EXTRACTION OF GINSENOSIDES FROM NORTH AMERICAN GINSENG USING SUPERCRITICAL FLUIDS

(Spine Title: Supercritical Fluid Extraction of Ginsenosides from Ginseng)

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By

Jeffery A. Wood

Graduate Program in Engineering
Department of Chemical and Biochemical Engineering

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Faculty of Graduate Studies
The University of Western Ontario
London, Ontario, Canada

THE UNIVERSITY OF WESTERN ONTARIO
FACULTY OF GRADUATE STUDIES

CERTIFICATE OF EXAMINATION

Joint-Supervisor	Examiners
_____	_____
Dr. Paul Charpentier	Dr. Argyrios Margaritis
	_____
Joint-Supervisor	Dr. Amin Rizkalla
_____	_____
Dr. Wan-Kei Wan	Dr. Ed Lui
Supervisory Committee	_____
_____	

The thesis by

Jeffery Alan Wood

entitled:

Extraction of Ginsenosides from North American Ginseng Using Supercritical Fluids

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ABSTRACT

The objective of this research was to study the effect on several process variables for the supercritical fluid extraction of ginsenosides from the root of North American ginseng (*Panax quinquefolius*) using supercritical carbon dioxide with various organic modifiers. Ginsenosides are a class of triterpene saponins which have various medicinal properties, including adaptogenic and aphrodisiac properties. The variables studied were pressure, temperature, modifier percentage and type, extraction time and extraction method (static or dynamic). The modifiers studied were methanol, dimethylsulfoxide and a 9:1 vol/vol mixture of aqueous ethanol and acetic acid. Supercritical fluid extraction with carbon dioxide is an emerging research field for natural product extractions, due to its reduction in organic solvent volume, decreased extraction time, and potential selectivity in extraction and fractionation of components.

The goal was to determine if conditions existed which could approach conventional solvent extraction techniques for total ginsenoside content, as well as to gain an understanding of what the primary variables governing the extraction process were. Experimental results show that SFE with carbon dioxide + modifiers can approach that of conventional solvent extraction techniques with reduced time and solvent volume and that the process is primarily desorption/mass transfer limited. The supercritical fluid technique was also able to extract ginsenosides not typically obtained in conventional extraction techniques.

Keywords: Supercritical fluids, carbon dioxide, ginseng, ginsenosides, extraction

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NOMENCLATURE

C_p	Heat Capacity at Constant Pressure
CO_2	Carbon Dioxide
DMSO	Dimethyl Sulfoxide
EtOH	Ethanol
H	Enthalpy
MeOH	Methanol
m_{CO2}	Mass of Carbon Dioxide
$m_{modifier}$	Mass of Modifier
MM	Molar Mass of Carbon Dioxide
MM	Molar Mass of Modifier
n_{CO2}	Number of moles of Carbon Dioxide
$n_{modifier}$	Number of moles of modifier
ODS	Octadecyl Silica
PEG	Poly(ethylene glycol)
PS-DVB	Poly(styrene divinylbenzene)
P	Pressure
Q_{CO2}	Flow rate of Carbon Dioxide
SCF	Supercritical Fluid
SFE	Supercritical Fluid Extraction
$scCO_2$	Supercritical Carbon Dioxide
T	Temperature
y	mole fraction (co-solvent)
V_{CO2}	Volume of CO2
Z	Compressibility Factor

Greek Letters

μ	Joule-Thomson Coefficient
ρ	Density

1. INTRODUCTION

The use of supercritical fluids as a replacement for traditional solvents has been explored in a wide range of fields over the past two decades, including extraction of natural products, fractionation/separation processes, particle design and as reaction media (Perrut, 2000). In particular, supercritical carbon dioxide has received a great deal of attention due to its many favourable properties, including: low critical temperature and pressure, low toxicity, inert nature and low cost. It is these properties which make supercritical carbon dioxide ($scCO_2$) an attractive "green" or environmentally friendly solvent (Wai, Gopalan, & Jacobs, 2003). Carbon dioxide (CO_2) is a linear molecule with no net dipole moment, meaning that it is a poor solvent for polar and ionic species (Raveendran & Wallen, 2003). For these types of species, CO_2 can be used in conjunction with a polar modifier or co-solvent to increase solubility. Typical modifiers for CO_2 include methanol, ethanol and acetone.

North American ginseng (*Panax quinquefolius*) is a widely used medicinal plant, with Canada being the largest grower (more than 60% of worldwide production) (Xiao, 2000). The medicinal properties are thought to be due to the presence of active components, one class of which are called ginsenosides (Nicol, Traquair, & Bernards, 2002). Ginsenosides are a series of triterpenoid saponins, each containing different sugar moieties. The medicinal properties associated with ginseng including anti-tumour and anti-diabetic effects (Ren & Chen, 1999). Conventional solvent extraction techniques for ginseng include Soxhlet, Ultrasound-assisted, and microwave assisted extraction. These types of

extractions are characterized by large solvent volumes, as well as longer extraction times and poor selectivity for extracted components.

A supercritical extraction technique for removing ginsenosides and other ginseng components from the ginseng plant may prove to be beneficial from an economic point of view, replacing the use of costly, potentially toxic solvents with benign CO_2 and modifiers. It could also greatly decrease the extraction time and solvent volume required due to the high diffusivity and low viscosity of supercritical fluids. In addition, the properties of supercritical fluids can be easily altered by changes in pressure and temperature, allowing potentially for the selective fractionation of desired components based on phase equilibria. The properties of supercritical fluids, in particular density, are tunable based on pressure and temperature. Since solubility is frequently related to density, the solubility of individual components in the supercritical fluid can be altered by changing pressure and/or temperature, allowing for selective fractionation based on the phase equilibria of the system.

Literature exists for supercritical extraction of ginseng using pure carbon dioxide for the purpose of removing pesticides (Perrut, 2000). Wang et al. (2001) have explored the extraction of Korean ginseng root hair using CO_2 + aqueous ethanol, but were able to only extract approximately 55% of the total ginsenoside content compared with conventional extraction techniques (Wang, Chen, & Chang, 2001). Development of a technique which is capable of extracting the bulk of ginsenoside content using

supercritical CO_2 + modifiers, could be an attractive alternative to existing methods for processing ginseng given the numerous potential benefits of supercritical fluid extraction.

2. BACKGROUND AND LITERATURE REVIEW

2.1 Supercritical Fluids and Supercritical Fluid Extraction

A supercritical fluid is a fluid that is placed under a pressure greater than its critical pressure, and a temperature greater than its critical temperature. A phase diagram of a supercritical fluid is shown in Figure 2.1.1. Under these conditions, a fluid exhibits a liquid-like density but maintains a gas-like diffusivity and viscosity. This means that a supercritical solvent has sufficient solvation power (from liquid-like density) and attractive mass transfer characteristics (gas-like diffusivity and viscosity). The physical properties (based on order of magnitude) of solvents in different states is given in Table 2.1.1. A supercritical fluid process can reduce the time required for extraction by orders of magnitude, and can be used for selective extractions or fractionations by altering pressure and/or temperature (tunable properties) (Lang & Wai, 2001). The critical properties of various fluids commonly used as supercritical solvents are shown in Table 2.1.2.

