angustifolia*, the presence of the alkylamide dodeca-2*E*,4*Z*,10*Z*-triene-8-ynoic acid isobutylamide has been confirmed by NMR, therefore this is the tentative assignment of the alkylamide eluting at 5.7 minutes (*24*).

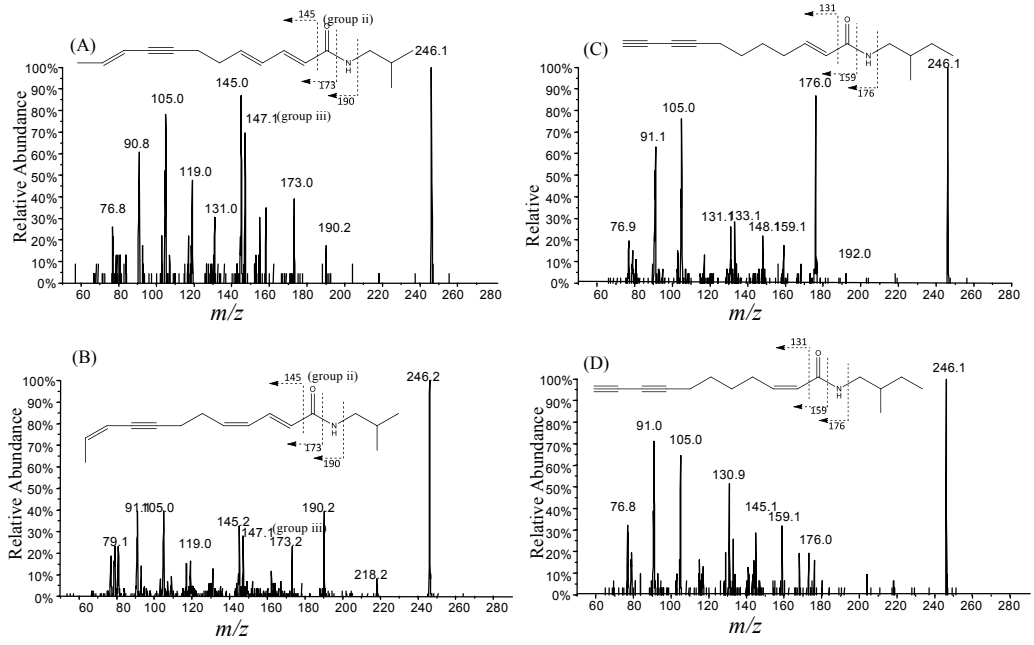

Figure 3-5. MS/MS spectra of isomeric pairs with the molecular ion of *m/z* 246. (A) an isobutylamide eluting at 5.5 minutes; (B) an isobutylamide eluting at 5.7 minutes; (C) a 2-methylbutylamide eluting at 6.1 minutes; (D) a 2-methylbutylamide eluting at 7.2 minutes.

Two other alkylamides with a molecular ion of *m/z* 246 $[M+H]^+$ were identified in *Echinacea angustifolia* roots with retention times of 6.1 and 7.2 minutes (peaks 10 and 14). As shown in Figure 3-5c-d, the fragment *m/z* 176 corresponds to the loss of the 2-methylbutyl group (-70 Da) and the fragment *m/z* 159 corresponds to the loss of the 2-methylbutyl amine (-87 Da). This confirms these alkylamides are 2-methylbutylamides and the alkyl chain contains 11 carbons. Since both alkylamides have similar fragmentation patterns, the

structures must differ by double bond configuration. The absence of the group iii fragments confirms these are monene alkylamides with one double bond and two triple bonds (*21*). For previously identified monene isomeric pairs (peaks 2 and 4 in Figure 3-3b), the elution order has been established as the *E* isomer eluting before the *Z* isomer of undeca-2-ene-8,10-diynoic acid isobutylamide. Therefore, the same elution order can reasonably be assigned for these alkylamides. Undeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide has been assigned to the alkylamide eluting at 6.1 minutes and undeca-2*Z*-ene-8,10-diynoic acid 2-methylbutylamide has been assigned as the alkylamide eluting at 7.2 minutes.

Two alkylamides with the molecular ion of *m/z* 300 $[M+H]^+$ were present with retention times of 7.2 and 11.6 minutes. The fragmentation pattern of the earlier eluting alkylamide had fragments of *m/z* 244 and *m/z* 227, which correspond to the loss of the isobutyl group and isobutyl amine, respectively. The fragment between carbons 1 and 2 of the alkyl chain produced a fragment at *m/z* 199. The molecular formula of $C_{20}H_{19}NO$ confirms an alkyl chain length of sixteen carbons and degree of unsaturation of seven. Therefore, the alkylamide hexadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide previously isolated by Bauer et al. (*24*) has tentatively been assigned to this alkylamide, although the double bond configuration cannot be confirmed solely using MS/MS fragmentation. The MS/MS fragmentation of alkylamide eluting at 11.6 minutes had the fragments 230 and 213 corresponding to the loss of the 2-methylbutyl group and 2-methylbutyl amine, thus confirming the structure as an 2-methylbutylamide. The molecular formula requires that this alkylamide contain an alkyl chain of fifteen carbons and a degree of unsaturation of seven. The tentative assignment of this alkylamide is pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid 2-methylbutylamide, which has also been isolated from *Echinacea angustifolia* by Bauer et al. (*14*). The presence of this alkylamide has also been reported in an *Echinacea* extract containing a mixture of *Echinacea angustifolia* and *Echinacea purpurea*, however no structural information was provided (*28*). For both of these alkylamides, the absence of the group iii fragments confirms that these alkylamides are not conjugated 2,4-dienes (*21*).

An alkylamide with a molecular ion of *m/z* 260 $[M+H]^+$ was present in the roots of both *Echinacea angustifolia* and *Echinacea purpurea*. It was characterized as a 2-methylbutylamide due to the presence of the fragments *m/z* 190 and *m/z* 173, which correspond to the loss of the 2-methyl butyl group and 2-methylbutyl amine. The molecular formula, $C_{17}H_{25}NO$, confirms a degree of unsaturation of six and an alkyl chain length of twelve, therefore this alkylamide was tentatively identified as dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide (*24*). The other alkylamide that was present in both root materials had a molecular ion of *m/z* 286 $[M+H]^+$. The MS/MS fragmentation confirmed the structure as an isobutylamide with the fragments *m/z* 230 and 213. The molecular formula, $C_{19}H_{27}NO$, requires an alkyl chain of fifteen carbons and a degree of unsaturation of seven. The alkylamide pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide previously isolated from *Echinacea angustifolia* roots meets these requirements, therefore this was the tentative assignment of this alkylamide (*24*). The last alkylamide eluting at 12.6 minutes had a molecular ion of *m/z* 266 $[M+H]^+$. The fragments *m/z* 196 and 179 confirm the structure as a 2-methylbutylamide. This alkylamide was identified as dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide, which was isolated using HSCCC in Chapter 2.

The alkylamide profiles were different between *Echinacea angustifolia* and *Echinacea purpurea*, which can be observed in Table 3-2. The UFLC-DAD chromatograms also had some distinct differences, where the intensity of the acetylenic alkylamides was much higher relative to the olefinic alkylamides in *Echinacea purpurea*. While in *Echinacea angustifolia*, the intensity of the acetylenic alkylamides was much lower relative to the main tetraenoic alkylamide and the other olefinic alkylamides. Two alkylamides, undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide and undeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide, were exclusive to *Echinacea purpurea*. There were several alkylamides, shown in Table 3-2, which are also exclusive to *Echinacea angustifolia* roots. This confirms that this method has the potential to differentiate between different *Echinacea* species and could be used as a diagnostic tool for

plant identification, although many more plants from different regions would need to be characterized to confirm this.

3.3.4 Identification of alkylamides in *Echinacea* dietary supplements

The commercial dietary supplements that were purchased from local grocery and natural food stores are listed in Table 3-3. The compositions of these products vary considerably since most products containing *Echinacea purpurea* are composed of blended roots and aerial parts. To further complicate the compositions, the amount of material used in one dose of these products also varies significantly between products.

Table 3-3. Label specifications of products analyzed for alkylamides using UFLC-DAD-MS/MS.

No.	Product Type	Contents
1	Liquid tincture	*Echinacea purpurea* flower and root
2	Liquid tincture	*Echinacea purpurea* flower and root
3	Liquid tincture	*Echinacea purpurea* and *Echinacea angustifolia* roots
4	Capsule, dried extract	Extract of *Echinacea purpurea* aerial parts
5	Capsule, dried extract	Extract of *Echinacea purpurea* roots
6	Softgel, extract	Extract of *Echinacea purpurea* aerial parts and root
7	Softgel, extract	Extract of *Echinacea purpurea* aerial parts and roots
8	Softgel, extract	Extract of *Echinacea angustifolia* roots
9	Softgel, extract	Extract of *Echinacea angustifolia*
10	Powdered crude extract	*Echinacea angustifolia* root extract (BCIT)

The alkylamides in the dietary supplements were identified based on the molecular ions, retention times and MS/MS fragmentation patterns previously identified in the root materials described in section 3.3.3. The isomeric pair of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobuytlamide was detected in eight of the nine commercial products evaluated. Based on the label of the product that did not contain this alkylamide, it is said to be composed of only the aerial parts of *Echinacea purpurea*. As shown in Table 3-4, it is evident that the alkylamide profiles of commercial *Echinacea* preparations vary considerably.

Table 3-4. Assignment of alkylamides in commercial *Echinacea* products and crude extracts.

	Presence in *Echinacea* Products									
Alkylamide	1	2	3	4	5	6	7	8	9	10
Undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	X	X	X	X	X	X	X	X	X	
Undeca-2*E*-ene-8,10-diynoic acid isobutylamide	X	X	X		X			X	X	X
Undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	X	X	X		X	X		X	X	X
Undeca-2*Z*-ene-8,10-diynoic acid isobutylamide			X					X	X	X
Dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	X	X	X		X			X		X
Undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide	X	X	X		X					
Dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide	X	X	X		X			X	X	X
Dodeca-2*E*,4*Z*,10*Z*-triene-8-ynoic acid isobutylamide			X					X	X	X
Dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	X		X		X					X
Undeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide										
Undeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide										
Trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide	X	X	X							
Dodeca-2*E*,4*Z*-diene-8,10-diynioc acid 2-methylbutylamide			X		X					
Undeca-2*Z*-ene-8,10-diynoic acid 2-methylbutylamide								X	X	X
Hexadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide										
Dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide			X							X
Dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide	X	X	X		X	X	X	X	X	X
Dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide	X	X	X		X	X	X	X	X	X
Dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide	X	X	X		X	X	X	X	X	X
Dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide	X	X						X	X	X
Pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide			X					X		X
Pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid 2-methylbutylamide								X	X	X
Dodeca-2*E*,4*E*-dienoic acid isobutylamide	X	X	X		X	X	X	X	X	X
Dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide								X	X	X
Dodeca-2*E*,4*E*,8*E*,10*Z*-tetraenoic acid isobutylamide	X	X	X							

An alkylamide in the commercial products, which was not identified in *Echinacea angustifolia* or *Echinacea purpurea* roots, was identified at a retention time of 9.2 minutes with a molecular ion of *m/z* 248 $[M+H]^+$. The fragment *m/z* 175 corresponds to the loss of the isobutyl amine. Two other fragments *m/z* 152 and *m/z* 166 represent the loss of seven and six carbons from the alkyl chain, respectively. This MS/MS fragmentation pattern was identical to the two main tetraenoic alkylamides, therefore the structure is in agreement with dodeca-2,4,8,10-tetraenoic acid isobuytlamide, with different stereochemistry. Previously, the alkylamide dodeca-2*E*,4*E*,8*E*,10*Z*-tetraenoic acid isobutylamide in *Echinacea purpurea* was reported, but was unable to separate it from its isomers

using reversed phase HPLC (*21*). This is the tentative assignment of this alkylamide, although the exact configuration of the double bonds cannot be confirmed using MS detection.

The labels of two dietary supplements (#8-9) claim that the products contain only the roots of *Echinacea angustifolia*. Using mass spectrometry as a potential diagnostic tool to determine the *Echinacea* species used in these products, the alkylamide profiles were compared to the root materials analyzed in this study. The alkylamide profiles in these products were identical to those in the roots of *Echinacea angustifolia*, giving a strong indication that *Echinacea angustifolia* roots were used in these two products. The two alkylamides exclusive to *Echinacea purpurea* were not present, while the alkylamides undeca-2*Z*-ene-8,10-diynoic acid isobuytlamide and dodeca-2*E*,4*Z*,10*Z*-triene-8-ynoic acid isobutylamide were present. Two other alkylamides, pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid 2-methylbutylamide and dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide were also identified in these roots materials.

The products containing *Echinacea purpurea* roots were usually blended with the aerial parts as well. One product contained only a root extract of *Echinacea purpurea* (#5), and the alkylamides identified in this product were consistent with those found in the roots of *Echinacea purpurea* summarized in section 3.3.3. The alkylamide undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide which is exclusive to *Echinacea purpurea* was present in this extract, while the other diagnostic alkylamide was not observed. Based on the range of the products in this study, the most common products available in the Canadian marketplace are preparations containing blended *Echinacea purpurea* roots and aerial parts, which modifies the presence and levels of alkylamides in the final products, complicating the utilization of this method as a diagnostic tool for identifying *Echinacea purpurea* roots.

3.4 Conclusions

In conclusion, UFLC-DAD-MS/MS method was developed for the qualitative analysis of alkylamides in *Echinacea* root materials and commercial products. This method is successful in separating and identifying alkylamides in

two different plant species, giving insight into the alkylamide profiles which could, in the future, be used as a diagnostic tool for species differentiation based on the presence or absence of alkylamides which are exclusive to that plant species. It will be necessary to analyze additional samples and build a database representing the chemical diversity of *Echinacea angustifolia* and *Echinacea purpurea* from several regions. The main drawback of this method is that double bond configuration cannot be verified by mass spectrometry, therefore only tentative assignments of alkylamides can be given. This method is superior to previously published methods due to the decreased analysis time, lower consumption of solvents but also because this method is the most extensive characterization of alkylamides in *Echinacea angustifolia* roots by LC-MS. Therefore, it can be used in future studies to characterize products used in biological activity and clinical trial reports. The sensitivity of the mass spectrometer allows for the identification of minor alkylamides, which would not otherwise be detected using LC-DAD analysis. Based on these results, the alkylamides present in commercial preparations containing pure root materials can be used as a potential diagnostic tool for species identification when compared with the alkylamide profiles of the respective root materials.

3.5 References

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Chapter 4. Quantitation of alkylamides in *Echinacea* plant materials and dietary supplements

4.1 Introduction

Detection and tentative assignments of alkylamides in *Echinacea* roots and NHPs was possible with UFLC-DAD-MS/MS as described in Chapter 3. Alkylamide profiles were examined for differences between the different species, which can potentially be used in species identification and alkylamide profiling in final NHPs for quality and authenticity purposes.