Figure 2.1.1 – Phase Diagram of a Supercritical Fluid
(Sui, 2005)

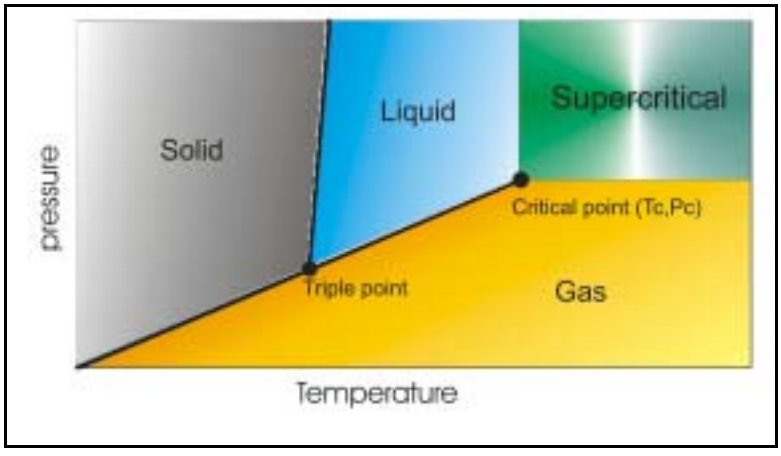


Table 2.1.1 – Physical Properties of Solvents in Different States
(Order of Magnitude) (Adapted from (Mukhopadhyay, 2000))

Property	Gas	Liquid	Supercritical Fluid
Density (g/cm^3)	10^{-3}	1	0.3
Diffusivity (cm^2/s)	10^{-1}	10^{-6}	10^{-3}
Viscosity (g/cm s)	10^{-4}	10^{-2}	10^{-4}

Table 2.1.2 – Critical Pressure and Temperature of Common Supercritical Solvents
(Adapted from (Mukhopadhyay, 2000))

Fluid	Critical Pressure (Psi)	Critical Temperature (°C)
Carbon Dioxide	1070.4	31.1
Ethane	707.8	32.2
Ethylene	731	9.3
Propane	616.4	96.7
Propylene	670.1	91.9
Toluene	596.1	318.6
Nitrous Oxide	1029.8	36.5
Ammonia	1636	132.5
Water	3198.1	374.2

The most commonly used supercritical fluid, particularly in the case of extractions, is supercritical carbon dioxide. This is due to a number of factors, such as the low critical values of CO_2 (T_c = 31.1°C and P_c = 1070.4 psi), the non-flammable and non-toxic nature of CO_2 and the low cost of CO_2 (Lang & Wai, 2001). Supercritical carbon dioxide, as

mentioned in the previous section, is a linear, non-polar molecule which provides poor solubility for polar or ionic compounds. Although the molecule has a zero dipole moment, it has a large quadrupole moment, and it is a charge separated molecule with partial charges on both the carbon (positive) and oxygen (negative). Hence, CO_2 can act as either an electron acceptor or electron donor, which is analogous to acting as a Lewis acid or Lewis base (Raveendran & Wallen, 2003). These properties make the solvent characteristics of CO_2 vary greatly from those of short alkyl-chain hydrocarbons, which have similar overall solubility parameters to CO_2. The variation of viscosity and diffusivity for carbon dioxide, at selected pressures and temperatures, are shown in Figures 2.1.2 and 2.1.3. The variation of density with reduced pressure and temperature for a supercritical fluid is given in Figure 2.1.4.

Figure 2.1.2 – Viscosity of Carbon Dioxide in the Supercritical State
(Mukhopadhyay, 2000)

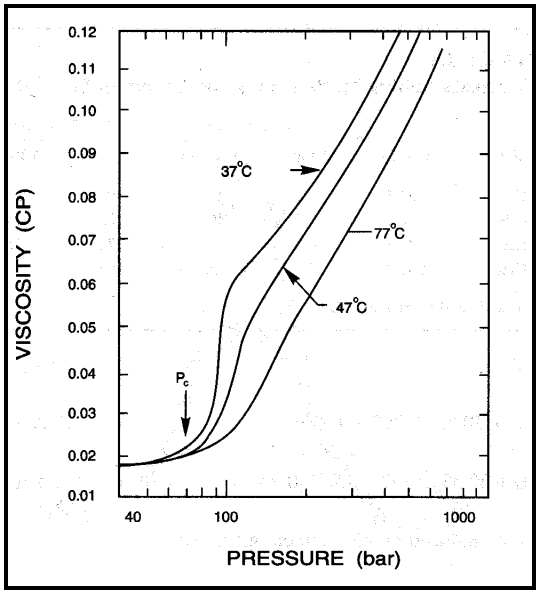
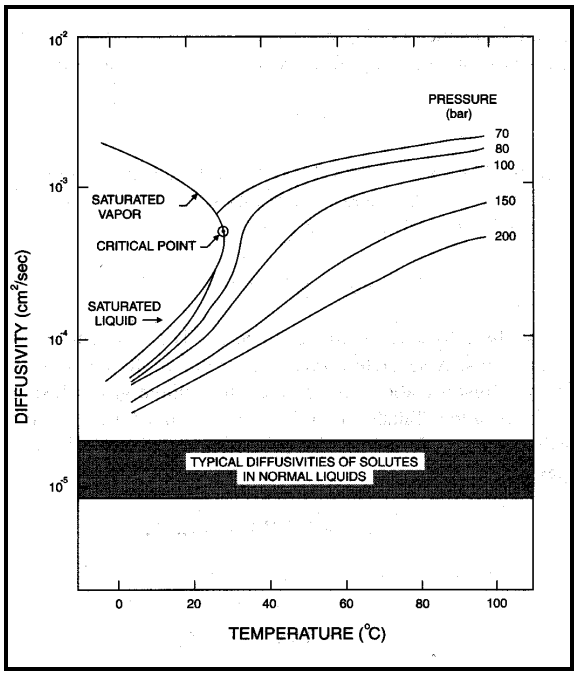


Figure 2.1.3 – Diffusivity of Carbon Dioxide at Various States
(Mukhopadhyay, 2000)



Nitrous oxide (N_2O) also has a relatively low critical temperature and pressure and has a small dipole moment, unlike CO_2. It is also better at displacing solutes from adsorption sites on matrices, which improves extraction efficiency. However, N_2O is not widely used for extractions since it supports combustion and tends to spontaneously combust under certain conditions. Ethylene also has a low critical temperature and pressure (9.3°C and 50.4 bar) and is used primarily in polyethylene polymerization, as both monomer and solvent (Alsoy & Duda, 1999).

In addition to the favourable attributes of CO_2 listed earlier, supercritical solvents in general have the benefit of having the solvation power being directly related to pressure and temperature. This means that for a given fluid, the solubility of a solute in the fluid

can be reduced or increased by increasing or decreasing pressure and/or temperature. This feature allows for a great deal of selectivity when extracting or separating compounds, which is particularly useful for extractions from plant materials due to the large number of components (Lang & Wai, 2001). A plot of reduced density vs. reduced pressure and temperature for a pure component supercritical fluid is shown in Figure 2.1.4 to illustrate the variation in density which can be achieved by pressure and/or temperature changes. When an extraction is performed using supercritical fluids, separation between the solute and the fluid can be achieved easily by dropping the pressure and collecting the material using some sort of trapping system (liquid-phase or solid-phase trap). A schematic of a typical supercritical fluid extraction unit is shown in Figure 2.1.5.