Although identification of alkylamides is an important aspect of *Echinacea* NHP quality control, quantitative analysis of alkylamide content is also necessary. As with the phytochemical profiles of NHPs, the levels of alkylamides depend on manufacturing processes and the quality of the initial plant material used (*1*). Alkylamides are known to be most abundant in *Echinacea angustifolia* roots and lower amounts are present in *Echinacea purpurea* roots (*2-4*). The aerial parts of *Echinacea purpurea* and *Echinacea angustifolia* also have minor levels of alkylamides (*4*). Therefore alkylamides in NHPs will depend significantly on the *Echinacea* species used in the final products.

The variability in *Echinacea* products will impact product quality and efficacy. Currently there is one product available (Echinilin), which has been standardized for caffeic acid derivatives, alkylamides and polysaccharides which has produced positive results in two double blind clinical studies (*5,6*). Most other commercial NHPs have not followed this type of standardization procedure. The levels of alkylamides and caffeic acids were evaluated in several products available in Australia with levels of alkylamides ranging from 0-1.4 mg/g, indicating a large amount of variation between similar products (*7*). This is typical in most NHPs, such as in gingko products where the levels of active components range from nothing to 400 times the reported levels (*8*). Based on these observations, it has been recommended that product labels contain quantitative reports of the phytochemical compositions (*7*).

Methods developed for the quantitation of active components in NHPs and plant extracts should undergo validation to ensure the methods are suitable for their intended use. Validation characteristics such as precision, accuracy, linearity,

and specificity are all essential to evaluate the performance of a method. Several HPLC methods for alkylamides and caffeic acid derivatives have been validated (*9-11*). As new methods are developed with different extraction and separation parameters, they should undergo validation to confirm their suitability.

The UFLC separation optimized in Chapter 3 has been used for the quantitation of alkylamides in a variety of *Echinacea* roots and NHPs. The quantitation was performed using the peak areas of the ultraviolet chromatograms, therefore mass spectrometric detection was not used. This method was validated for accuracy, precision, linearity and detection limits and the stability of the alkylamide standards were evaluated under ideal storage conditions.

4.2 Experimental

4.2.1 Solvents and reagents

The solvents water, methanol, and acetonitrile were HPLC grade and purchased from Fisher Scientific (Ottawa, ON). The solvents used as mobile phases were filtered using a millipore filtration system with 0.2 µm nylon filters. Formic acid (>99%) was analytical grade and purchased from Fischer Scientific. The alkylamide standards dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (alkylamide 1), dodeca-2*E*,4*E*,8*Z*-trienoic acid isobuytlamide (alkylamide 2) and dodeca-2*E*,4*E*-dienoic acid isobuytlamide (alkylamide 3) were isolated using HSCCC as described in section 2.2.6.1. The purities, as determined by qNMR are 92%, 94% and 99.5% respectively, which was analyzed externally.

4.2.2 Plant materials and extraction

The plant materials and dietary supplements are summarized in section 3.2.2. The extraction parameters vary depending on the type of material being quantified. All extraction parameters have been summarized in section 3.2.3. The plant materials, extracts and dietary supplements were extracted and injected in duplicate.

4.2.3 Preparation of calibration standards

Stock solutions of the three alkylamide standards were prepared at a concentration of 1 mg/mL taking into account the purity determined by qNMR.

Working stock solutions were prepared for each standard, 500 µg/mL alkylamide 1, 200 µg/mL alkylamide 2 and 200 µg/mL alkylamide 3. The standards were then combined at the concentrations described in Table 4-1 for the construction of the high concentration calibration curve. Due to the large variability in the concentrations of alkylamides between different matrices, a second calibration curve is required for alkylamide 2 and 3. A secondary working stock solution containing 10 µg/mL of both alkylamides was prepared and used for the construction of the low concentration calibration curve. The concentration of the standards is shown in Table 4-2. These standards are analyzed in duplicate using the UFLC-DAD where calibration curves were obtained by plotting the peak area of the standards versus the concentration.

Table 4-1. Concentration of the alkylamides in each of the standard mix solutions.

Calibration Standard	Alkylamide 1 (µg/mL)	Alkylamide 2 (µg/mL)	Alkylamide 3 (µg/mL)
Standard 1	100	50	50
Standard 2	75	30	30
Standard 3	50	20	20
Standard 4	25	10	10
Standard 5	10	5	5
Standard 6	5	2	2
Standard 7	1	0.5	0.5

Table 4-2. Concentration of the alkylamides in each of the standard mix solutions for the low concentration calibration curve.

Calibration Standard	Alkylamide 2 (µg/mL)	Alkylamide 3 (µg/mL)
Low Standard 1	1	1
Low Standard 2	0.75	0.75
Low Standard 3	0.5	0.5
Low Standard 4	0.25	0.25
Low Standard 5	0.1	0.1
Low Standard 6	0.075	0.075
Low Standard 7	0.05	0.05

4.2.4 UFLC-DAD quantitation of alkylamides

The LC separation was performed using the UFLCXR Prominence liquid chromatograph as described in section 3.2.4, with the exception that the instrument was not coupled with the mass spectrometer. The quantitative analysis

was performed using the UV chromatogram at 254 nm. The data acquisition was performed using LCSolution software (Shimadzu).

4.2.5 Method validation

4.2.5.1 Extraction efficiency

Two replicate samples of *Echinacea angustifolia* roots (AB source, 200 mg), a capsule product (300 mg) and one softgel product (300 mg) were extracted with 8 mL of 70% methanol according to the methods described in section 3.2.3. Half of the supernatant was removed (4 mL) from each of the samples, and 1 mL of each was filtered using a 0.2 μm nylon filter to be analyzed with the UFLC. A fresh aliquot of 4 mL of 70% methanol was added to the extracts and this procedure was repeated 2 more times. This method was used to confirm the extraction efficiency for the methods described above, where if the amount extracted the second time was higher than 50% complete extraction was not achieved in the first extraction.

4.2.5.2 Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were determined according to the method described in the ICH guidelines: Validation of analytical procedures: text and methodologies *(12)*. The standard deviation of the blank was extrapolated from the plots of the standard deviations of samples injected in triplicate at concentrations in the range of the LOD. This standard deviation was then used to determine LOD and LOQ.

4.2.5.3 Precision

Three solutions were prepared in triplicate to evaluate repeatability and intermediate precision. The high concentration solution contained 50 μg/mL of alkylamide 1, and 10 μg/mL of alkylamides 2 and 3. The medium concentration contained 10 μg/mL of alkylamide 1 and 2 μg/mL of alkylamides 2 and 3. The low concentration contained 2 μg/mL of alkylamide 1 and 0.4 μg/mL of alkylamides 2 and 3. Repeatability was reported as the relative standard deviation of the triplicate analyses within a single run. Intermediate precision was reported as the relative standard deviation of the back calculated concentrations on three

separate days. The precision for extraction of alkylamides was evaluated using different product matrices from six products extracted in triplicate on three separate days. Repeatability and intermediate precision were determined the same way as for the standards.

4.2.5.4 Accuracy

Standard addition analysis was performed in order to evaluate the method accuracy and recovery. Two standard solutions containing all three standards at 8 μg/mL and 80 μg/mL were made. Two spike levels were evaluated for each standard and three replicates were prepared for each. 200 mg of *Echinacea angustifolia* (Naturex) was weighed into centrifuge tubes. 1 mL of standard solution was added to each replicate (3 of 8 μg/mL, 3 of 80 μg/mL). 7 mL of 70% methanol was added to each solution and vortexed for 1 minute. These samples were extracted using sonication at room temperature for 30 minutes. 1 mL aliquots were removed and filtered with 0.2 μm nylon filters and analyzed using UFLC. The expected concentration increases for the two levels are 1 and 10 μg/mL. The percent recovery was determined by the ratio of the calculated to expected concentration increase. The standard error of the method was also evaluated from the standards analyzed in section 4.2.5.3 by back calculating the concentration using the calibration curves.

4.2.5.5 Stability of alkylamide standards

The alkylamide standards 1 and 3 were dried and stored in amber containers in nitrogen atmosphere at -20 °C. Stock solutions were prepared at concentrations of 1 mg/mL, taking into account their purity using 5 mL volumetric flasks. The initial standard solutions were used for the construction of the calibration curves throughout the stability study. The calibration curves were constructed with concentration ranges of 0.2-100 μg/mL for alkylamide 1 and 0.1-50 μg/mL for alkylamide 3. The dry standards were prepared at concentrations of 1 mg/mL in 5 mL volumetric flasks every two weeks for 12 weeks. A final evaluation was performed at 19 weeks. Once the standards were weighed, the containers were purged with nitrogen and stored at -20 °C. These stocks were

diluted to 10 and 25 µg/mL and analyzed using the UFLC to determine the calculated concentration compared to the expected concentration. During the study, the initial solution was also evaluated for concentration according to the above parameters to determine if there is an effect on the concentration in both liquid and dried alkylamide standards. This solution was also purged with nitrogen after opening and stored at -20 °C between analyses.

4.2.5.6 Stability of alkylamides in plant materials and liquid extracts

Three plant materials were selected to study the stability of alkylamides in ground plant materials: *Echinacea angustifolia* roots (AB source), *Echinacea purpurea* roots (AB source) and *Echinacea angustifolia* extract (BCIT source). These materials were evaluated for stability of alkylamides 1 and 3 present in the plant materials stored at -20 °C and for their stability in the extracted solutions stored at -20 °C for 12 weeks. The plant materials and dried extracts were extracted according to the methods described in sections 3.2.3.1 for the plant materials and 3.2.3.2 for the dried extract. The plant materials were extracted on the initial day of the stability study and analyzed for alkylamide content. These samples were stored and analyzed every two weeks for the remainder of the study. Every two weeks, new samples of the plant materials were extracted to determine the stability of these two alkylamides in the plant materials during storage.

4.3 Results and Discussion

4.3.1 Alkylamide standards and calibration curves

Echinacea alkylamide standards are unavailable from phytochemical reference standard producers and distributers; therefore isolation of standards was required. The three major olefinic alkylamides purified in Chapter 2 have been selected as standards in this work. The quantitation of total alkylamides is unavailable due to the lack of knowledge of response factors for all alkylamides, limiting the quantitation to the three alkylamides which were purified.

The alkylamides 2 and 3 have been found to be present in lower levels in *Echinacea purpurea*, and products containing this plant material. This required the use of two calibration curves for quantitation of these alkylamides in both plant materials and commercial products. The high calibration curves ranged from

0.5-50 µg/mL and the low calibration curves ranged from 50-1000 ng/mL. This allowed for quantitation of all three alkylamides in a variety of products.

Alkylamide 1 was present in much higher abundance compared to the other olefinic alkylamides in most products, with the exception of one product which did not contain alkylamides. Therefore only a high calibration curve was required for this alkylamide. The concentration of this calibration curve ranged from 1-100 µg/mL.

The calibration curves for the high concentrations of the alkylamides can be found in Figure 4-1. After visual inspection of the curves, the calibration standard number 4 was removed to improve the linearity, this maintained the number of standards above 5, therefore maintaining within requirements set by ICH calibration standards (*12*). The correlation coefficients for all calibration curves was greater than 0.999, indicating successful linearity over the desired concentration ranges (*13*). Plots of the residuals for all standards confirmed random patterns with a mean of zero, further confirming the linearity of these calibration curves. Table 4-3 summarizes the calibration data for all three standards.

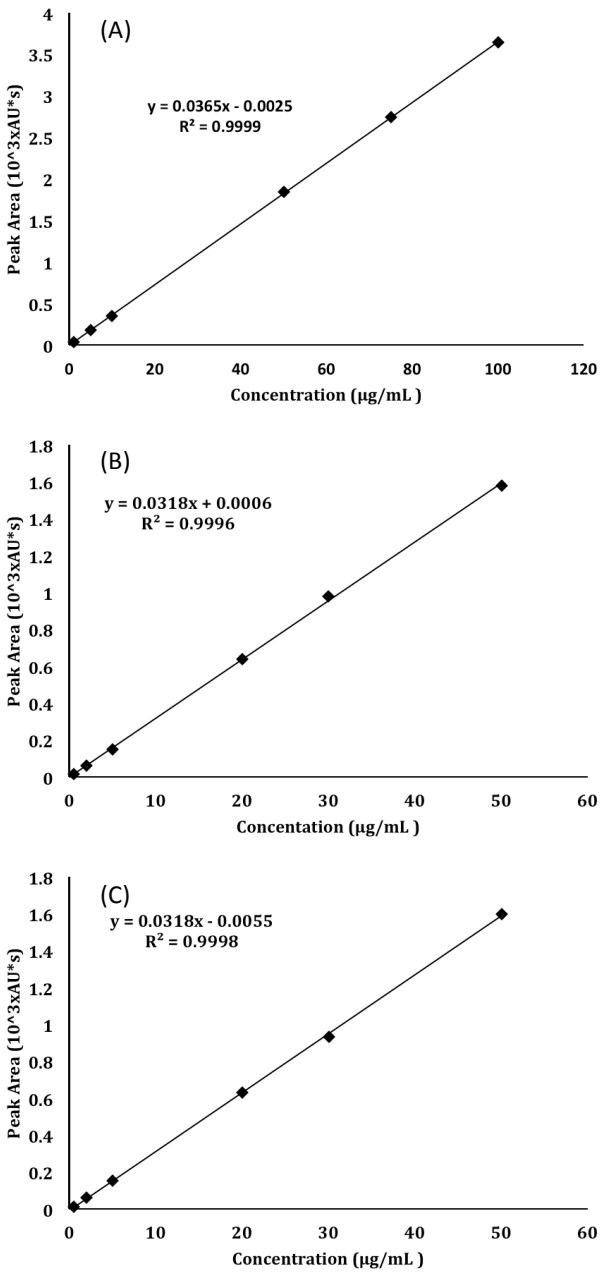

Figure 4-1. High calibration curves for the three alkylamide standards: (A) alkylamide 1 with a concentration range of 1-100 µg/mL, (B) alkylamide 2 with a concentration range of 0.5-50 µg/mL and (C) alkylamide 3 with a concentration range from 0.5-50 µg/mL.

Table 4-3. Calibration data for the three alkylamides at from the calibration curves using high concentrations.

Compound	Slope	Intercept	Correlation coefficient (r^2)	Standard error	Concentration range (µg/mL)
Alkylamide 1	0.0365	-0.0025	0.99995	0.01213	1-100
Alkylamide 2	0.0318	-0.0055	0.9998	0.00994	0.5-50
Alkylamide 3	0.0318	0.0006	0.9996	0.01395	0.5-50

The calibration curves for the low concentrations of alkylamides 2 and 3 can be found in Figure 4-2. Based on the visual inspection and correlation coefficients greater than 0.999 these curves are linear. These calibration curves are successful for quantifying alkylamides from 50 to 1000 ng/mL. Plots of the residuals confirmed a random pattern with a zero mean, confirming the linear fit of the data. The calibration data is summarized in Table 4-4.

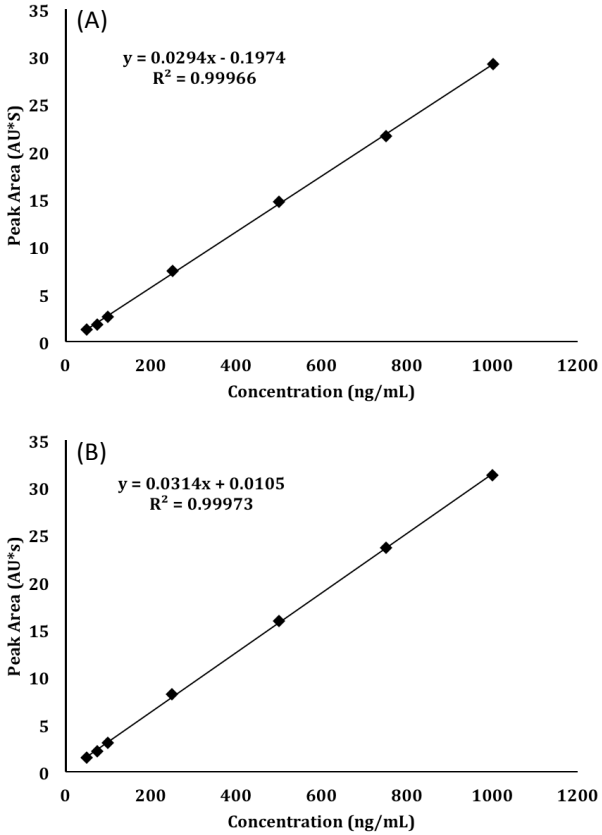

Figure 4-2. Calibration curves for the low concentrations of the olefinic alkylamides. (A) alkylamide 2 with a concentration range from 50-1000 ng/mL (B) alkylamide 3 with a concentration range from 50-1000 ng/mL.

Table 4-4. Calibration data for the low calibration curves.

Compound	Slope	Intercept	Correlation coefficient (r^2)	Standard error	Concentration range (ng/mL)
Alkylamide 2	0.0294	-0.1974	0.99966	0.22277	50-1000
Alkylamide 3	0.0314	0.0105	0.99973	0.21077	50-1000

4.3.2 Quantitation of alkylamides in *Echinacea* roots and commercial products

The olefinic alkylamides were quantified in *Echinacea* roots and commercial products using external calibration with the calibration curves in section 4.3.1. The commercial products have been summarized in Table 4-5 including the matrix and plant materials used in the products. There are a wide variety of products found in the Canadian marketplace, and the ones analyzed in this study are just a select few of what is available.

Table 4-5. Commercial NHPs available in the Canadian marketplace which were evaluated for alkylamide content.

No.	Product Type	Contents
1	Capsule, dried extract	Extract of *Echinacea purpurea* aerial parts
2	Capsule, dried extract	Extract of *Echinacea purpurea* roots
3	Capsule, dried extract	Extract of *Echinacea purpurea*
4	Softgel, extract	Extract of *Echinacea purpurea* roots and aerial parts
5	Softgel, extract	Extract of *Echinacea purpurea* roots and aerial parts
6	Softgel, extract	Extract of *Echinacea angustifolia* roots
7	Softgel, extract	Extract of *Echinacea angustifolia*
8	Softgel, extract	Extract of *Echinacea purpurea* roots and aerial parts
9	Softgel, extract	Extract of *Echinacea purpurea* roots and aerial parts
10	Softgel, extract	Extract of *Echinacea purpurea*
11	Liquid tincture	*Echinacea purpurea* flower and roots
12	Liquid tincture	*Echinacea purpurea* flower and roots
13	Liquid tincture	*Echinacea purpurea* roots and herb
14	Liquid tincture	*Echinacea purpurea* flowers and roots, *Echinacea angustifolia* roots
15	Liquid tincture	*Echinacea purpurea* roots and aerial parts
16	Liquid tincture	*Echinacea purpurea* flowers

The quantitative results are summarized in Table 4-6. Alkylamides are reported in mg/g of material, except for tinctures where they are reported as mg/mL of tincture. The samples are grouped according to the matrix in order to compare the alkylamide content between similar products. The coefficient of variation (%RSD) is reported from the analysis for each alkylamide in each sample.

Table 4-6. Alkylamide levels measured in *Echinacea* roots and NHPs.

	Alkylamide 1		Alkylamide 2		Alkylamide 3	
Product	amount (mg/g)	RSD (%)	amount (mg/g)	RSD (%)	amount (mg/g)	RSD (%)
Plant materials						
Echinacea angustifolia roots (CAN)	2.79	0.25	0.27	0.86	0.44	0.74
Echinacea angustifolia roots (USA)	0.18	0.86	0.20	0.91	0.33	1.08
Echinacea purpurea roots (CAN)	1.58	0.33	0.07	2.95	0.03	5.58
Echinacea purpurea roots (USA)	0.27	0.59	0.02	1.10	0.007	2.78
Commercial products - capsules						
1	n/d		n/d		n/d	
2	0.05	0.60	0.004	3.30	0.002	3.32
3	0.05	2.34	0.002	3.30	0.002	6.51
Commercial products - softgels						
4	0.08	0.22	0.002	4.75	0.002	3.57
5	0.22	2.76	0.005	2.45	0.004	5.16
6	0.73	1.57	0.18	0.79	0.20	1.53
7	0.57	0.20	0.18	0.65	0.16	0.26
8	0.33	1.53	0.008	1.94	0.005	5.05
9	0.22	1.67	0.005	2.95	0.003	5.47
10	0.34	0.60	0.008	2.27	0.004	2.18
Commercial Products - tinctures	(mg/mL)		(mg/mL)		(mg/mL)	
11	0.07	4.48	0.002	5.24	0.0006	6.48
12	0.07	1.95	0.002	3.53	0.0006	8.52
13	0.02	0.81	0.001	1.06	0.0004	1.26
14	0.01	0.99	0.001	5.72	0.0006	6.91
15	0.07	0.72	0.013	0.79	0.001	3.87
16	0.01	0.28	0.0004	3.72	0.0005	5.36

The *Echinacea* plant materials are used as starting materials for natural health products, therefore the variability in the plant materials alone has a significant influence on the content of the final products. The two *Echinacea angustifolia* roots analyzed in this study contain significantly different levels of alkylamide 1, while the other two alkylamides are present at more similar levels. Similarly, there are large differences between the levels of all three alkylamides in the two *Echinacea purpurea* roots.

As previously shown in the MS/MS identification of alkylamides in Chapter 3, there was one capsule product that only contained the first eluting acetylenic alkylamide and none of the olefinic alkylamides. This was confirmed in the quantitative analysis for product number 1. The two other capsule products contain similar levels of the three alkylamides, which are much lower when compared with the plant materials containing *Echinacea purpurea*. Typically,

extracts in capsules are diluted with fillers such as cellulose, which may reduce the phytochemical content in final products compared to the initial plant material content. The blending of aerial parts with roots of *Echinacea purpurea* can also reduce alkylamide content, as the aerial parts are known to contain lower levels of alkylamides compared to the roots (*3,4*).

Softgels containing *Echinacea* are widely available in the marketplace, and contain varying levels of alkylamides. Two products (6-7) contain *Echinacea angustifolia*, with higher levels of alkylamides 2 and 3 compared with the other commercial products. This is consistent with these two alkylamides in the *Echinacea angustifolia* roots.

The softgels containing *Echinacea purpurea* had similar alkylamide contents, with the exception of product 4. All three alkylamides in this product were lower when compared with the other products (5,8-10). Similar to capsules, these products are filled with oils and other fillers, which dilute the alkylamide content relative to the initial concentration in the *Echinacea* roots. Although the alkylamides 2 and 3 are at similar levels compared to the capsules, alkylamide 1 is in higher abundance in softgels. One possibility for this difference may be a protective effect of the oils or lower oxygen levels inside the softgels compared with the dried extracts in the capsules for this alkylamide, where degradation may have occurred during storage.

The tinctures have low levels of alkylamides. The typical dosage of tinctures, as recommended on the bottles, is around 1 mL three times daily, while capsules and softgels are approximately 100-200 mg in weight, with recommended doses of 1 capsule three times daily. Therefore, dosages may actually be quite similar in terms of alkylamide content. The label of product 14 claims to contain both *Echinacea purpurea* and *Echinacea angustifolia*. Due to the addition of *Echinacea angustifolia*, one might expect the alkylamide content to increase, especially for alkylamides 2 and 3. This is not the case as this product contains a lower abundance of these alkylamides compared with the products containing only *Echinacea purpurea*.

As shown in Table 4-6, there is a significant amount of variability in alkylamide content in different commercial products in the Canadian marketplace. One issue with natural health products is the quality of starting materials and the *Echinacea* species chosen for the products. *Echinacea angustifolia* contains higher levels of alkylamides when compared with *Echinacea purpurea*, especially for the alkylamides dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide and dodeca-2*E*,4*E*-dienoic acid isobutylamide. This study also confirms that the alkylamide contents within the same species can also vary significantly, which can be related to growing conditions, harvest time, storage conditions, and product handling (*7,14-16*).

The need for analytical methods for analysis of products used in clinical trials and molecular assays is increasing, as the alkylamide content may have an impact on results (*17*). We have confirmed that variability is an issue in commercial products, which needs to be addressed in NHP research and industry. Implementation of quantitation methods is necessary to ensure product variability is reported in future *Echinacea* studies to increase understanding of why results from clinical trials are so variable. They are also necessary for manufactures to ensure product quality control and reduce product variability in the future.

4.3.3 UFLC-DAD method validation

Linearity is one of the validation characteristics required in quantitative analysis, as the degree of fit will impact the final quantitative results. ICH validation protocol requires reporting the correlation coefficient, without defining a specific amount of correlation, while AOAC International (AOAC Int) validation protocol requires methods to have correlation coefficients greater than 0.999 and random residuals with an average of zero (*12,13*). The requirements set up by the AOAC Int are much more restrictive compared with ICH protocols, which were utilized in this method validation. As described in section 4.3.1, the high and low calibration curves meet the requirements for linearity.

Extraction efficiency was evaluated using two methods. This first method was performed in Section 3.3.1, where the complete solvent was removed and samples were re-extracted with fresh solvent. A second method to confirm

extraction efficiency was performed where half of the extraction solvent is replaced with fresh solvent, as described by Brown et al. in their *Echinacea* validation protocol (*18*). The peak area for alkylamide 1 was evaluated for extraction efficiency. The results for these extractions are summarized in Table 4-7, where 50% of alkylamide 1 was extracted in the first and second extractions in three matrices, confirming a complete extraction.

Table 4-7. Extraction efficiencies of alkylamide 1 determined by replacing half of the solvent three times using three different products.

Sample	1st Extraction (%)	2nd Extraction (%)
E. angustifolia roots	49.7	50.3
Capsule (product 2)	49.5	46.9
Softgel (product 4)	48.2	45.9

The LOD and LOQ for each alkylamide were evaluated by linear extrapolation of the standard deviation of the blank according the ICH method 6.3 and 6.3.2 using calibration curves of low concentrations and standard deviations of those analyses (*12*). The LOD and LOQ are reported in Table 4-8 as both the concentration of the standard and the level of each alkylamide in a plant material (200 mg), a capsule or softgel (300 mg) and a liquid tincture (1 mL). The limits of quantitation and detection are in the ng/mL range. One advantage of UPLC/UFLC equipment is the increased sensitivity resulting in lower detection limits compared with HPLC equipment (*19*).

Table 4-8. Limits of detection and quantitation for all three alkylamides. The corresponding concentrations in several matrices were calculated based on these results.

Standard	Concentration		Amount in *Echinacea* roots		Amount in capsules, softgels		Amount in tinctures	
	LOD (ng/mL)	LOQ (ng/mL)	LOD (µg/g)	LOQ (µg/g)	LOD (µg/g)	LOQ (µg/g)	LOD (µg/mL)	LOQ (µg/mL)
Alkylamide 1	7.3	19.8	0.29	0.79	0.19	0.53	0.01	0.04
Alkylamide 2	8.5	51.3	0.34	2.1	0.22	1.4	0.02	0.1
Alkylamide 3	5.7	18.4	0.23	0.74	0.15	0.49	0.01	0.04

Standard addition at two concentrations was performed to determine the method recovery and accuracy. The two concentrations, 1 µg/mL and 10 µg/mL were added to *Echinacea angustifolia* roots as a solution and the increase in sample concentration was determined for accuracy. This was performed in

triplicate. The recovery of the alkylamide additions are shown in Table 4-9. The total recovery is calculated as the average of the two determinations. According to the AOAC Int validation protocol, recovery for 10 ppm should be within 85-115% and for 1 ppm should be within 80-120%, confirming that adequate recovery for all three alkylamides was achieved (*13*).

Table 4-9. Percent recoveries of all three alkylamides at different addition levels to *Echinacea angustifolia* roots.

Standard	Recovery 1 µg/mL (%)	Recovery 10 µg/mL (%)	Average recovery (%)
Alkylamide 1	96.0	104.6	100.3
Alkylamide 2	84.7	93.9	89.3
Alkylamide 3	86.6	92.8	89.7

Another method for determining method accuracy is to analyze three concentration of each standard in triplicate and determine the relative error in the measurements (*12*). As this analysis was performed for precision measurements, the relative error (residuals) has also been included, as shown in Table 4-10. The percentage of error ranges from 0.2 to 6.8%, which is significantly lower than a previously published alkylamide quantitation method (*9*).

Table 4-10. Precision data for all three alkylamides at three concentration levels analyzed on three separate days.

Standard	Theoretical concentration (µg/mL)	Measured concentration (µg/mL)	Residuals (%)	Repeatability (%)	Intermediate precision (%)
Alkylamide 1	50.05	51.67	3.1	2.8	1.5
	10.01	10.18	1.6	2.1	2.0
	2.00	2.03	1.5	2.1	6.1
Alkylamide 2	10.00	9.67	-3.5	3.4	3.1
	2.00	1.87	-6.8	2.1	7.5
	0.400	0.42	4.3	2.9	4.5
Alkylamide 3	10.01	9.99	-0.2	2.8	1.3
	2.00	1.96	-1.9	2.3	5.2
	0.400	0.42	5.2	1.7	1.9

Precision was evaluated using three standard solutions analyzed in triplicate. The repeatability was measured as the relative standard deviation of the three measurements on one day, and the intermediate precision was calculated as the relative standard deviation of the average results from three separate days. The precision data is summarized in Table 4-10. The repeatability ranges from 1.7-

3.4%. The intermediate precision RSDs were higher compared to the repeatability, ranging from 1.5-7.5%, as variability is expected to increase between different days.

Precision was also evaluated using *Echinacea* plant materials and commercial products, which evaluates the precision of results taking into account sample preparation, extraction, etc. These results are summarized in Table 4-11. The repeatability of these extractions was comparable with the repeatability of the standards, while higher RSDs were observed in between day analysis in some samples. The intermediate precision was higher compared to the standards, ranging from 1.3-10.5%, indicating that extraction and sample preparation increase the variability in results.

Table 4-11. Precision of each alkylamide evaluated in different *Echinacea* matrices analyzed on three separate days.