Figure 2.1.4 – Variation of Reduced Density for a Pure Component SCF
(Mukhopadhyay, 2000)

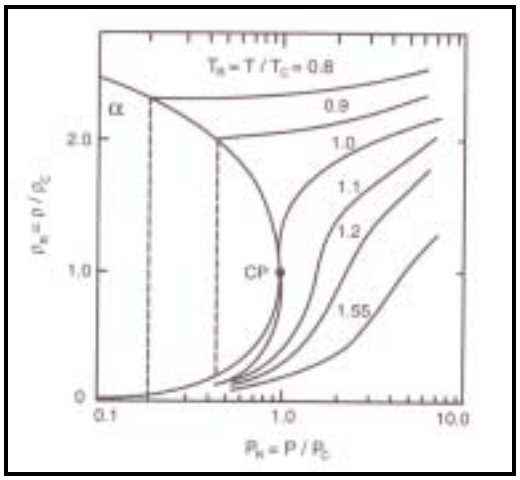
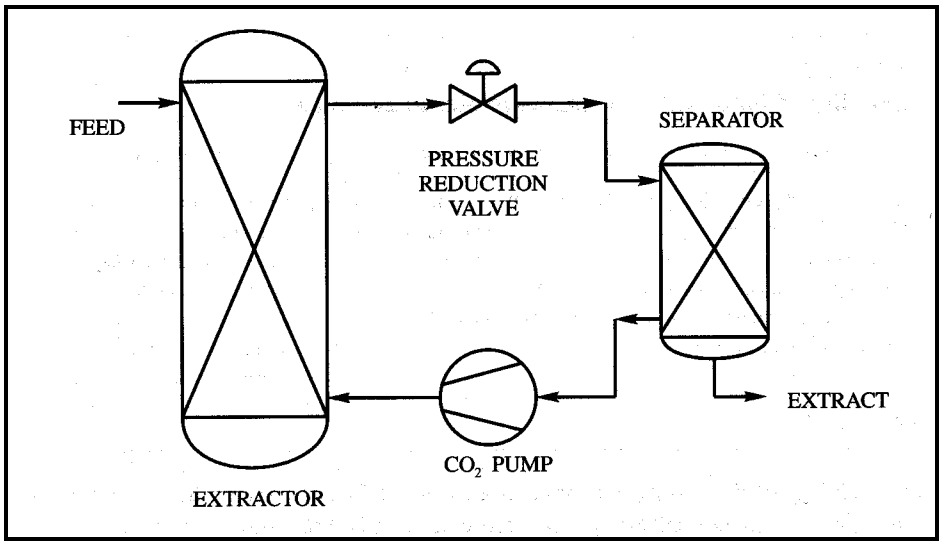


Figure 2.1.5 – Schematic of Typical Supercritical Fluid Extraction Unit
(Mukhopadhyay, 2000)



The main disadvantage of using supercritical CO_2 alone for extractions is the poor solubility of polar compounds. The solubility of various classes of components based on polarity and molecular weight is given in Table 2.1.3. In order to overcome this lack of solubility, modifiers (also called co-solvents) are required in order to increase the solubility of materials in the supercritical fluid mixture. The use of modifiers increases the operating cost of a supercritical fluid process, as well as increasing the difficulties associated with collecting materials and potentially decreasing the selectivity of extraction. Some examples of modifiers used for supercritical carbon dioxide include methanol, ethanol and acetone. Methanol is the most commonly used modifier for supercritical fluid extraction using carbon dioxide, however, it is less suitable for extracting natural products for medicinal purposes due to its toxicity, but when developing a process for extracting these natural products, methanol can be very useful for exploring system dynamics (Mannila, Lang, Wai, Cui, & Ang., 2003).

In addition to increasing solute solubility, co-solvents can decrease the crossover pressure for a system. The crossover pressure is the boundary between density effects and vapour pressure effects on solubility. For solubility, increasing temperature at a constant pressure will decrease the solvent density, which decreases solute solubility, but will increase the solute volatility, which increases solute solubility. At a pressure higher than the crossover pressure, increasing temperature will increase solubility due to volatility effects dominating. At a pressure lower than the crossover pressure, increasing temperature will decrease solubility due to density effects dominating (Mukhopadhyay, 2000).

Table 2.1.3 – Solubility of Various Classes of Natural Products in $scCO_2$
(Adapted from (Mukhopadhyay, 2000))

Very Soluble	**Moderately Soluble**	**Almost Insoluble**
Non-polar and slightly polar low M.W. Organics (<250) (e.g. acetic acid, glycerol, thiazoles)	Higher M.W. organics (<400) (e.g. water, oleic acid, saturated lipids up to C_{12})	Organics with M.W. above 400 (e.g. sugars, carotenoids, nitrates, amino acids)

Supercritical fluid processes involving CO_2 and other solvents, are currently restricted to limited applications due to economics or perceived economics. The cost of high-pressure equipment is considered prohibitive by many companies in comparison with low-pressure traditional equipment, leading to the perception that these types of processes should be restricted to high value products, such as pharmaceuticals. However, most of the existing successful commercial supercritical processes involve large volume, low value material, such as caffeine extraction from coffee beans. For supercritical processes, generally,

capital costs grow slowly compared to increasing unit sizes, making investment in large scale units attractive as well as providing low operating costs due to low solvent cost (particularly in the case of carbon dioxide) (Perrut, 2000).

In terms of issues relating to natural product extraction, the major issues are solute solubility in the supercritical fluid. In the case of CO_2, modifier use is recommended for polar solutes since the quadrupole moment allows CO_2 to dissolve only moderately polar compounds, such as alcohols. Methanol is the most common modifier, due to its high miscibility with CO_2. As well, high percentages of methanol are believed to be able to disrupt bonding between solutes and plant matrices, decreasing the mass transfer resistance for extractions. To completely extract desired components, staged extractions with various modifiers may be desirable. In addition, extraction with supercritical fluids can increase the activity of extracts due to the lack of exposure to air and light during extractions (Lang & Wai, 2001).

Langenfeld et al. (1994) attempted to relate modifier effectiveness to solvatochromic parameters for the extraction of PCBs from river sediment and PAHs from particular matter in air. Methanol, dichloromethane, toluene, hexane, acetonitrile, aniline, diethylamine and acetic acid were studied as modifiers and characterized by potential solvent interactions (induced dipole, hydrogen bonding, dispersion and π- π) as well as dipole moment and whether the modifier was a Brønsted acid or base (Langenfeld, Hawthorne, Miller, & Pawilszyn, 1994). Experiments were run at lower modifier concentrations to ensure that the only possible effect was matrix disruption. For PCBs,

the hydrogen bonding and acid/base effects of modifiers seemed to be dominant for modifying the matrix; while for PAHs, the trend was more difficult to discern. The authors found that the modifier played an important role in interacting with the solute/matrix complex to facilitate desorption of solute from the matrix and prevent readsorption by enhancing the solubility of solutes in the supercritical fluid (Langenfeld et al., 1994).

There are 3 common methods for injecting modifier into a system:1) sequential addition, 2) pre-mixed fluids, and 3) direct spiking on solid surfaces. Direct spiking on solid surfaces is generally the easiest, most reproducible, and most economical method. In addition, direct spiking allows for a static stage where modifier is given time to modify the solid surface. This modification can greatly reduce the necessary extraction time to achieve a given amount of material. Sequential addition of CO_2 and modifier is useful when the system is known to be solubility limited, while pre-mixed fluids tend to be ineffective due to the change in co-solvent concentration over time due to shifting equilibrium in the cylinder or tank of pre-mixed fluid (Lang & Wai, 2001).