Plant Material	Alkylamide	Repeatability (%)	Intermediate Precision (%)
E. angustifolia roots (USA)	1	1.6	5.9
	2	2.0	5.6
	3	1.7	6.7
E. purpurea roots (Can)	1	3.6	4.9
	2	4.9	5.0
	3	4.9	7.7
Capsule (product 2)	1	2.0	10.5
	2	0.9	8.0
	3	3.2	5.4
Softgel (product 4)	1	0.7	4.2
	2	1.4	5.7
	3	3.0	3.5
Softgel (product 7)	1	3.6	3.0
	2	3.9	0.8
	3	3.7	4.6
Tincture (product 11)	1	1.0	1.3
	2	1.1	5.1
	3	2.0	6.2

Information pertaining to the stability of alkylamide standards is not available in scientific literature, although comments have been stated on some alkylamides quickly turning yellow after drying, while in solution, the alkylamides appear to remain stable (*11,20*). An evaluation of alkylamide stability was performed on the two alkylamides, which were available in high quantities (alkylamide 1 and 3). The assumption that alkylamides in solution were stable

was made in order to compare solutions prepared after the first week, this assumption was based on results by previous groups stating that the peak area of the alkylamides did not change during storage (*20*). The calibration curves from each week were constructed using the same solution prepared on the initial week of the study, and peak areas were compared to determine if stability was low. Although detectors are known to have variable responses over time, the peak areas for the alkylamides remained stable throughout the evaluation.

The alkylamides were evaluated at two concentrations (10 and 25 µg/mL) from both a dry sample of each alkylamide and the alkylamides in solution. The peak areas obtained were back calculated according to the calibration curve to determine the experimental concentration. The relative abundance was the ratio of the calculated versus expected concentration of each alkylamide. A plot of the average of the two concentrations for the dry and liquid standards were plotted versus time in Figure 4-3 for alkylamides 1 and 3.

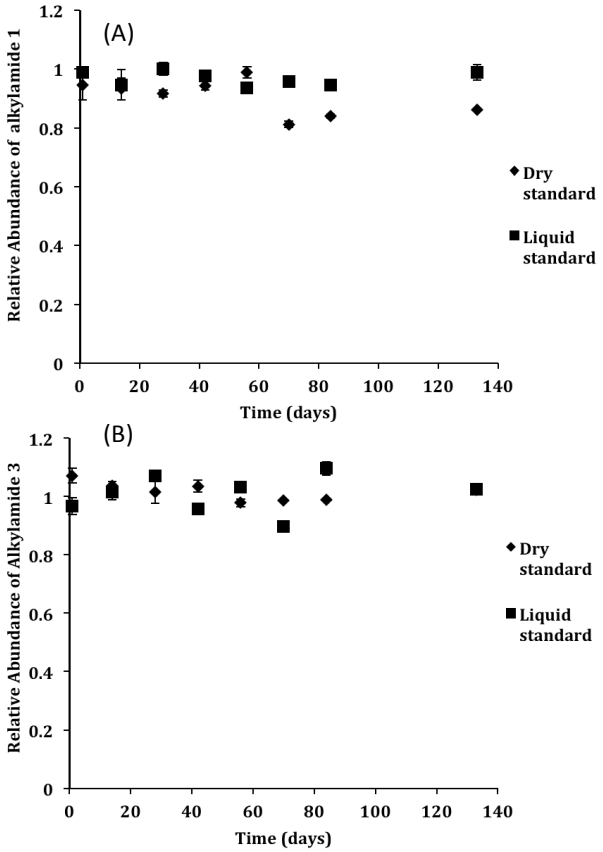

Figure 4-3. Stability of alkylamides 1 and 3 in stored as dried powders and in solution at -20°C. (A) alkylamide 1 (B) Alkylamide 3.

The alkylamide 1 contains more conjugated double bonds in its structure compared with alkylamide 3, which may result in increased oxidation of these double bonds. For the first eight weeks of this stability study, this alkylamide remained at relative abundances around 90-100%. After this time, the relative abundance of the dry standard decreased to approximately 85% for the remainder of the study. The liquid standard remained in the 90-100% range, confirming that the stability of this alkylamide is reduced when stored dry versus in solution, although even as a dry standard, the stability is still acceptable after 133 days (85% of original concentration). Analysis of 200 μg/mL solutions of this alkylamide using UFLC-MS/MS did not produce any information on degradation byproducts. On the final day of the stability study, the colour of the solution had changed to yellow, although this did not impact the calculated concentration. Absorption of water during storage may also have occurred with these alkylamides, which would decrease the alkylamide concentration without degradation.

The alkylamide 3 was originally stored as a dry powder at -20°C five months before the stability study was initiated. Contrary to the storage of alkylamide 1, this alkylamide remained white in colour throughout this storage and the stability study. Monitoring the concentration of this alkylamide as a dried powder and in liquid confirmed the stability of this alkylamide under ideal conditions, as shown in Figure 4-3b. The dried standard did not decrease in concentration during storage, confirming that this alkylamide is more stable compared to alkylamide 1. Based on these results, it may be practical for phytochemical standard companies to supply these alkylamides in solution, therefore maintaining their stability for alkylamide quantitation.

The contents of alkylamides 1 and 3 were evaluated in dried roots and extracts in both the powdered samples and liquid extracts stored at -20°C. The concentrations versus time are plotted for both alkylamides in Figure 4-4. The alkylamide contents remained stable for all samples during this storage for both dried plants and solutions. This confirms that liquid extracted samples stored

under these conditions are stable for up to 12 weeks without significant alkylamide degradation.

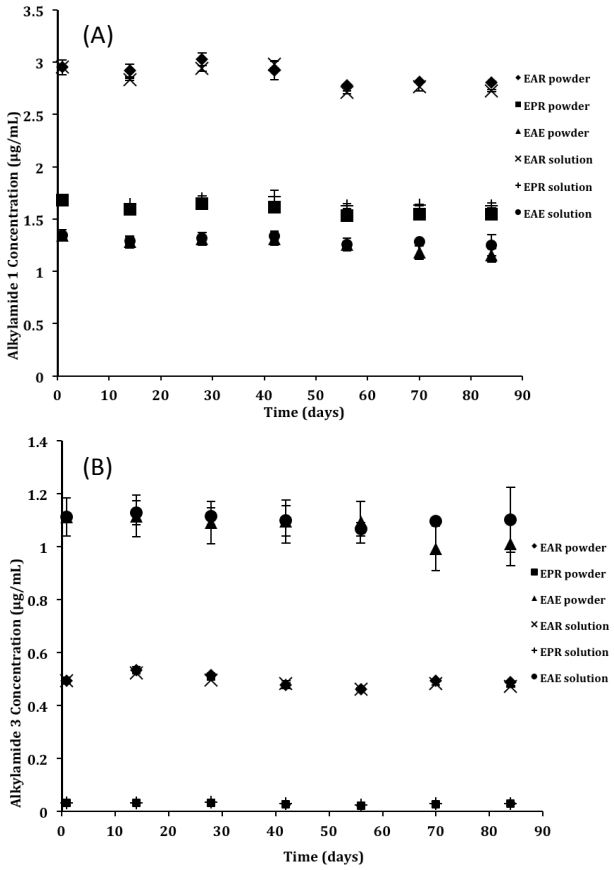

Figure 4-4. Stability of alkylamides 1 and 3 in *Echinacea* extracts (in solution) and in powdered root materials stored at -20°C. (A) Alkylamide 1 (B) Alkylamide 3.

4.4 Conclusions

The UFLC-DAD method was used to quantify three alkylamides in *Echinacea* roots and NHPs found in the Canadian marketplace. The alkylamide contents vary considerably between the same species grown and harvested in different regions, as well as between different commercial products. This method has been validated according to ICH protocols for recovery, precision, linearity, detection limits and stability of reference standards. Based on the results of the stability studies for reference standards, it is recommended that alkylamides be made available to purchase and sold in liquid forms which maintain alkylamide

stability. Due to the limited availability of alkylamide standards this quantitation was restricted to the alkylamides isolated previously, therefore comparisons of acetylenic alkylamide abundances were not possible at this time.

4.5 References

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Chapter 5. Isolation and identification of phenylalkanoids from *Rhodiola rosea* roots

5.1 Introduction

The roots of *Rhodiola rosea* contain several classes of compounds including cyanogenic glycosides, phenylpropanoid glycosides, phenyethanoid derivatives and monoterpene glycosides. This results in a very diverse phytochemical composition of the roots, in which increased variety is observed from plants grown in different regions (*1*). It has even been found that the salidroside and rosavin contents vary depending on the gender of the individual plants (*2*).

The main components in *Rhodiola rosea* roots, salidroside and the rosavins, are used as marker compounds in quality assessments of initial root materials and final NHPs (*3,4*). Typically, product labels claim that the contents of these components are 0.8 and 3% respectively, although there is no reference as to what they are the percentage of, therefore allowing for variations in the levels of these components in commercial products. Initial quality of *Rhodiola rosea* was reported only as salidroside content, but since salidroside is not restricted to this species of *Rhodiola*, adulteration with other *Rhodiola* species occurred (*3*). As rosavins are only present in the roots of *Rhodiola rosea*, they are used both as quality markers and for plant authentication (*3*).

Interest in the other phenylalkanoid glycosides and monoterpene glycosides identified in *Rhodiola rosea* roots is increasing as they may also have biological activity. As with alkylamides from *Echinacea*, these compounds are not readily available from phytochemical standard producers, therefore limiting the evaluations of their activity. The standards that are available to be purchased are also expensive and available in very small amounts and their actual purity may be lower than what is claimed by the supplier (*5*).

Several methods for rosavin and salidroside isolation and purification have included semi-preparative HPLC, and HSCCC (*6-8*). Original phytochemical profiling of *Rhodiola* began in the 1980's. Zapesochnaya and Kurkin (1982), were the first to discover the presence of the rosavins in *Rhodiola rosea* roots, which were isolated using multiple steps including column chromatography with Al_2O_3,

silica gel and Sephadex (*9*). The HSCCC separations have been limited to the separation of salidroside from two species of *Rhodiola*, *Rhodiola sachalinensis* and *Rhodiola crenulata* (*7,8*). The method isolating salidroside from *Rhodiola crenulata* required a two-step separation, whereas for *Rhodiola sachalinensis* salidroside was purified in one step (*7,8*). These methods are limited to the isolation of only one component, although the isolation of multiple components is possible with HSCCC, which could increase the significance of this technique.

Methods for the separation and identification of components in *Rhodiola* methanol extracts typically utilize reversed-phase HPLC coupled to ultraviolet and/or mass spectrometric detection (*1,4,10-12*). The methods using UV detection typically use aqueous phosphoric acid solution and either methanol or acetonitrile as the mobile phase (*1,4,13*). Methods using mass spectrometric detection use an acidic aqueous solvent with a volatile acid and methanol, acetonitrile or a combination of the two (*1,11*). Several of these methods, including one fast HPLC method of 22 minutes, are ended after the elution of the rosavins, therefore later eluting compounds were not detected (*11*). As it is necessary to identify more components in *Rhodiola rosea* extracts after HSCCC separation, a longer run is necessary.

The objective of this study was to develop a UFLC method for the separation and identification of components in the methanolic extracts of *Rhodiola rosea*, which can be used to profile the phytochemical variation in different *Rhodiola rosea* roots and NHPs available in the Canadian marketplace. A fraction of *Rhodiola rosea* roots was separated using HSCCC to isolate individual phenylalkanoid glycosides and monoterpene glycosides, in which the new UFLC-MS method was used to analyze the fractions for purity. Due to the complexity of the *Rhodiola* extract, semi-preparative HPLC was used to further purify co-eluting components from the HSCCC separation.

5.2 Experimental

5.2.1 Solvents and reagents

The organic solvents hexane, 1-butanol, and ethyl acetate were analytical grade and purchased from Fisher Scientific (Ottawa, ON). Methanol, acetonitrile

and water were HPLC grade and purchased from Fischer Scientific. The solvents used as mobile phases in the UFLC were filtered in a millipore filtration system with 0.2 μm nylon filters. HPLC grade acetic acid, glacial was purchased from Fisher Scientific. Polyamide for column chromatography was purchased from Sigma Aldrich (St. Louis, MO).

The standards of rosavin (99%), rosin (93%), rosarin (98%), salidroside (99%) and *p*-tyrosol (100%) were purchased from Phytolab (Vestenbergsgreuth, Germany). Cinnamyl alcohol (98%) was purchased from Sigma Aldrich. Lotaustralin standard (reagent grade) was purchased from Chromadex (Irvine, CA).

5.2.2 Plant materials and dietary supplements

The *Rhodiola rosea* roots were provided as a powdered material from Three Feather Farms (Sherwood Park, AB). Whole, fresh *Rhodiola rosea* plants were purchased from Midmore Farms (Morinville, AB). The roots were cleaned and dried at 40°C. These materials were ground into a fine powder using a coffee grinder. A biomass reference material was purchased from Chromadex. All plant materials were stored at -20°C before analysis. Several dietary supplements, capsules and tinctures, were purchased from local natural health stores throughout Edmonton, AB. These products were stored at room temperature.

5.2.3 Analytical-scale extraction of *Rhodiola rosea* products

5.2.3.1 Extraction of plant materials and capsule dietary supplements

The contents of 10 capsules were combined and mixed thoroughly before analysis. 150 mg of plant material or capsule material was extracted with 15 mL of 80% methanol. These samples were mixed using a vortex mixer for 1 minute and placed in a sonicating bath for 20 minutes at room temperature. The samples were allowed time to settle before the clean-up procedure, described in section 5.2.3.3.

5.2.3.2 Extraction of tincture dietary supplements

Tinctures were mixed before analysis. 1 mL of tincture was extracted with 14 mL of 80% methanol. The samples were mixed using a vortex mixer for 1

minute. These samples were then cleaned-up according to the procedure in section 5.2.3.3.

5.2.3.3 Solid-phase extraction clean-up procedure

The samples were diluted to 10% methanol by mixing 1 mL of the extracts prepared above with 7 mL of water. The cartridge, Waters Sep-pak 6CC 1.0 g C_{18}, was conditioned with methanol, followed by water. The sample was added to the cartridge and the eluate was discarded. Two column volumes (4 mL) of water were passed through the column to waste. The phenylpropanoids were collected in a round bottom flask with 5 mL of methanol. The sample was dried under vacuum and re-dissolved in 1 mL of methanol. These samples were filtered with 0.2 µm nylon filters into HPLC vials for analysis.

5.2.4 UFLC-DAD-MS analysis of *Rhodiola rosea*

The UFLC separation was performed on a Shimadzu (Toyko, Japan) Prominence UFLCXR liquid chromatograph system equipped with a CBM-20A communication bus module, two LC-20AD XR pumps, a DGU-20A3 vacuum degasser, a SIL-20AC XR autosampler, a CTO-20AC column oven and a SPD-M20A diode array detector. The UFLC was coupled to a 4000 QTRAP MS/MS system (ABSciex). The chromatographic separation was performed using a VisionHT C18 P with 50 x 2.0 mm, 1.5 µm particle size (Mandel Scientific, Guelph, ON). The mobile phase was composed of A: 0.5% acetic acid in water and B: 50:50 methanol:acetonitrile using a gradient elution as follows: 0-3 min (1-6% B), 3-7 min (6-15% B), 7-11 min (15-25% B), 11-15 min (25-40% B), 15-16 min (40-95% B), 16-17 min (95% B), 17-17.5 min (95-1% B), 17.5-22 min (1% B). The mobile phase flow rate was 0.4 mL/min and the column oven was maintained at 25°C. The injection volume was 1 µL. The UV spectra were collected from 190-800 nm and rosavins were monitored at 254 nm and salidroside was monitored at 280 nm. The mass spectrometer was equipped with electrospray ionization operating in positive mode. Nitrogen gas (>99%) was used as nebulizing gas (Gas 1, GS1) and collision gas (Gas 2, GS2). The sensitivity of the mass spectrometer was optimized using the *Rhodiola* standards: rosavin, rosarin, rosin, salidroside, tyrosol, lotaustralin and cinnamyl alcohol for signal

intensity of the molecular ions and fragmentation using full scan and product ion scans, respectively. The values for optimum spray voltage, source temperature, GS1, GS2 and curtain gases were +5.0 kV, 550°C, 50, 40 and 15 psi, respectively. The declustering potential was 60.0 V, the collision energy was 20.0 eV with a collision energy spread of 15.0 eV, the entrance potential was 10 V and the collision exit potential was 10 V. The spectra were obtained over a mass range of 100 to 600 amu. MS/MS analyses were performed using the information-dependent acquisition (IDA) method where the mass spectrometer alternates between the enhanced MS (EMS) scan for the full scan and the enhanced product ion (EPI) scan and generates MS/MS data on the eight most intense peaks. This allows for the identification of both the major and minor components eluting from the column. The scan rate for EMS was 1000 amu/s and for EPI was 4000 amu/s. The data acquisition was performed using Analyst 1.5 software (ABSciex).

5.2.5 Crude extraction of phenylpropanoids from *Rhodiola rosea* roots

5.2.5.1 Crude extraction of *Rhodiola rosea* roots

The *Rhodiola rosea* roots obtained from Three Feather Farms were defatted with hexane using a Soxhlet extraction. 13.50 g of root material was extracted with 150 mL of hexanes for 6 hours. The yield of the hexane soluble fraction was 0.79%. The defatted material was then dried and extracted for six hours in a Soxhlet apparatus with 200 mL of methanol. The yield of the methanol fraction was 39.1% (5.08 g).

5.2.5.2 Butanol fractionation of *Rhodiola* extract

2.0 g of the methanol extract was dissolved in 100 mL of water. In a separatory funnel, 100 mL of butanol was added. The two phases were separated and the lower phase was re-extracted 2 times with butanol. The butanol fractions were combined and dried under vacuum. The yield of the butanol fraction was 25.2% (0.51 grams).

5.2.5.3 Polyamide fractionation of Butanol fraction

A 2 g polyamide column was washed with methanol and conditioned with water. 100 mg of the butanol fraction was dissolved in 1 mL of water and added

to the column. The phenylalkanoids and monoterpenes were washed from the column with 30 mL of water, and then the column was washed with 30 mL of methanol. The two fractions were dried. The yield of the aqueous polyamide fraction was 42% (42 mg).

5.2.5 Solvent system selection for HSCCC

Solvent systems were screened based on partition coefficients of the main components in *Rhodiola rosea* roots: rosavin, rosarin, rosin and salidroside. The other properties screened were the settling time and solvent phase ratio. The settling time and solvent phase ratios were evaluated by preparing 10 mL of each solvent system in test tubes. Initial solvent systems evaluated were according to the HEMWat system where no partition was observed. Therefore, the ethyl acetate-butanol-water solvent systems were evaluated. In a test tube, 2 mg of the butanol fraction was combined with 2 mL of the upper and lower phases of the solvent systems and mixed thoroughly using a vortex mixer. When the systems settled into two distinct phases, 200 µL of each phase was separated and dried under nitrogen to remove the butanol. They were re-dissolved in 200 µL of methanol and analyzed using the UFLC-DAD method described in section 5.3.4, without the MS detector. The partition coefficient was determined using the ratio of the peak areas in the upper and lower phases for the components of interest.

5.2.6 Isolation of phenylpropanoids from *Rhodiola rosea* roots

5.2.6.1 Instrumentation

The HSCCC instrument used was the model TBE-300B high-speed counter-current chromatograph (Tauto Biotech). The components used with this instrument are summarized previously in section 2.2.5. The semi-preparative HPLC separation was performed using the Agilent 1200 series degasser and pump as described in section 2.2.7. The separation was performed on a Synergi-Hydro RP C_{18} column with 250 x 21.2 mm i.d., 4 µm particle size. The outlet of the column was connected to the fraction collector described in section 2.2.5. Fractions were analyzed using the Shimadzu UFLC-DAD-MS was described in section 5.2.4.

5.2.6.2 HSCCC separation

The solvent system composed of ethyl acetate-1-butanol-water (3:2:5) was prepared and mixed. The solution equilibrated at room temperature overnight and the two phases were separated and degassed shortly before use. The head-to-tail elution mode was employed, therefore the upper phase was used as the stationary phase and the lower phase was used as the mobile phase.

The sample was prepared by mixing 100 mg of the polyamide fraction in 5 mL of both phases. The column was filled completely with the stationary phase. The column was rotated at 1000 rpm and the mobile phase was introduced at a flow rate of 1.5 mL/min. Once the mobile phase eluted from the column, the sample was injected. The eluate was monitored at 254 nm to detect the rosavins. The temperature of the column was maintained at 20°C. The fractions were collected at a rate of 3 min/tube (4.5 mL total per tube). The mobile phase was switched to the upper phase after one column volume eluted, at 200 minutes. The separation was continued for another 200 minutes, with the total separation time of 400 minutes and 133 tubes collected. 100 µL of each fraction was dried under nitrogen in order to remove butanol and were re-dissolved in 100 µL of methanol and subject to UFLC analysis. Fractions containing pure components were combined and structures were confirmed with UFLC-MS and NMR spectroscopy.

5.2.6.3 Semi-preparative HPLC separation

Fraction 101-107 obtained from the HSCCC contained two components, one of which did not absorb at 254 or 280 nm. This fraction required further purification using semi-preparative HPLC. Several HSCCC runs described in section 5.2.6.2 were performed in order to accumulate large quantities of this fraction. The separation was performed on the synergi-hydro column described in section 5.2.6.1. The mobile phase composition was A: 0.5% acetic acid in water and B: methanol. The flow rate applied was 4 mL/min with a gradient elution as follows: 0-30 min (35-40% B), 30-60 min (40-100% B), 60-65 min (100% B). The sample injected was 100 µL of methanol containing 5 mg of the above fraction. The fractions were collected at a rate of 1 min/tube. No detector was connected to the outlet of the column, therefore fractions were analyzed using

UFLC-MS and pure components were combined and structures were confirmed with UFLC-MS and NMR spectroscopy.

5.2.7 Structure elucidation of phenylpropanoids

The pure fractions obtained from the HSCCC separation were analyzed using the UFLC-DAD-MS analysis described in section 5.2.4. ^{1}H and ^{13}C NMR spectra were collected using a Varian VNMRS 500 NMR spectrometer with $^{13}C/^{1}H$ dual cold probe (Agilent Technologies, Santa Clara, USA) at 499.82 and 125.69 MHz, respectively. The solvent methanol (CD_3OD) was used with tetramethylsilane (TMS) as the internal standard.

5.3 Results and Discussion

5.3.1 Extraction optimization of *Rhodiola rosea* roots

Publications describing extraction of *Rhodiola rosea* roots are inconsistent on the percentage of methanol used and the ratio of plant material to solvent (*1,4,12*). Therefore, optimization to obtain complete extraction from *Rhodiola rosea* roots in one step was assessed. The optimal percentage of methanol was determined by extracting 150 mg of *Rhodiola rosea* roots with 15 mL of methanol ranging from 20-100% aqueous methanol. The peak areas of the four main components, rosavin, rosarin, rosin and salidroside were plotted versus percentage methanol as shown in Figure 5-1. The main phenylpropanoid, rosavin, was the only one that depended significantly on the percentage of methanol. Therefore the range for optimal methanol concentration was between 70-100% methanol and 80% methanol was chosen for the extractions.

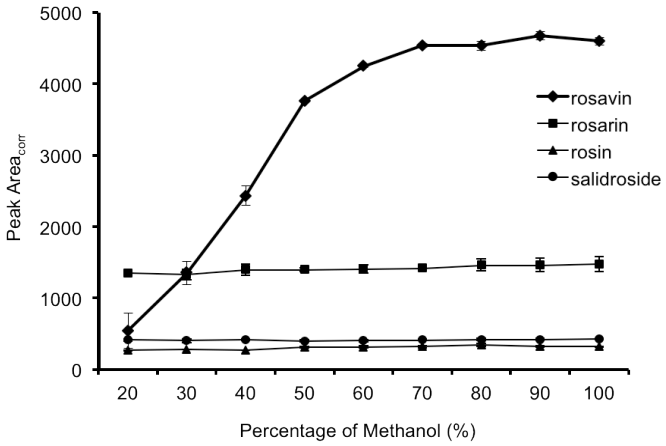

Figure 5-1. Level of salidroside, rosarin, rosin and rosavin extracted from *Rhodiola rosea* roots with methanol concentrations ranging from 20-100%.

In order to confirm that the extraction was complete, half of the solvent was removed and fresh solvent was added to the samples. The fresh extraction solvent is expected to extract any unextracted components if a complete extraction was not obtained, therefore a peak area greater than 50% in the second extraction would confirm an incomplete extraction (*14*). The second extraction resulted in recoveries of less than 50% for all of the main components in *Rhodiola rosea*: rosavin (49.7%), rosarin (49.1%), rosin (48.0%), and salidroside (48.9%).

5.3.2 UFLC-DAD-MS analysis of *Rhodiola rosea* roots and dietary supplements

Traditional HPLC methods for analysing *Rhodiola* phenylpropanoids are limited to the separation of the rosavins, salidroside and sometimes rosiridin, while other components are not considered (*4,12,15*). Typical run times range from 22 minutes up to 55 minutes including re-equilibration times. The benefit of a UFLC method is that these components elute faster from the column, but also the long re-equilibration times are reduced (*16*). The other later eluting components, which may be of interest in product profiling will also elute earlier from the column, reducing the run time. The current method developed using UFLC has a run time of 22 minutes, which includes the column re-equilibration time. This method is an improvement over previous fast LC methods because the

rosavins elute around the middle of the run, while other later eluting components are considered.

Several columns were assessed in the separation of the components in *Rhodiola rosea* extracts including Synergi Hydro-RP C18, Vision HT HL C18, Kinetex PFP and Vision HT C18-P columns. The Synergi hydro-RP C18 and Vision HT C18-P columns were both successful in separating the rosavins, while the later produced baseline separation between the rosavins and was therefore selected for this method. As phosphoric acid is commonly used in HPLC-DAD analysis of *Rhodiola*, which is not volatile, it was replaced with acetic acid in the mobile phase for MS detection. Several organic modifiers were tested including 100% methanol, 100% acetonitrile and 50:50 methanol:acetonitrile. Using the Vision HT C18-P column, 50:50 methanol:acetonitrile allowed for the best separation. There is co-elution between rosin and cinnamyl alcohol, resolution of 1.9, but the low abundance of cinnamyl alcohol in the plant materials and dietary supplements does not interfere with the analysis.

A solution containing seven *Rhodiola* standards was used to optimize the separation. This mixture contained lotaustralin, p-tyrosol, salidroside, cinnamyl alcohol, rosavin, rosarin and rosin. The optimal gradient and parameters are described in section 5.2.4 and the resulting chromatograms at 254 and 280 nm are shown in Figure 5-2. Lotaustralin does not contain a chromophore, therefore the MS was used to confirm the retention time.

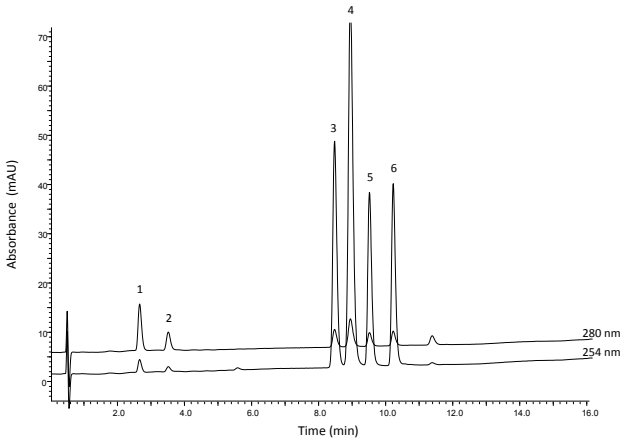

Figure 5-2. UFLC-DAD chromatogram extracted at 254 and 280 nm for the standard solution containing lotaustralin, tyrosol (1), salidroside (2), rosin (3), cinnamyl alcohol (4), rosarin (5) and rosavin (6) at 50 µg/mL.

Due to the high concentrations of sugars and other unwanted polar components in *Rhodiola rosea* methanol extract, a clean-up step using solid-phase extraction was developed. This clean-up procedure prolonged the life of the UFLC columns, where column efficiency decreased rapidly otherwise. The UFLC-DAD chromatograms for these cleaned-up extracts are shown in Figure 5-3 for the root materials obtained from Three Feather Farms, Midmore Farms and the biomass reference material from Chromadex. The main component in all of these extracts according to their UV chromatograms is rosavin eluting at 9.4 minutes.

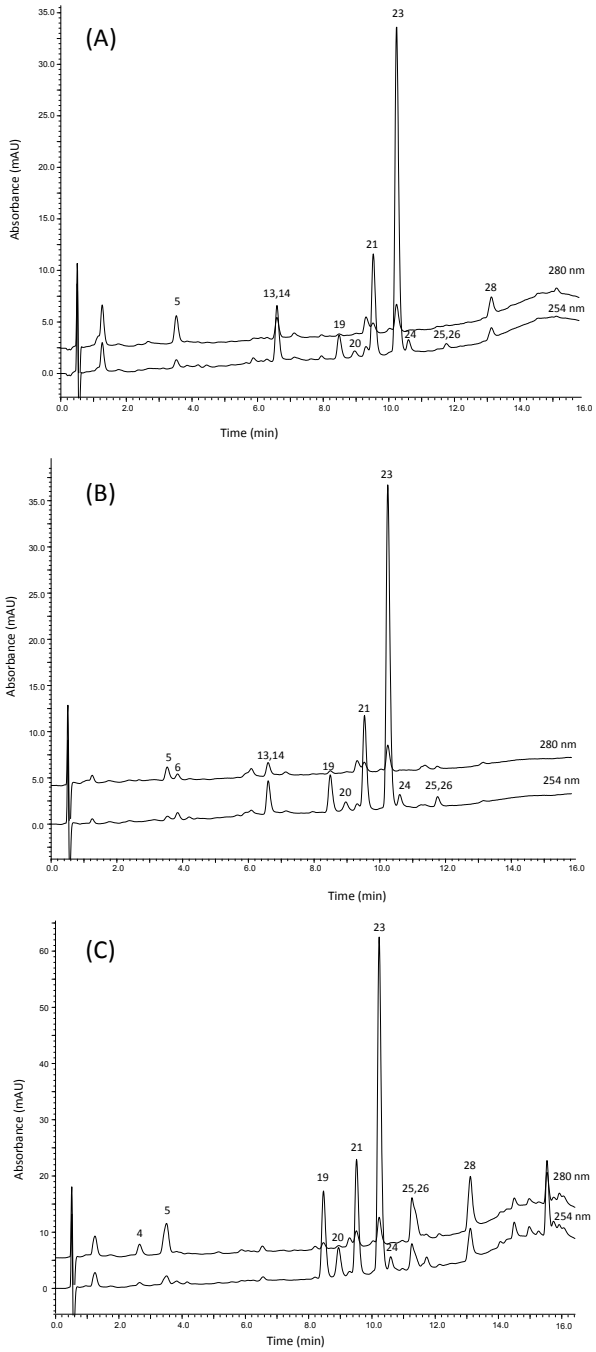

Figure 5-3. UFLC-DAD chromatograms of *Rhodiola* extracts obtained at 254 nm for the rosavins and 280 nm for salidroside. (A) Midmore Farms Rhodiola rosea roots (B) Three Feather Farms Rhodiola rosea roots (C) Chromadex biomass reference material. Peak detected in the UV chromatograms are labeled according to peak numbers in Table 5-5.