The solute-modifier effect is not well understood, and is generally treated in a matrix-free method by accounting for dipole-dipole, hydrogen bonding and other polar forces. This type of solubility enhancement in the supercritical fluid can be accounted for by use of an equation of state with an appropriate mixing rule. The Peng-Robinson equation of state with the classic van der Waal mixing rules is very popular for single solute systems, although more empirical methods are required for multicomponent systems. Use of a

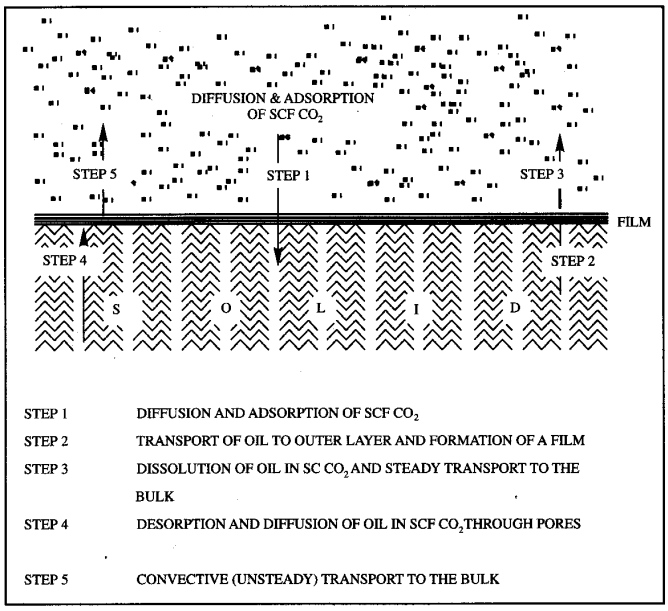
modifier also can lead to poor selectivity for extractions, although the fractionation ability of supercritical fluids helps to offset this disadvantage (Lang & Wai, 2001). For ideal selectivity, it is generally preferable to perform extractions at conditions just above the point where the desired component(s) become soluble in the supercritical fluid. This will minimize the extraction of other, potentially undesirable components. For compounds with low volatility, higher densities are required. For low solubility, higher modifier percentages which means higher temperatures are required (Lang & Wai, 2001).

When performing supercritical extractions with CO_2, it is important to control the moisture content of the sample. Water has a very low solubility in CO_2, which can lead to mechanical problems during extraction, such as plugging during collection, due to ice formation. Use of a drying agent, such as Na_2SO_4 or diatomaceous earth, can help to avoid these types of problems. In general, it is preferable to insure that the sample is dry prior to extraction, whether through freeze drying, vacuum drying or oven drying (Lang & Wai, 2001).

Sample size is another critical parameter for supercritical fluid extraction. Larger particles are usually subject to diffusional control, while small particles can lead to difficulty in maintaining flow rate and may lead to channeling of the fluid flow. The use of rigid, inert particles (such as glass beads or sand) can help to maintain flow rate, avoid channeling of the fluid (preferential flow through one path rather than uniformly throughout the bed) and prevent the solid from pressing into an impermeable plug (Lang & Wai, 2001).

The overall extraction process can be described, as illustrated in Figure 2.1.6, as a five step process where: 1) the supercritical fluid (shown as CO_2 in the figure) diffuses and adsorbs to the surface of the solid, 2) the solute (oil in Figure 2.1.6) is transported to the outer layer of the solid, 3) the solute dissolves in the supercritical fluid, 4) the fluid undergoes desorption from the solid matrix, and 5) there is convective transport into the bulk fluid (Mukhopadhyay, 2000).

Figure 2.1.6 – Extraction Steps for SFE of a Natural Product from a Solid Matrix
(Mukhopadhyay, 2000)



For extraction from plant materials, diffusion is typically the limiting resistance. The diffusion rate from the sample can be affected by three primary factors (Lang & Wai, 2001):

1) Occupation of matrix sites by the supercritical fluid
2) Dissolution of the solute into the supercritical fluid (related to density)
3) Temperature effects (volatility of solute)

Collection of extracts during supercritical fluid extraction is also a major area of concern. During typical collection processes, the fluid is brought from a high-pressure state to a low-pressure state where the fluid or gas (depending on pressure and temperature) will flow into a trapping vessel containing either a solid phase trap or a liquid phase trap (Lang & Wai, 2001). The process of going from a high-pressure fluid to a low-pressure gas is generally considered to be an isenthalpic process (constant enthalpy). During this type of process, for a real fluid, there is a substantial change in temperature. This is referred to as the Joule-Thomson effect, with the direction of the temperature change being governed by the sign of the Joule-Thomson coefficient. If the Joule-Thomson coefficient is positive, the temperature decreases and if it is negative, the temperature increases (Smith, Van Ness, & Abbott, 2001).

Equation 2.1.1

$$\mu = \left(\frac{\partial T}{\partial P}\right)_H$$

(Smith et al., 2001)

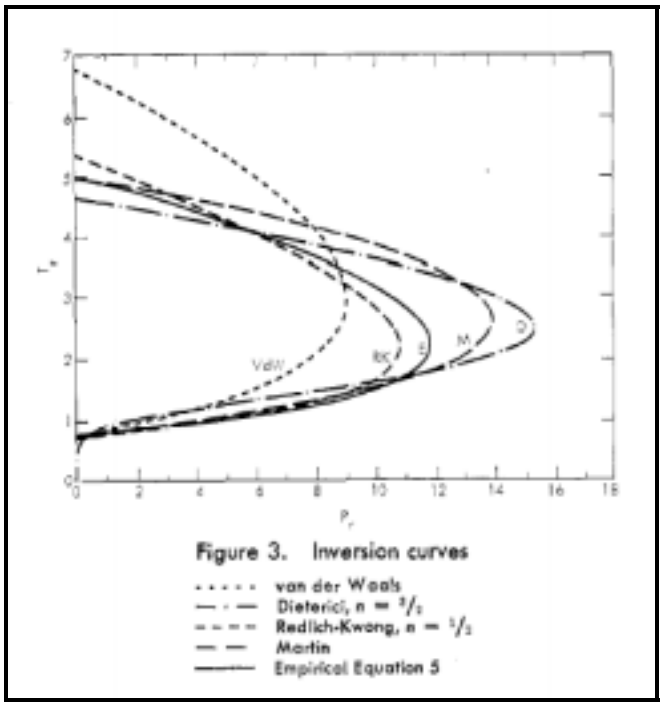
Equation 2.1.2

$$\mu = \frac{RT^2}{C_p P}\left(\frac{\partial Z}{\partial T}\right)_P$$

where μ is the Joule-Thomson coefficient, T is the temperature, P is the pressure, H is enthalpy, Z is compressibility and C_P is heat capacity at constant pressure.

If throttling results in a temperature decrease, the gas is known as cryogenic. Carbon dioxide is a cryogenic gas, as shown in Figure 2.1.7 which plots the regions of μ being positive and negative vs. reduced pressure and temperature, based on several equations of state (Miller, 1970).

Figure 2.1.7 – Joule-Thomson Inversion Curve for Several Equations of State
(Adapted from (Miller, 1970))



For the conditions being studied in this project, throttling of CO_2 will result in a decrease in temperature. This can lead to difficulties in collection for two primary reasons: 1) plugging of the restrictor due to ice formation from moisture present in the sample, and 2) plugging of the restrictor due to loss of solvation power from the decrease in both fluid density and temperature. In order to overcome these difficulties, the sample can be dried to remove excess moisture, and in order to limit the effect of solute deposition along the restrictor length, heating is used.

In addition to the Joule-Thomson effect on temperature of the fluid at the exit of the restrictor, there are a number of other variables effecting trapping efficiency/collection of extracts. For liquid traps, the decompressed gas is bubbled directly through a liquid solvent to collect extracted materials. For liquid trapping, several variables need to be optimized including: type and volume of the organic solvent, depth of the restrictor in trapping vessel, geometry of the trapping vessel, restrictor and trapping solvent temperature, and the flow rate (Moore & Taylor, 1995). A flow rate of 1 mL/min in the liquid state corresponds to ~500 mL/min in the decompressed gas state. Hence, low flow rates must be used to insure proper collection since higher flow rates will result in sample loss due to venting and purging of the gas. In addition, higher flow rates will reduce the residence time in the trapping solvent as well as increase the bubble size, reducing mass transfer between the gas and liquid phases (Chaudot, Tambute, & Caude, 1998). The trapping solvent should be chosen to have high solubility for the components of interest as well as a relatively high viscosity to reduce bubble size and increase residence time in the trapping vessel (Berg, Turner, Dahlberg, & Mathiasson, 2000). Solvents typically

chosen for liquid phase trapping include dichloromethane, ethyl acetate, methanol, ethanol and hexane (Lang & Wai, 2001).

In terms of optimizing liquid trapping, widely varying behaviour is observed for different analytes. For collection of kava lactones, it was found that keeping the vial emersed in water at room temperature enhanced recovery, while for other samples, trapping in liquid nitrogen (-170°C) was more effective than methanol-dry ice at -15°C (Lang & Wai, 2001). Lowering the temperature will not necessarily always increase trapping, as it can also cause a decrease in trapping efficiency due to a loss of solvation power of the trap solvent. Depth of trapping solvent in the trap vessel also plays an important role, as it has been found that using narrower vials tends to lead to higher trapping efficiencies, than wider vials for the same solvent volume (Lang & Wai, 2001).

Solid phase trapping is the alternative to liquid phase trapping. There are two types of solid phase traps, those with inert materials and those with adsorbents. For solid phase trapping with inert materials, glass beads, glass wool or stainless steel beads are frequently used. Ideally, solutes precipitate on these materials and can be collected for analysis by rinsing the inert material with an appropriate solvent. These types of systems are generally unsuitable for trapping of volatile solutes (Chaudot et al., 1998). Using an adsorbent instead of an inert material can improve trapping efficiency, particularly of more volatile compounds. Octadecyl silica (ODS) is a frequent choice for supercritical fluid extractions. Temperature can also play an important role in trapping efficiency for

both types of solid traps, but there are also many systems for which temperature has no significant effect on trapping efficiency (Moore & Taylor, 1995).

The major drawback to using solid phase traps for trapping of natural product extracts is the fact that the use of a modifier tends to greatly reduce trapping efficiency. This analyte loss is believed to be a result of aerosol formation due to expansion of the gas through the restrictor. For these types of collections, increasing trap temperature was found to increase trapping efficiency as the trap boiled away modifier (Moore & Taylor, 1995). For certain systems, trapping with modifier using a solid phase trap is unfeasible at any temperature. The alternative is then to use a solid sorbent with high retention power, even in the presence of modifier (Chaudot et al., 1998).

Chaudot et al. (1998) have studied the use of solid sorbents with a higher specific surface area than conventional ODS. They were able to obtain approximately 90% recovery at room temperature using a styrene divinylbenzene (PS-DVB) copolymer sorbent at up to 10% v/v methanol as a modifier. At methanol levels higher than 10% v/v, a single solid phase trap was unsuitable for quantifiable recovery, and a solid phase trap in tandem with liquid trapping was needed. The authors noted that the higher the methanol concentration, the more effective collection in a liquid phase trap became (Chaudot et al., 1998).

Supercritical fluid extraction has been utilized for a number of natural products, including: St. John's Wort (Mannila et al., 2003), ginger (Zancan, Marques, Petenate, &

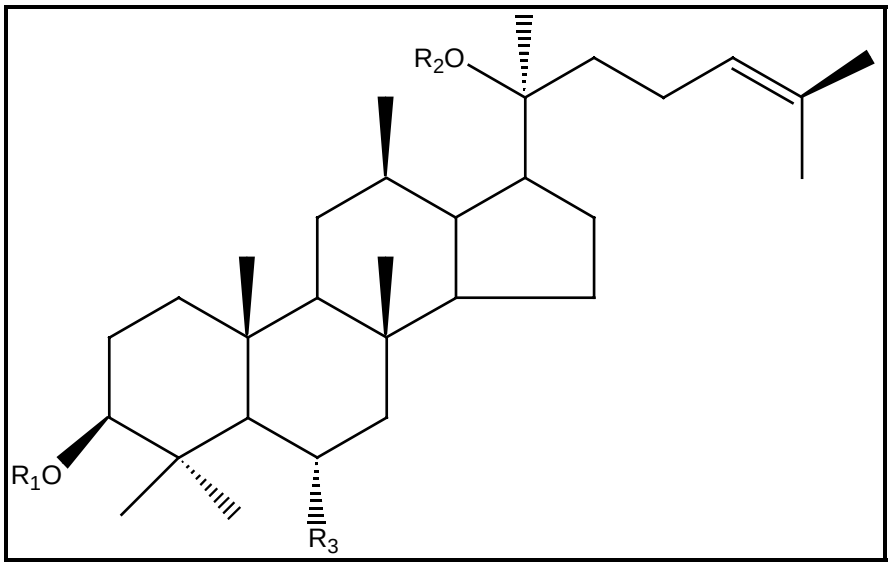
Meireles, 2002), β-carotene from carrots and other products (Subra, Castellani, Jestin, & Aoufi, 1998), nimbin from neem seeds (Tonthubthimthong et al., 2004) and nicotine from tobacco (Fischer & Jefferies, 1996). There are numerous more examples, as demonstrated in the reviews by Lang and Wai (2001) and Reverchon (1997) (Lang & Wai, 2001; Reverchon, 1997). The review by Lang and Wai (2001) also covers most of the common issues and pitfalls associated with SFE of natural products.

2.2 Uses and Properties of North American Ginseng

Panax quinquefolius (North American ginseng) is a medicinal plant used in traditional herbal medicine which is grown in the eastern part of North America as well as British Columbia. Native Americans used this plant as a medicine to reduce fever, stomach pain and hemorrhage (Assinewe, Arnason, Aubry, Mullin, & Lemaire, 2002). The extracts of this plant have been studied and reported to provide a number of medicinal benefits, including: pharmaceutical effects on the central nervous, cardiovascular, endocrine and immune systems (Teng et al., 2004), adaptogenic properties (Nocerino, Amato, & Izzo, 2000), hypoglycaemic activity (increase in insulin release and number of insulin receptors (Nocerino et al., 2000), anti-cancer and anti-tumour properties (Popovich & Kitts, 2004), as well as aphrodisiac type properties (natural viagra) (Nocerino et al., 2000). Nutraceuticals are substances which are considered food or part of a food and also offer health or medical benefits, such as preventing or treating diseases (Ferrari, 2004). Vitamins, minerals, plant extracts (*Ginkgo biloba*, *Panax ginseng*) and animal extracts (chitosan, carnosine) are examples of nutraceuticals (Ferrari, 2004). Ginseng extracts used in nutritional supplements can therefore be valuable nutraceuticals.

The adaptogenic properties of ginseng mean that ginseng helps the body maintain a state of homeostasis, that is helps the body react to stresses (either chemical, physical or biological). The aphrodisiac properties of ginseng are well known, as ginseng has been used in traditional Chinese medicine as a treatment for impotence. Both of these properties are attributed to the ginsenosides found in the plant (Nocerino et al., 2000). Ginsenosides are a series of triterpenoid saponins, each containing different sugar moieties. Over 30 ginsenosides have been isolated from the various plants of the *Panax* family, leading to a large volume of work over the last 30 years to develop reliable methods for analysis and quantification of ginsenosides (Kitts & Hu, 2000). The structure of a typical ginsenoside is shown in Figure 2.21.

Figure 2.2.1 – Structure of a Typical Ginsenoside
(Adapted from (Nicol et al., 2002))



The various R groups available for some of the more commonly found ginsenosides in North American ginseng are given in Table 2.2.1.