Over 30 compounds have been identified in the roots of *Rhodiola rosea*, allowing for a significant amount of variability in the phytochemical profiles (*17*).

Although the main components, salidroside and rosavins are present in all *Rhodiola rosea* roots, the other components have not always been identified (*1*). An additional complication is that the majority of the other components do not have chromophores, do not absorb at the two wavelengths of interest or are present in very small abundances. The use of mass spectrometery allows for the detection and identification of minor components and those without UV absorption.

Previous reports have confirmed that electrospray ionization in positive mode is the most sensitive ionization method for identification of components from *Rhodiola rosea* roots (*11*). Under these conditions, the molecular ions form as sodium adducts, which are very stable in the mass spectrometer and do not undergo significant fragmentation (*11*). Components containing two sugar units fragmented while components with only one sugar unit did not undergo fragmentation (*11*).

Table 5-1 summarizes all of the components present in the *Rhodiola rosea* roots from Three Feather Farms. Peak assignments for the seven components that standards were purchased were accomplished based on retention times, molecular ions and fragmentation patterns. All other components were tentatively identified by comparisons with molecular ions and fragmentation patterns of components in *Rhodiola rosea* roots previously reported. Since several components do not fragment, only tentative identification can be given as only molecular ions are detected.

Table 5-1. Tentative assignments of components in *Rhodiola rosea* roots from Three Feather Farms (Alberta, CA) using UFLC-DAD-MS/MS.

No.	Retention time (min)	Molecular ion $[M+Na]^+$ or $[M+H]^+$	Fragments (*m/z*)	Identification
1	1.2	284	185	Lotaustralin
2	1.4	282		Rhodiocyanoside A
3	2.4	323		unknown
4	2.7	121		*p*-tyrosol
5	3.5	323		Salidroside
6	3.7	293	233	Benzyl-*O*-β-glucopyranoside
7	4.7	373	357, 271	Rhodiolcyanoside D
8	5.5	425	293	Benzyl-*O*-α-arabinofuranosyl-β-glucopyranoside
9	5.9	371	355, 269	Rhodioloside A
10	5.9	337	318	viridoside
11	5.9	369	327	unknown
12	5.9	373	357, 271, 193	Sachaloside VI
13	6.7	335	201	Sachaliside 1/triandrin
14	6.7	365	263	Coniferin
15	6.4	425	293	Benzyl-*O*-α-arabinopyranosyl-β-glucopyranoside
16	7.2	439	395, 307	2-Phenylethyl-1-*O*-α-arabinofuranosyl-β-D-glucopyranoside
17	7.7	331		unknown
18	8.0	439	307	2-Phenylethyl-1-*O*-α-arabinopyranosyl-β-D-glucopyranoside
19	8.5	319	201	Rosin
20	8.5	139	117	Cinnamyl alcohol
21	9.0	451	333, 319	Rosarin
22	9.5	355		Rosiridin
23	9.9	451	333, 319, 117	Rosavin
24	10.6	451	333	Cinnamyl-(1-*O*-β-xylopyranosyl)-*O*-β-glucopyranoside
25	11.2	489	335, 333	Rhodioloside E
26	11.3	487	333, 417	Rhodioloside F
27	11.8	481	333	4-Methoxycinnamyl (6'-*O*-α-arabinopyranosyl)-*O*-β- glucopyranoside
28	13.2	469	333	Sachaloside II
29	14.6	469	451, 333, 323	Mongrhoside
30	15.7	471	453, 333	Geranyl-1-*O*-α-arabinopyranosyl-β-glucopyranoside

The main phenylpropanoids rosavin and rosarin had identical fragmentation patterns as their structures vary only by the second sugar unit being an arabinopyranoside in rosavin and arabinofuranoside in rosarin. Therefore, identification was confirmed by comparing the retention time to the rosavin and rosarin standards, as well as the molecular ions and the fragmentation patterns. The main fragments produced by collision-induced dissociation occur between the phenylpropene group and the two sugar units producing the fragments *m/z* 333 and *m/z* 117. Fragmentation also occurs between the 1" carbon and oxygen bonding the two sugar units producing a fragment at *m/z* 319. The fragmentation

pattern of rosavin is shown in Figure 5-4. Based on these results, the elution order was confirmed as rosarin (arabinofuranoside) followed by rosavin (arabinopyranoside).

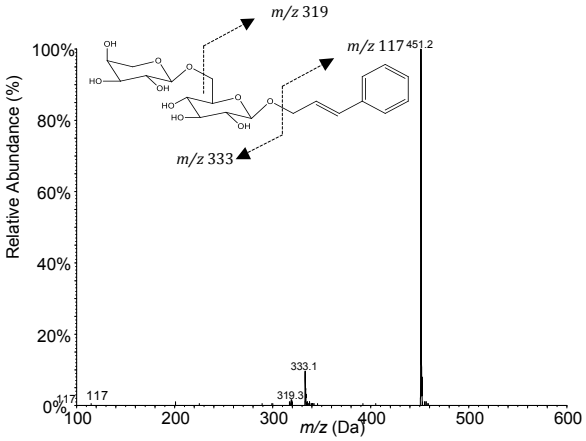

Figure 5-4. MS/MS fragmentation spectrum for rosavin from *Rhodiola rosea*.

Due to the low abundance of fragmentation using these conditions, there are three components which could not be identified. For the ones which have chemical assignments, the majority are tentative as unequivocal identification cannot be performed using these MS conditions. The results confirmed the typical fragmentation patterns, with the exception that if phenylpropanoids containing one sugar unit also contain a double bond in the carbon chain linking the phenyl group to the sugar, then fragmentation occurs between the two groups, such as with rosin as shown in Figure 5-5. This fragmentation also occurs with phenylpropanoids with two sugar units and a double bond.

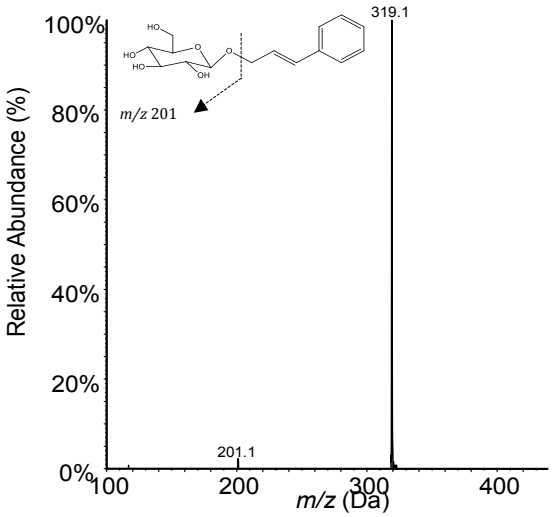

Figure 5-5. MS/MS fragmentation spectrum for rosin from *Rhodiola rosea* roots.

There are several components in *Rhodiola rosea* roots that have the same molecular ion and vary only by the second sugar unit. Two examples are: benzyl-*O*-α-arabinofuranosyl-β-glucopyranoside and benzyl-*O*-α-arabinopyranosyl-β-glucopyranoside and 2-phenylethyl-1-*O*-α-arabinopyranosyl-β-D-glucopyranoside and 2-phenylethyl-1-*O*-α-arabinofuranosyl-β-D-glucopyranoside. The fragmentation patterns of the arabinopyranosyl components are shown in Figure 5-6. Fragmentation typically occurs before the first sugar unit and between the oxygen and first carbon of the second sugar unit, similar to rosavin. The fragmentation patterns of the arabinopyranosyl and arabinofuranosyl components are identical. Although unequivocal identification cannot be assigned to these components using this MS/MS method, the retention order can be assumed to be the arabinofuranosyl eluting prior to the arabinopyranosyl group, which is based on the elution order of rosarin and rosavin. The elution order is opposite of what was observed previously (*18*), which may be due the components interactions with the different column chemistry and/or mobile phase composition.

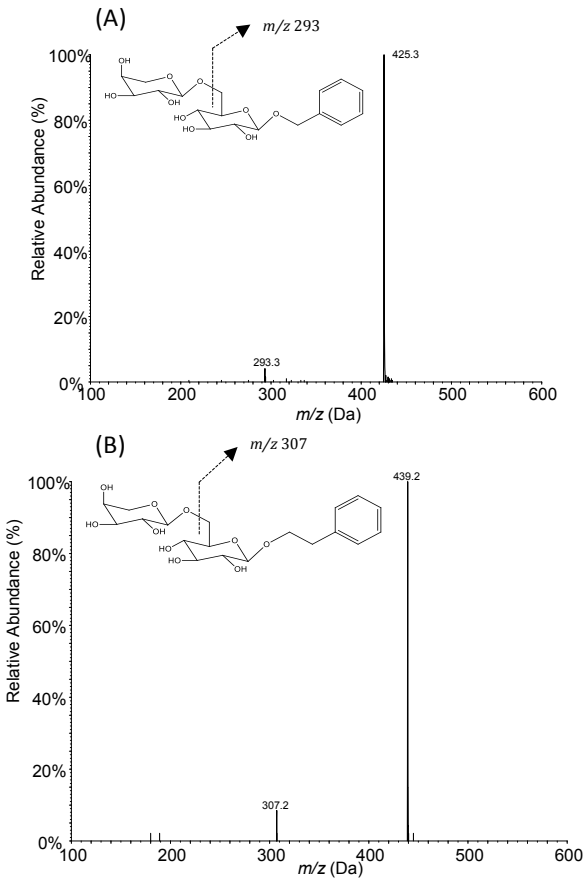

Figure 5-6. MS/MS fragmentation patters for two phenylalkanoids. The main fragmentation occurs between the two sugar units. (A) benzyl-*O*-α-arabinopyranosyl-β-glucopyranoside (B) 2-Phenylethyl-1-*O*-α-arabinopyranosyl-β-D-glucopyranoside.

This method was primarily developed for the analysis of fractions eluting from HSCCC but was also used to determine the phytochemical profiles of *Rhodiola rosea* roots and dietary supplements. The products analyzed are listed in Table 5-2. Table 5-3 summarizes the presence of the components identified in Table 5-1 in all of the products analyzed.

Table 5-2. *Rhodiola* roots and NHPs evaluated for phytochemical profiles using UFLC-DAD-MS/MS.

No.	Product Type/Source	Contents
1	Three Feather Farms roots	*Rhodiola rosea* roots
2	Midmore Farms roots	*Rhodiola rosea* roots
3	Chromadex BRM	*Rhodiola rosea* roots
4	Tincture	*Rhodiola rosea* roots
5	Tincture	*Rhodiola rosea* roots
6	Capsule	*Rhodiola*
7	Capsule	*Rhodiola rosea* roots
8	Capsule	*Rhodiola rosea* root extract
9	Capsule	*Rhodiola rosea* roots
10	Capsule	*Rhodiola* root extract

Table 5-3. Phytochemical compositions of the all products determined using UFLC-DAD-MS/MS.

	Presence in *Rhodiola rosea* roots and Commercial Products									
Component	1	2	3	4	5	6	7	8	9	10
Lotaustralin	X	X	X	X	X	X	X	X	X	X
Rhodiocyanoside A	X	X	X	X	X	X	X	X	X	X
323*	X	X	X	X		X	X	X	X	X
p-tyrosol	X	X	X	X	X	X	X	X	X	X
Salidroside	X	X	X	X	X	X	X	X	X	X
Benzyl-*O*-β-glucopyranoside	X	X	X	X	X		X	X	X	X
Rhodiolcyanoside D	X	X	X	X	X	X	X	X	X	X
Benzyl-*O*-α-arabinofuranosyl-β-glucopyranoside	X	X	X	X			X	X	X	X
Rhodioloside A	X	X	X	X	X		X	X	X	X
viridoside	X	X	X	X			X	X	X	X
369*	X	X								
Sachaloside VI	X	X	X	X			X			X
Sachaliside 1/triandrin	X	X						X	X	
Coniferin	X	X							X	
Benzyl-*O*-α-arabinopyranosyl-β-glucopyranoside	X	X	X	X	X		X	X	X	X
2-Phenylethyl-1-*O*-α-arabinofuranosyl-β-D-glucopyranoside	X	X	X	X	X		X	X	X	X
331*	X	X				X				
2-Phenylethyl-1-*O*-α-arabinopyranosyl-β-D-glucopyranoside	X	X	X		X			X	X	X
Rosin	X	X	X	X	X		X	X	X	X
Cinnamyl alcohol	X	X	X	X	X	X	X	X	X	X
Rosarin	X	X	X	X	X	X	X	X	X	X
Rosiridin	X	X	X	X	X	X	X	X	X	X
Rosavin	X	X	X		X	X	X	X	X	X
Cinnamyl-(1-*O*-β-xylopyranosyl)-*O*-β-glucopyranoside	X	X	X	X	X	X	X	X	X	X
Rhodioloside E	X	X	X	X	X	X	X	X	X	X
Rhodioloside F	X	X	X		X		X	X	X	X
4-Methoxycinnamyl (6'-*O*-α-arabinopyranosyl)-*O*-β-glucopyranoside	X	X	X	X		X	X	X	X	X
Sachaloside II	X	X		X			X	X		X
Mongrhoside	X	X	X			X	X	X		
Geranyl-1-*O*-α-arabinopyranosyl-β-glucopyranoside	X	X	X	X	X	X	X	X	X	X
Geranyl-1-*O*-α-arabinofuranosyl-β-glucopyranoside (14.2 min)									X	X

* Components could not be tentatively assigned due to low fragmentation and not previously found in *Rhodiola rosea* roots

As with all dietary supplements, there is a large amount of variability in the phytochemical compositions of these different dietary supplements. With the

exception of two products, the rosavins and salidroside were present in all dietary supplements. One product did not contain rosavin (#4), while another did not contain rosin (#6).