Table 2.2.1 – R groups for different ginsenosides found in *Panax quinquefolius*
(Adapted from (Nicol et al., 2002))

Ginsenoside	**R_1**	**R_2**	**R_3**
Rb_1	-Glc[2 -> 1]Glc	-Glc[6 -> 1]Glc	H
Rb_2	-Glc[2 -> 1]Glc	-Glc[6 -> 1]Ara(p)	H
Rc	-Glc[2 -> 1]Glc	-Glc[6 -> 1]Ara(f)	H
Rd	-Glc[2 -> 1]Glc	-Glc	H
Re	H	-Glc	-O-Glc[2 -> 1]Rha
Rg_1	H	-Glc	-O-Glc

* Glc – glucose; Ara(p) – Arabinose in pyranose form; Ara(f) – Arabinose in furanose form; Rha – Rhamnose;

The mechanism by which ginsenosides act as adaptogens is believed to be related to ginsenosides augmenting the production of corticosteroids in the adrenal glands by indirectly acting on the pituitary gland (Nocerino et al., 2000). This proposed mechanism is shown in Figure 2.2.2. The immune response has been shown to be due to acidic polysaccharides in ginseng (Assinewe et al., 2002). Some of the adaptogenic properties of ginseng, as reported in the literature, are given in Table 2.2.2.

Figure 2.2.2 – Proposed Mechanism for Ginseng to Act as an Adaptogen
(Nocerino et al., 2000)

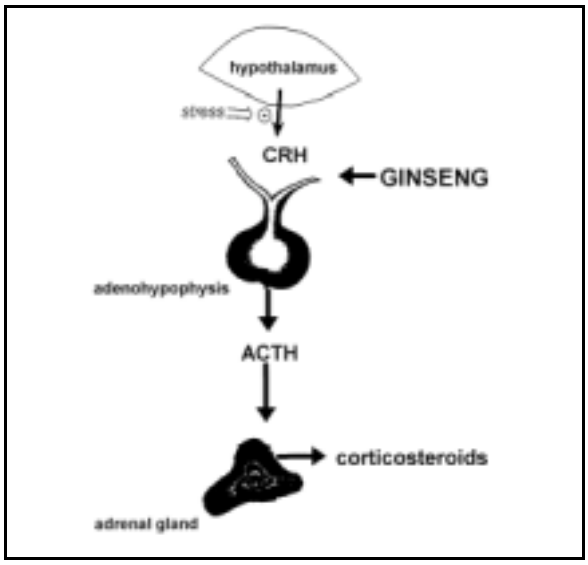


Table 2.2.2 – Reported Adaptogenic Responses of Ginseng Extracts
(Adapted from (Kitts & Hu, 2000))

		Observed Effect
Physiological System	Metabolic	- Enhanced Oxygen Uptake - Enhanced Cellular Glucose Uptake - Activates DNA polymerase - Stimulatory effect on brain neuronal activity - Lowers blood glucose in non-insulin diabetic patients
	Endocrine	- Enhanced adrenocorticotrophin secretion - Rb_1, Rc and Rd-induced increase in plasma corticosterone
	Immune	- Enhanced function of peripheral blood mononuclear cells in immune compromised subjects - Rg_1-induced increase in T-helper cells - T-cell and macrophage cytokine induction - Immunostimulatory activity in aged subjects
Chronic Disease Condition		
	Cancer	- Specific anti-mutagenic and anti-tumour activity - Protection for radiation-induced DNA damage - Rb_2-induced inhibition of tumour metastatis
	Cardiovascular	- Enhanced recovery of brain ischaemia injury - Inhibition of platelet aggregation - Enhanced recovery from cardiac ischaemia injury

There are numerous ginseng species, including *Panax ginseng* (Korean or Asian ginseng), *Panax quinquefolius* (American ginseng), *Panax notoginseng* (Tienchi or Sanchi ginseng), *Panax japonicus* (Japanese ginseng) and *Panax vietnamensis*

(Vietnamese ginseng) (Fuzzati, 2004). Each of these species contains different ginsenosides and different ginsenoside compositions, and therefore different medicinal values and properties. There are three primary types of ginsenosides, based on the structure: 1) 20(S) – protopanaxadiol type (Rb_1, Rc, Rb_2, Rd), 2) 20(S)-protopanaxatriol type (Rg_1, Rf, Re), and 3) oleanic acid type (R_O). Both Korean and North American ginseng contain Rg_1, Re, Ro, Rb_1, Rc, Rb_2 and Rd. Ginsenoside Rf is present in Korean ginseng but not in North American ginseng, and North American ginseng contains the pseudoginsenoside F_{11} which is not found in Korean ginseng (Chan et al., 2000).
For the same species, ginsenoside content can vary depending on which part of the plant is processed. For instance, the ginsenoside content in the leaves of *Panax quinquefolius* has been found to vary between 1.9 and 4.2% of the dry weight, while in the root it can vary between 3 and over 7% of dry weight (Nicol et al., 2002). The composition of the ginsenosides can also vary between leaves and root, as the roots of *Panax quinquefolius* contained primarily Re and Rd where the roots were primarily Rb_1 and Re (Assinewe, Baum, Gagnon, & Arnason, 2003).

Ginsenosides are thermally unstable, in particular a class of ginsenosides known as malonyl-ginsenosides. Malonyl ginsenosides have a malonyl group attached at the 6 location of the glucosyl group of the unit labeled R1 in Figure 2.2.1. Malonyl ginsenosides are easily demalonylated under heating, with the rate constant for degradation being somewhere between 3 to 60 times that of the corresponding neutral ginsenosides (Ren & Chen, 1999). As another example of thermal instability, the ginsenosides Rh_2, which has been shown to reduce proliferation of cancer cells, and Rg_3,

which has been shown to have anti-tumour applications, are not found naturally in North American ginseng, but are in fact the breakdown products of the thermal degradation of the ginsenosides Rb_1 and Rc (Ren & Chen, 1999). Due to this thermal instability, the composition of ginsenosides in an extract is sensitive to the extraction technique used to obtain them.

Although there exists a great quantity of literature about the medicinal benefits of ginseng, there have been a number of studies that have questioned the benefits ginseng proponents claim through statistical examination of clinical results. Vogler et al. (1999) reviewed 16 double-blind randomized controlled trials using ginseng (*Panax ginseng*, *Panax quinquefolius* and *Eleutherococcus senticosus*). For trials involving physical performance, no improvement was noted with trials involving all three ginseng species. Trials involving psychomotor function and cognitive abilities showed subjects did experience significant improvements when using *Panax ginseng* (Vogler, Pittler, & Ernst, 1999). The authors reviewed two studies related to the effect of ginseng on the immune system and found one study showed a significant increase in both the number and activity of T lymphocytes, while another showed no significant changes after ingestion of standardized *Panax ginseng* (Vogler et al., 1999). Reviews of studies performed using ginseng for treatment of diabetes also showed improvements with patients newly diagnosed with type II diabetes mellitus taking 200 mg of ginseng daily (Vogler et al., 1999). The authors concluded that there was contradictory evidence for ginseng to improve physical performance and immunological response, and that further investigation was required.

Kitts and Hu (2000) reviewed the use of standardized extracts of *Panax ginseng* and *Panax quinquefolius* in mostly in vitro and animal studies, with emphasis placed on the possible mechanisms by which ginseng functions. They reviewed the work of other authors regarding the adaptogenic properties of ginseng, and found that there was a great deal of uncertainty regarding the composition of extracts being tested for medicinal purposes, which prevented more definitive conclusions on efficacy and safety as well as making confirmation of findings impossible (Kitts & Hu, 2000). This stresses the need for more standardized extracts to perform rigorous, randomized tests on the medicinal effects of ginseng. The authors also reviewed the antioxidant behaviour of ginseng, noting that several studies both in vitro and in vivo have characterized a number of potential mechanisms. Oxidative damage through exposure to free radicals is believed to be the source of damage leading to numerous chronic diseases, such as cancer and atherosclerosis, making potential antioxidant behaviour of ginseng another attractive medicinal benefit (Kitts & Hu, 2000).