Two dietary supplements contained another monoterpene glycoside with a retention time of 14.2 minutes. The molecular ion was *m/z* 471, which is the sodium adduct of the molecular weight of 448. This corresponds to an isomer of geranyl-1-*O*-α-arabinopyranosyl-β- glucopyranoside (15.7 minutes). The fragments *m/z* 454 and 333 were identical to this component and with this component eluting before the arabinopyranosyl, its structure was tentatively assigned to the arabinofuranosyl isomer: geranyl-1-*O*-α-arabinofuranosyl-β-glucopyranoside.

5.3.3 HSCCC solvent system selection for phenylpropanoids in *Rhodiola rosea* roots

HSCCC has been limited to the isolation of salidroside, where as separation of phenylpropanoids has not been considered. Due to the high polarity of the phenylpropanoids, the more polar solvent systems were assessed containing ethyl acetate-butanol-water. The partition coefficients of salidroside and the rosavins in these solvent systems are shown in Table 5-4, and a partition coefficient of 1 for rosarin was used for the solvent system selection (3:2:5, number 12).

Table 5-4. Partition coefficients of the main phenylpropanoids in *Rhodiola rosea* used to evaluate several solvent systems composed of ethyl acetate-butanol-water.

Solvent system	Composition	$K_{U/L}$ salidroside	$K_{U/L}$ rosarin	$K_{U/L}$ rosavin	$K_{U/L}$ rosin
11	4 EtOAc- 1 BuOH – 5H_2O	0.53	0.37	0.14	0.86
12	3 EtOAc- 2 BuOH – 5 H_2O	0.65	1.03	0.47	1.62
13	2 EtOAc- 3-BuOH – 5 H_2O	n/a§	n/a§	n/a§	n/a§
14	1 EtOAc-4 BuOH – 5 H_2O	0.88	2.02	1.13	2.70
17	2 EtOAc -3BuOH – 5 0.5% NaCl	0.85	1.60	0.81	2.95
18	3 EtOAc -2BuOH – 5 0.5% NaCl	0.67	1.06	0.48	2.22
19	1 EtOAc -4BuOH – 5 0.5% NaCl	0.82	1.93	1.03	3.40
21	3 EtOAc -2BuOH – 5 0.1% NaCl	0.62	0.89	0.41	1.91
22	3 EtOAc -2BuOH – 5 0.075% NaCl	0.78	1.14	0.54	2.49

§ not calculated due to strong emulsion formed

The methanol extract of the *Rhodiola rosea* roots contained a large amount of sugars and other unwanted components, requiring clean-up procedures to

remove them. Based on the partition coefficients found in Table 5-4, the phenylpropanoids transferred to the butanol phase when a partition between water and butanol was performed, retaining sugars in the water phase using the liquid-liquid partition as described in section 5.2.5.2. Both the methanol extract and butanol fraction formed strong emulsions when assessing the partition coefficients using test tubes, which could be broken when salt was added to the water phase of the solvent system. The removal of salt after the HSCCC run lead to the development of a second clean-up step using a polyamide column was used to fractionate the butanol fraction prior to the HSCCC run. The resulting faction did not produce an emulsion using the test tube method and eliminated the requirement for salt in the water phase and the post run clean-up. The UFLC-DAD profile of the polyamide fraction is shown in Figure 5-7.

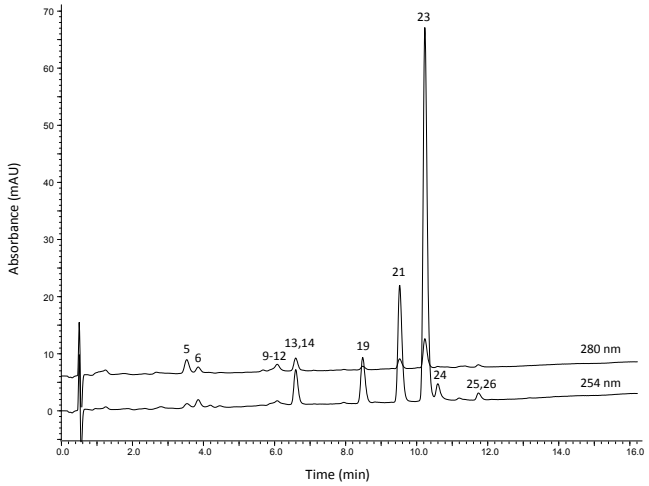

Figure 5-7. UFLC-DAD chromatogram of the polyamide fraction used in the HSCCC separation. The peaks detected in the UV chromatograms at 254 and 280 nm are labeled according to peak number in Table 5-5.

5.3.4 Isolation of phenylalkanoids from *Rhodiola rosea* roots

Initial attempts to isolate the phenylpropanoids were performed using 3 mL/min. The higher flow rate resulted in the peaks eluting closer together, therefore insufficient separation was achieved. The retention of the stationary phase at this flow rate was 38.3%, which does not meet the requirements for a suitable solvent system (*19*). There are several factors that affect the retention of the stationary phase including the solvent system composition, the flow rate of the

mobile phase, the revolution speed of the column or the temperature of the column (*19*). The most efficient is reducing the flow rate of the mobile phase. Therefore, in an attempt to improve the retention of the stationary phase and the separation of the components the flow rate was reduced to 1.5 mL/min, which increased the stationary phase retention to 49%.

Using 100 mg of the polyamide fraction in the HSCCC separation with 3:2:5 (ethyl acetate-butanol-water) and a flow rate of 1.5 mL, six main fractions were obtained and are summarized in Table 5-5. The UV absorbance was monitored at 254 nm and the chromatogram is shown in Figure 5-8. The seven components isolated are shown in Figure 5-9. The fractions 40-45 contained the cyanogenic glycosides, lotaustralin and rhodiocyanoside A, eluting together with several other polar compounds detected in *Rhodiola rosea* roots. Therefore these components were not able to be isolated using this solvent system with HSCCC.

Table 5-5. Main fractions obtained from the HSCCC separation of the polyamide fraction of *Rhodiola rosea* roots with the solvent system 3:2:5 (ethyl acetate-butanol-water).

No.	Fractions	Retention time (min)*	Peak Area (%)	Weight (mg)	Compound	$[M+Na]^+$	Fragments (m/z)
1	48-49	10.2	97%	3.4	Rosavin	451	333, 319, 117
2	54-55	3.5	90%	0.5	Salidroside	323	n/a
3	57-59	3.7	85%	1.2	Benzyl-*O*-β glucopyranoside	293	n/a
4	64-66	9.5	99%	1.3	Rosarin	451	333
5	72-74	9.9	92%	1.8	Rosiridin	355	n/a
6	101-107	8.5 15.7	100%	3.3§	Rosin Impurity	319 471	201 454, 333

*Retention time observed in the UFLC-DAD-MS
§Total weight of fraction from HSCCC separation

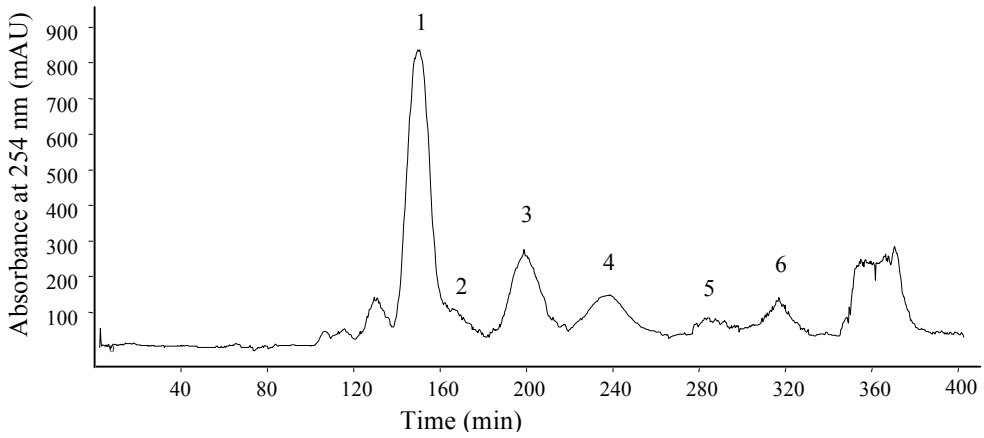

Figure 5-8. HSCCC chromatogram of the separation of the aqueous polyamide fraction from *Rhodiola rosea* roots at 254 nm using the solvent system 3:2:5 (ethyl acetate-butanol-water). Peaks are labeled according to fractions summarized in Table 5-5.

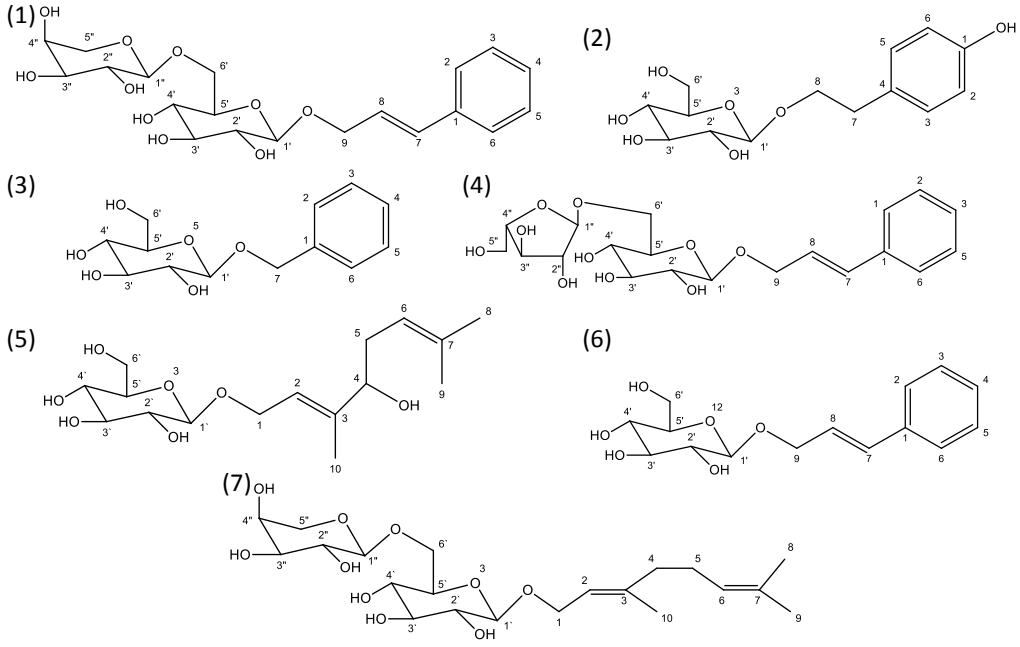

Figure 5-9. Structures of the seven components isolated from the roots of *Rhodiola rosea* using HSCCC and semi-preparative HPLC. (1) rosavin (2) salidroside (3) benzyl-*O*-β-glucopyranoside (4) rosarin (5) rosiridin (6) rosin (7) geranyl-1-*O*-α-arabinopyranosyl(1→6)-β-glucopyranoside.

Fractions 48-49 (3.4 mg) contained the most abundant phenylpropanoid rosavin, with a purity of 97%, calculated using the UV chromatogram at 254 nm. The UFLC-MS purity was in agreement with the UV purity. The UFLC-MS spectra confirmed the sodium adduct of rosavin with a *m/z* 451, and the same

retention time as rosavin compared with the purchased standard. The fragments *m/z* 333, 319 and 117 are characteristic of rosavin. The ^{1}H and ^{13}C NMR data are in agreement with those previously published on rosavin and are summarized in Table 5-6 (*20,21*).

Table 5-6. ^{1}H and ^{13}C shifts for rosavin, salidroside, benzyl-*O*-β-glucopyranoside and rosarin isolated using HSCCC.

	Rosavin		Salidroside		Benzyl-*O*-β-glucopyranoside		Rosarin	
Carbon No.	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)
1		138.3		156.8		133.9		138.1
2/6	7.42 *d* (7.5)	127.5	6.66 *m*	116.1	7.42 *m*	129.3	7.41 *d* (7.5)	127.4
3/5	7.30 *t* (7.5)	129.6	7.03 *m*	130.7	7.33 *m*	129.2	7.29 *t* (7.5)	129.5
4	7.22 *t* (7.5)	128.7		130.9	7.21 *m*	139.1	7.21 *t* (7.5)	128.6
7	6.70 *d* (16.5)	133.7	2.82 *m*	36.4	4.93 *d* (11.5)* 4.66 *d* (11.5)*	71.75	6.69 *d* (16.0)	133.8
8	6.37 *dt* (16.0,6.0)	126.7	3.17 *m*	72.1			6.36 *dt* (16.0,6.0)	126.5
9	4.51 *ddd* (12.5,6.5, 1.5) 4.31 *m*	70.9					4.50 *ddd* (12.5,6.5, 1.5) 4.31 *ddd* (12.5,6.5, 1.5)	70.8
1’	4.37 *d* (7.5)	103.5	4.28 *d* (7.5)	104.4	4.35 *d* (7.5)	103.3	4.36 *d* (7.5)	103.2
2’	3.24 *m*	75.1	3.24 -3.28 *m* (3H)	75.1	3.25-3.35 *m* (4H)	75.2	3.22 *m*	75.0
3’	3.36 *m* (2H)	78.0		77.9		78.0	3.34 *m*	77.9
4’		71.7		71.7		71.73	3.29 *m*	71.9
5’	3.46 *m*	77.0	4.02 *m*	78.1		78.1	3.45 *m*	76.7
6’	4.11 *dd* (11.0,2.5) 3.74 *dd* (11.5,6.0)	69.5	3.85 *dd* (12.0,2.5) 3.69 *m*	62.8	3.89 *dd* (12.0,2.5) 3.69 *dd* (12.0,6.0)	62.8	4.15 *dd* (12.0,2.5) 3.62 *m*§	68.0
1”	4.34 *d* (7.0)	105.2					4.98 *d* (1.5)	109.9
2”	3.61 *dd* (11,7.0)	72.4					4.01 *dd* (3.3,1.5)	83.1
3”	3.53 *m*	74.3					3.83 *dd* (6.0,3.5)	78.8
4”	3.80 *m*	69.5					3.98 *ddd* (5.0,5.0,3.5)	85.8
5”	3.87 *dd* (12.5,3.5) 3.52 *m*	66.7					3.74 *dd* (11.5,2.5) 3.62 *m*§	63.0

Measured in CD_3OD
* peaks have coupling reported based on interpretation as an AB pattern
§ overlapping signals

Fraction 55 (0.5 mg) contained salidroside with a purity of 90%, calculated using the UV chromatogram at 276 nm. The sodium adduct of *m/z* 323 was observed in the UFLC-MS spectrum, with the identical retention time

compared with the purchased salidroside standard. The ^{1}H and ^{13}C NMR data are in agreement with previously published data on salidroside and are summarized in Table 5-6 (*7,22*).

The fractions 57-59 (1.2 mg) contained benzyl-*O*-β-glucopyranoside. The purity was estimated by performing extracted ion chromatograms of the main components present in the fractions, which had sodium adducts of *m/z* 293 and *m/z* 451. The *m/z* 293 corresponded to benzyl-*O*-β-glucopyranoside which had a purity of 85%, while the *m/z* 451 is suspected to correspond to the compound cinnamyl-(1'-*O*-β-xylopyranosyl)-*O*-β-glucopyranoside, which had a retention time of 10.6 minutes. The ^{1}H NMR signals confirmed the presence of another co-eluting component, while the main component was benzyl-*O*-β-glucopyranoside. The ^{1}H and ^{13}C NMR signals corresponding to this compound are summarized in Table 5-6. These are consistent with previously published data on this component (*23*).