The toxicity of using ginseng has been reported only in a few cases. Animal studies with dogs showed no adverse effects from taking ginseng on body weight or blood chemistry. In mice the LD_{50} ranged from 10 to 30 g per kg body weight, while in a human study 14 out of 133 subjects reported negative side effects such as hypertension, insomnia, nervousness and gastrointestinal disturbances over a 2-year period with up to 15 g per day doses (Kitts & Hu, 2000). These observations are difficult to evaluate since, a) no attempt was made to use a placebo, b) subjects were not controlled for other bioactive substances (such as caffeine), and c) the ginsenoside content of the ginseng used in the

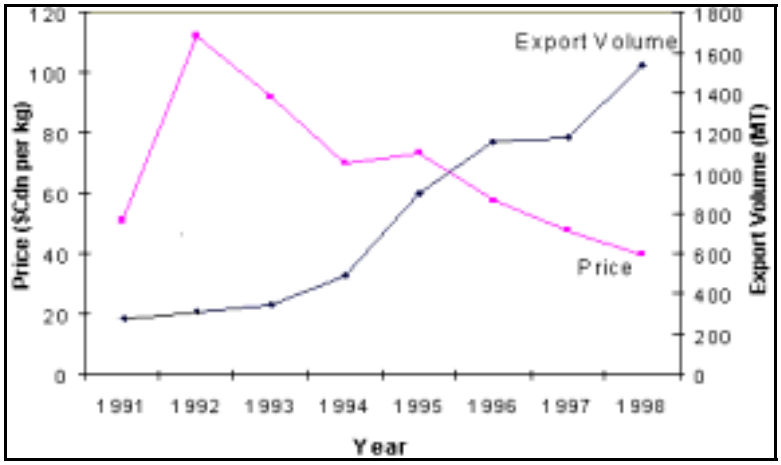
study was not determined (Kitts & Hu, 2000). Subjects who consumed greater than 15 g per day showed symptoms of confusion and depression, although this quantity is far greater than the recommended daily dose of 1 – 2 g per day with 4 – 5% ginsenosides (Kitts & Hu, 2000). A patient taking a 25 g of *Panax ginseng* dose experienced extreme headache, nausea and cerebral arteritis (Vogler et al., 1999). Ginseng-drug interactions have also been observed in some cases, with phenelzine and warfarin (Vogler et al., 1999).

To identify and quantify different ginsenosides, many analytical techniques have been used, including: thin layer chromatography, gas chromatography, high performance liquid chromatography, capillary electrophoresis, near infrared spectroscopy and enzyme immunoassay. Of these methods, the most popular is high performance liquid chromatography (HPLC) due to its speed, sensitivity and suitability for non-volatile polar compounds (Fuzzati, 2004). Numerous papers have been written on the use of HPLC for analysis of ginsenosides ((Court, Hendel, & Elmi, 1996; Ji et al., 2001; Reeleder, 2003)) are some of the more recent examples) and the procedure is well established in the literature for determining ginsenoside content accurately. In the case of identifying non-common or unknown ginsenoside and ginsenoside-like structures, liquid chromatography with tandem mass spectrometry can be employed (Kite, Howes, Leon, & Simmonds, 2003; van Breeman et al., 1995).

As mentioned in the introduction, North American ginseng is an important commercial crop in Canada. Canada exports most of the ginseng it produces, mainly to Asian

markets (Hong Kong), and the crop was worth over $75 million dollars (Cdn.) from exports in 2002 according to Agriculture and Agri-Food Canada (Agriculture and Agri-Food Canada, 2003). Over the last 15 years, there has been a decrease in price and an increase in production volume, as shown in Figure 2.2.3. The price of ginseng fell from an average of $112 per kg in 1992 to $40 per kg in 1998, with the current price range in the high $20s to mid $30s (Reeleder, 2003; Xiao, 2000). Commercial formulations based on the roots and marketed as dietary supplements accounted for 15 – 20% of the U.S. market share in 1997 (Li et al., 2000)

Figure 2.2.3 – Price/Export Volume of North American Ginseng over Time
(Xiao, 2000)



Due to the increased popularity of ginseng extracts as food or dietary supplements, there has been increased scrutiny from regulatory agencies such as Health Canada or the US FDA. This leads to the need for reproducible, standardized methods for meeting ginsenoside content to prevent fraudulent or misrepresentative advertising for products (Harkey, Henderson, Gershwin, Stern, & Hackman, 2001). A review of 50 commercial

ginseng products available worldwide in 1994 found that there was variation of between 1.9 wt% ginsenosides to 9 wt% ginsenosides, with several products containing negligible amounts of ginsenosides (Cui, Garle, Bjorkhem, & Eneroth, 1994). Li and Fitzloff (2002) examined 21 commercially available ginseng products using HPLC to determine ginsenoside content and found that ginsenoside content in the products ranged from 1.53 to 9.96 wt% (Li & Fitzloff, 2002).

The wide variability of composition in ginseng and in other herbal medicines caused Health Canada to create the Natural Health Products Directorate, which is charged with regulating natural health products in Canada. As of January 1st, 2004 the Natural Health Products Regulations were introduced, requiring that all manufacturers, importers, packagers and labelers of substances classified as natural products meet good manufacturing practices (GMPs) and obtain site licenses within 2 years, as well as shifting over existing drug identification numbers for natural products previously classified as drugs to new natural product numbers (Health Canada, 2003). Natural health products are defined by the regulation as vitamins and minerals, herbal remedies, homeopathic medicines, traditional medicines (such as traditional Chinese medicines), probiotics and other products such as amino acids and essential fatty acids (Health Canada, 2003). Due to the new, stricter regulation from both the government and the need for consistent extracts with well defined ginsenoside content for clinical studies, it is important to develop techniques of ginseng extraction which yield consistent, known quantities of ginsenosides both for commercial and research use.