The fractions 64-66 (1.3 mg) contained rosarin with a purity of 99%, calculated based on the UV chromatogram of these fractions at 254 nm. The UFLC-MS was used to confirm the sodium adduct of *m/z* 451 for rosarin, while the retention time was compared with the purchased standard to confirm identity. The NMR data are summarized in Table 5-6 and are consistent with previously published data available (*24*).

The fractions 72-74 (1.8 mg) contained the monoterpene rosiridin, which does not contain a chromophore, therefore the purity was determined using the extracted ion chromatograms of all components present in the fractions. The main sodium adduct, *m/z* 355, corresponded to rosiridin, *m/z* 335 and *m/z* 337 were also present at low levels. The purity of rosiridin was calculated at 92%. The ^{1}H and ^{13}C NMR data are summarized in Table 5-7. These confirm the component as rosiridin when comparing with previously published data (*25*).

Table 5-7. ^{1}H and ^{13}C shifts for rosiridin, rosin and geranyl-1-*O*-α-arabinopyranosyl-β-glucopyranoside isolated using HSCCC and semi-preparative HPLC.

	Rosin			Rosiridin		Geranyl-1-*O*-α-arabinopyranosyl-β-glucopyranoside	
Carbon No.	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)	Carbon No.	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)	δ ^{1}H (ppm); multiplicity; *J* (Hz)	δ ^{13}C (ppm)
1		138.3	1	4.36 *dd* (12.5,6.0)* 4.30 *dd* (12.5,7.0)*	66.1	4.35 *dd* (12.5,6.5)* 4.23 *ddd* (12.0,12.0,8.0)*	66.6
2/6	7.40 *d* (7.5)	127.5	2	5.56 *t* (6.5)	122.9	5.37 *t* (6.5)	121.5
3/5	7.29 *t* (7.5)	129.6	3		142.9		141.8
4	7.21 *t* (7.5)	128.7	4	3.96 *t* (6.5)	78.0	2.04 *m*	33.1
			5	2.23 *t* (6.8)	34.8	2.12 *m*	27.4
			6	5.11 *t* (7.5)	121.6	5.10 *m*	125.1
7	6.68 *d* (16.1)	133.7	7		134.1		132.5
8	6.36 *dt* (16.1,5.6)	126.7	8	1.61 *s*	18.0	1.67 *s*	25.9
9	4.52 *ddd* (12.6,6.5,1.5) 4.32 *ddd* (12.6,6.5,1.5)	70.8	9	1.65 *s*	26.0	1.60 *s*	17.8
10			10	1.80 *s*	12.0	1.68 *s*	16.6
1’	4.36 *d* (7.5)	103.3	1’	4.29 *d* (7.5)	102.9	4.29 *d* (7.5)	102.9
2’	3.32-3.38 *m* (4H)	75.1	2’	3.17 *t* (7.8)	75.1	3.17 *m*	75.1
3’		78.0	3’	3.20-3.36 *m* (3H)	78.0	3.34 *m* (3H)	77.9
4’		71.7	4’		71.7		71.6
5’		78.1	5’		78.1		76.7
6’	3.87 *dd* (12.6,2.5) 3.67 *dd* (11.9,5.6)	62.8	6’	3.86 *dd* (12.5,2.5) 3.66 *dd* (12.0,6.0)	62.8	4.08 *m* 3.79 *m*	69.3
			1”			4.32 *d* (6.5)	105.1
			2”			3.52 *m*§	72.4
			3”			3.59 *m*	74.2
			4”			3.72 *dd* (12.5,5.5)	69.4
			5”			3.86 *dd* (12.5,3.5) 3.52 *m*§	66.5

Measured in CD_3OD

* peaks have coupling reported based on interpretation as an ABX pattern

§ overlapping signals

The fractions 101-107 (3.3 mg) contained a mixture of rosin and another component with a sodium adduct of *m/z* 471. Since the component with the *m/z* of 471 did not contain a chromophore, this fraction appeared to contained rosin at 100% purity. NMR confirmed the presence of the other component. These two components were isolated in larger abundances using several HSCCC runs and then separated using semi-preparative HPLC according to the method described in section 5.2.6.3. 13 mg of the mixture was separated using the semi-preparative

HPLC, where the fractions 56-57 contained rosin and the fractions 65-67 contained the component with *m/z* 471. The semi-preparative HPLC fractions 56-57 yielded 1.2 mg of rosin at 95% purity. The ^{1}H and ^{13}C NMR, summarized in Table 5-7, were used to confirm the structure of rosin comparing the data to previously published NMR data (*22,65*).

The semi-preparative HPLC fractions 65-67 yielded 6.5 mg of the component with a sodium adduct of *m/z* 471, which corresponds to a molecular weight of 448. Two previously identified monoterpenes from *Rhodiola rosea* have the same molecular weight and are: geranyl-1-*O*-α-arabinofuranosyl(1→6)-β-glucopyranoside and geranyl-1-*O*-α-arabinopyranosyl(1→6)-β-glucopyranoside. These two structures cannot be differentiated using MS, therefore NMR was used to confirm the structure of the second sugar moiety. The signals for the two protons on the fifth carbon of the second sugar unit were 3.86 *dd* and 3.52 *m* confirming the structure as an arabinopyranosyl moiety. If the structure were an arabinofuranosyl, the shifts would have been 3.72 *dd* and 3.62 *dd*. Therefore, with the aid of literature containing the NMR data for this structure and this ^{1}H shift of the fifth carbon in the second sugar, this structure was confirmed as geranyl-1-*O*-α-arabinopyranosyl(1→6)-β-glucopyranoside (*27,28*). The NMR data is summarized in Table 5-7. The MS fragmentation is shown in Figure 5-10.

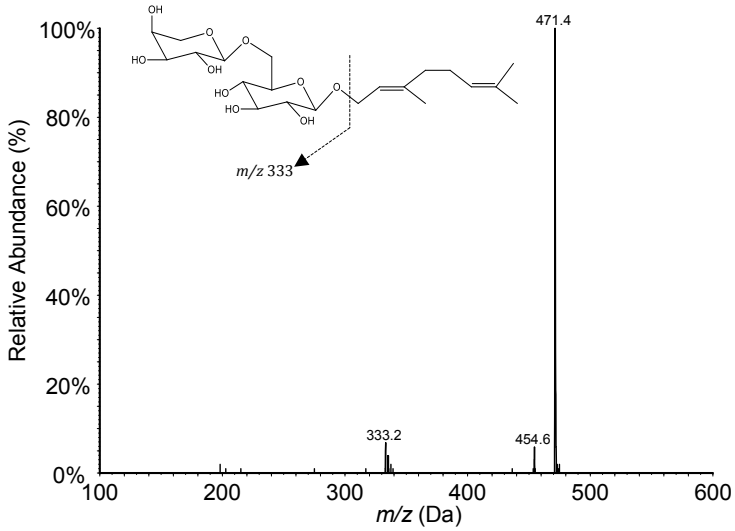

Figure 5-10. Fragmentation of the monoterpene glycoside isolated using HSCCC prefractionation and semi-preparative HPLC. This component was identified as geranyl-1-*O*-α-arabinopyranosyl(1→6)-β-glucopyranoside.

The structures of the phenylalkanoids and monoterpenes isolated using HSCCC and semi-preparative HPLC are shown in Figure 5-9. Although the *Rhodiola* extracts contain a large variety of components, many of which are in very small abundances compared to the components obtained in this study. Two monoterpenes were isolated which are currently unavailable commercially, therefore making their isolation of interest for future studies related to isolated components from *Rhodiola rosea*. The use of HSCCC as a fractionation step before purification using semi-preparative HPLC was required for two components. Semi-preparative HPLC could also be used in the future to isolate the less abundant phenylpropanoids present in these extracts.

5.4 Conclusions

The development of the UFLC-DAD-MS/MS method allowed for the characterization of 27 components, although due to the lack of fragmentation several of these components have only been tentatively identified and three could not be identified. This method reduced analysis time and solvent consumption for fractions obtained from HSCCC separation, in which seven components were isolated from *Rhodiola rosea*. Two were purified using a second isolation step with semi-preparative HPLC. Due to the high cost for isolated phytochemicals and the low availability of the two monoterpene glycosides, these isolated components can be used as standards in analytical method development, as quality and authenticity markers in raw materials and final products and to evaluate the bioactivity of individual standards. The phytochemical composition of *Rhodiola rosea* hydroalcoholic extracts is complex, therefore monitoring all components using pure standards and MS/MS detection is essential to ensure product quality and authenticity is guaranteed for the health and safety of consumers.

5.5 References

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Chapter 6. Summary and future work

6.1 Summary of Thesis

Echinacea sp. and *Rhodiola rosea* are medicinal herbs grown and sold in Canada as natural health products. *Echinacea* products are primarily used for their immunomodulatory activity in the treatment and prevention of colds and flus, while *Rhodiola rosea* products are used for their adaptogenic properties by increasing non-specific resistance to stress. The plants and their respective commercial products contain pharmacologically active components, which should be controlled to ensure public safety and product efficacy. Conventional methods to monitor the active compounds require isolation prior to chromatographic separation. The methods are extremely time consuming and require large amounts of solvents. In this thesis fast and efficient methods have been developed for isolation and identification of alkylamides in *Echinacea* and phenylpropanoids and monoterpenes in *Rhodiola rosea* using high-speed counter-current chromatography and ultra-fast liquid chromatography.

HSCCC has been used successfully in the purification of alkylamides from the roots of *Echinacea angustifolia* and the main phenylalkanoids and monoterpene glycosides from *Rhodiola rosea* roots. This technique allows for the optimization of solvent systems that are selective to components in an extract, therefore separation of components with a wide range of polarities is possible. Four alkylamides were isolated from *Echinacea angustifolia* roots with a four hour separation time, with over 35 mg of the main isomeric pair dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobuytlamide and the isolation of a new, minor alkylamide dododeca-2*E*,4*E*-dienoic acid 2-methylbutylamide. A second solvent system was used to isolate the most abundant acetylenic alkylamides, where 10 fractions were collected from the first separation, and one alkylamide, undeca-2*E*,4*E*-diene-8,10-dynoic acid isobutylamide, was isolated that has not previously been found in *Echinacea angustifolia* roots. The HSCCC separation of the *Rhodiola rosea* extract yielded six fractions containing the rosavins, salidroside and monoterpene glycosides. The final fraction was further purified using semi-prep HPLC to yield two other pure components, therefore seven components were

isolated. HSCCC offers several benefits over conventional isolation techniques, such as no waste generated from used column packing, lower solvent consumption, and faster isolation of several components simultaneously. These isolated components can be used as standards in analytical method development and component quantitation as well as assessments of individual components in biological assays.

Analytical methods for separation, identification and quantification of components in most natural health products have traditionally used HPLC-DAD-MS, which have high consumption of solvents, long analysis times, low sample throughput and lower MS sensitivity compared to newly developed equipment: ultra fast liquid chromatography (UFLC). UFLC uses columns containing smaller particle sizes, which maintain resolution in shorter analysis times. This technique was used in the separation and identification of alkylamides in *Echinacea* roots and commercial products and in the identification of components in alcoholic extracts of *Rhodiola rosea*. In the development of these methods, clean-up procedures were established for both *Echinacea* dried extracts and all *Rhodiola rosea* extracts using SPE cartridges. A total of 24 alkylamides were identified in the roots of *Echinacea angustifolia* and *Echinacea purpurea*, and alkylamide fingerprints were shown to have the potential to be used for species identification and detection of product adulteration. Due to the use of several species of *Echinacea* and the blending of roots and aerial parts in commercial NHPs, species identification was more difficult when comparing the alkylamide profiles. A total of 30 components were detected in the *Rhodiola rosea* roots, although due to the limited fragmentation only tentative assignments were given to several components. Species identification was not possible with *Rhodiola rosea* as analysis was limited to two root samples with identical phytochemical profiles. These two methods had shorter run times compared to those previously published, with improved or similar resolution between components.

The UFLC methods were used to compare phytochemical profiles of the medicinal herbs, the monitoring of fractions from the *Rhodiola* HSCCC separation and quantitation of alkylamides in *Echinacea* plant materials and

NHPs. The quantitation was performed using the three alkylamide standards isolated using HSCCC. The levels of these components varied significantly between different *Echinacea* species, but also plants grown in different regions. The variability was also high between different commercial NHPs, which is consistent with current knowledge of variability in NHPs. The variability of the plant materials has the most impact on phytochemical contents in final products, therefore quality control evaluations should be performed before large scale extractions to determine the expected levels of phytochemicals. This UFLC-DAD method was validated according to several validation parameters described by ICH, including linearity, accuracy, limits of detection and quantitation, precision and stability of standards.

This thesis has demonstrated the use of HSCCC and UFLC-DAD-MS/MS for the isolation and identification of components in *Echinacea* and *Rhodiola rosea* extracts. Both techniques reduce separation/analysis time considerably from traditional methods and also reduce their environmental impact, as smaller amounts of solvents are required. The UFLC methods are currently limited as very few reference standards are available for these products, therefore isolation of a larger number of components would allow for improved phytochemical profiling of products. The implementation of these techniques will improve the quality of natural health products, increase knowledge of the effects of phytochemical variation on clinical efficacy and potentially reduce product adulteration.

6.2 Future work

There are several research projects that could be continued from this work. They are summarized below.

1. Semi-preparative HPLC was used for the fractionation of two components that co-eluted in the HSCCC separation. This technique can be used to isolate the pre-fractionated components from HSCCC runs of *Echinacea* alkylamides or *Rhodiola rosea* to isolate and confirm the structures of these components in the UFLC-DAD-MS/MS analyses. Therefore, unequivocal identification could be

given to an increased number of components, allowing for more extensive and accurate phytochemical profiling of these materials.

2. The phytochemical profiles can be used for species identification and determination of product adulteration. The analysis of a significantly higher number of materials from a variety of sources are required and could be used to build a database for species variations throughout different regions and potentially be used to differentiate between plant species. The database could potentially be used by growers, manufacturers and regulatory bodies to ensure product safety and quality of NHPs.

3. The validation of the alkylamide method was limited to a partial validation, which, if used by regulatory bodies, requires full validation either by single laboratory validation and/or collaborative study. This would determine the reproducibility of the method by different analysts, instruments and other varying factors that could impact alkylamide quantitation. The AOAC International has protocols for dietary supplements and methods committees, therefore the full validation of this method may be possible with collaboration with this committee.

4. The UFLC method for *Rhodiola* may also be used in the future for the quantitation of phenylalkanoids and monoterpene glycosides with these components isolated from HSCCC and semi-preparative HPLC. The quantitation would also require this method be validated, similar to the method for alkylamides.

5. The incorporation of the UFLC-DAD-MS/MS methods for phytochemical profiling for products used in clinical trials and biological assays would increase understanding in the variable efficacy of these products.