2.3 Literature Review

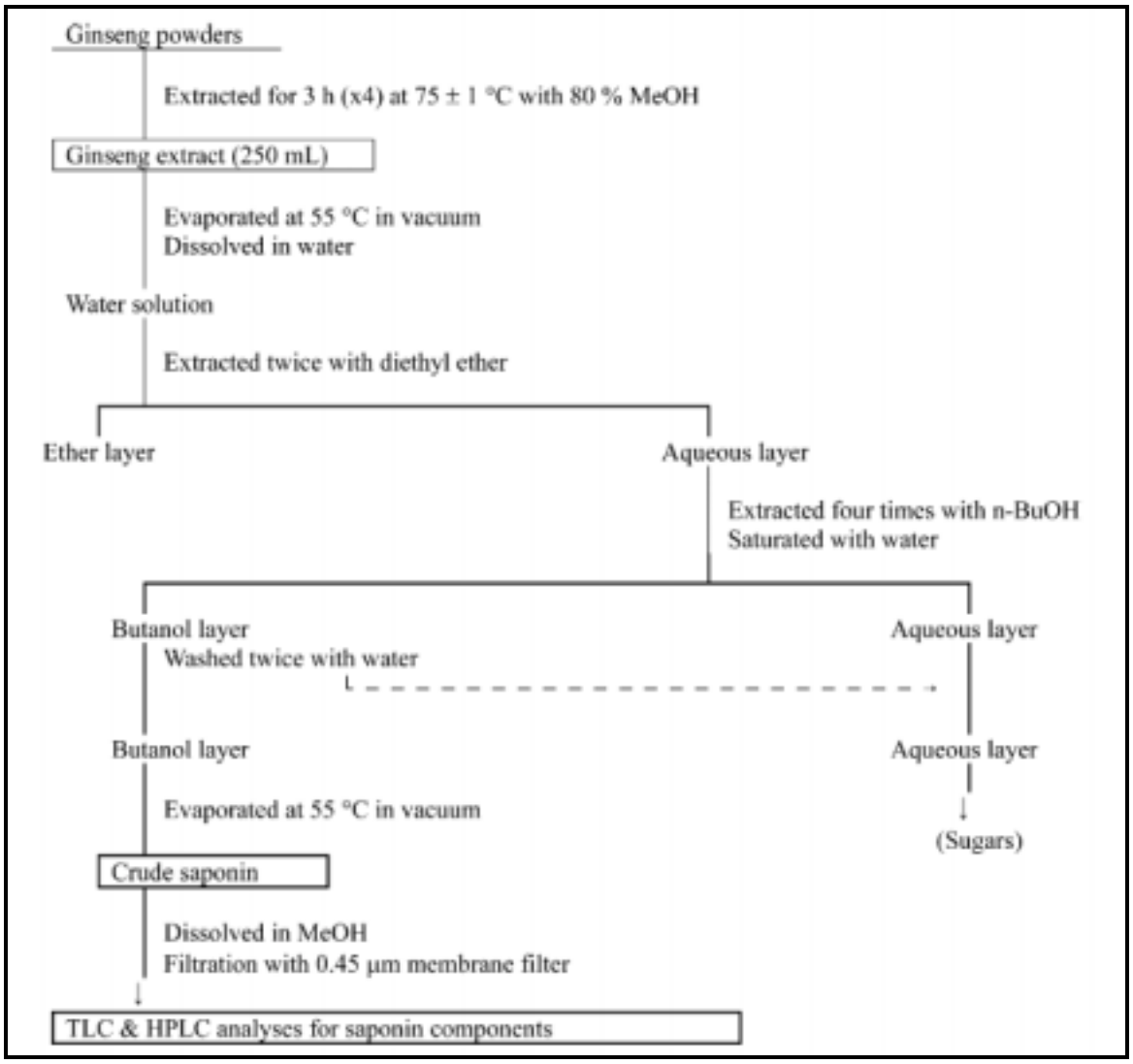
For conventional solvent extraction of ginseng, there are several established methods for extraction of ginsenosides. These include Soxhlet extraction, microwave-assisted extraction, ultrasound-assisted extraction and pressurized liquid extraction. In Soxhlet extraction, a solvent is boiled in a boiling flask and the vapours pass through the system and recondense at the top by cooling water. The recondensed vapours fall by gravity directly onto a solid sample which is placed inside of a thimble (cellulose or glass with a frit) in a glass apparatus between the boiling flask and the cooling section. Eventually, the solvent height will be sufficient to drain the solvent back into the boiling flask. As the solute of interest is far less volatile than the solvent, only fresh solvent will be vapourized and recondensed for further extraction. The advantages to using the Soxhlet technique are ease of use, reproducibility and inexpensive equipment for operation. The disadvantages are large solvent volumes and long extraction times required.

In microwave- and ultrasound-assisted extraction, conventional solvent extraction is enhanced by the addition of external energy. In the case of microwave-assisted extraction, microwave energy is used to create localized superheating and raise solvent temperature. This increases diffusion and extraction rates, reducing time and solvent consumption (Yang, Chen, Zhang, & Guo, 2004). Ultrasound-assisted extraction is more effective than conventional solvent extraction for plant materials primarily due to the mechanical effects of acoustic cavitation, which enhances solvent penetration into

samples, as well as intercellular material release due to the disruption of cell walls (Wu, Lin, & Chau, 2001).

Soxhlet extraction is a frequently used technique to extract and quantify ginsenoside content in *Panax* species. Court et al. (1996) used pure methanol as an extraction solvent in a 20 mL: 1 g solvent to solid ratio to extract the saponin content from *Panax quinquefolius* with a 20 hour extraction, leading to full conversion of malonyl ginsenosides into neutral ginsenosides (Court et al., 1996). Gafner et al. (2004) studied the extraction of *Panax quinquefolius* roots using 50% v/v aqueous ethanol, 20%-40%-40% ethanol-glycerin-water and 65% aqueous glycerin and found that for 5:1 solvent to solid ratio that aqueous ethanol provided the highest saponin yields (Gafner et al., 2004). Glycerin was found to have enzymatic activity, leading to a reduction in total ginsenoside content and an increase in gypsenoside XVII and ginsenoside F_2 (Gafner et al., 2004). The Korean Ginseng & Tobacco Research Institute uses a standardized method of Soxhlet extraction using a 10:1 solvent to solid ratio for 4 extractions, each 3 hours long (Kwon, Bélanger, Paré, & Yaylayan, 2003). This method is shown in Figure 2.3.2.

Figure 2.3.2 – Standardized Extraction Method for *Panax ginseng*
(Kwon et al., 2003)



Microwave-assisted extraction using the MAP™ process (Environment Canada, Ottawa, ON, Canada) has been used as an alternative to conventional solvent extraction. The MAP™ technology is based on using solvents which are relatively transparent to microwaves compared with the target solutes, allowing the liquid to act as both a solvent and as a coolant (Kwon et al., 2003). MAP™ was used to extract ginsenosides from Korean ginseng (*Panax ginseng*), ground to pass through a 60 mesh screen, and

compared to an established conventional solvent technique used by the Korean Ginseng & Tobacco Research Institute, as described above. MAP™ was tested at several different sample quantities (2.5, 5, 10 g) with 50 mL of 80% methanol and several irradiation times (20, 40 and 60 seconds) and power levels (75, 150, 225, 300 W). The authors found that total saponin content and relative ginsenoside concentrations similar to conventional solvent techniques could be obtained using 4 repeats of a 30 second, 300 W microwave-assisted extraction as shown in Table 2.3.1 (Kwon et al., 2003).

Table 2.3.1 – Ginsenoside Content for Conventional and MAP Process
(Kwon et al., 2003)

Extraction method	Ginsenoside (%, d.b.)						
	Rb_1	Rb_2	Rc	Rd	Re	Rg_1	Total
MAP[a]	0.849[d]	0.574	1.164	0.530	0.414	0.099	3.630
Conventional reflux[b]	0.846	0.570	1.170	0.528	0.420	0.094	3.624
Accredited value[c]	0.850	0.572	1.171	0.531	0.409	0.092	3.625

[a] Microwave-assisted process was performed at full power (300 W) for 30 s on a mixture consisting of 5 g of sample and 50 ml of 80% methanol. The residue was taken back and re-extracted four times using fresh solvent each time and above conditions.
[b] Conventional reflux at 75±1 °C for 3 h on a mixture consisting of 5 g of sample and 50 ml of 80% methanol. This method was also repeated four times on the residues obtained.
[c] Values from Korea Ginseng and Tobacco Research Institute.
[d] Mean of triplicates experiments.

The analyzed ginsenosides (Rb_1, Rb_2, Rc, Rd, Re and Rg_1) were found to be sufficiently stable under the studied conditions that the use of the microwave-assisted extraction led to no significant degradation of ginsenoside components (Kwon et al., 2003). This result is consistent with the work of Ren and Chen (1999) who found that microwave-assisted extraction had no greater thermal degradation on ginsenoside contents in ginseng than from longer conventional extraction processes (Ren & Chen, 1999). Although there was no greater degradation than conventional extraction, there is still thermal conversion of the malonyl-ginsenosides which are 3 to 60 times more sensitive to heat than neutral

ginsenosides (Ren & Chen, 1999). These types of reactions (conversion of malonyl ginsenosides) may not be undesirable, for instance Rg_3 has been shown to have anti-tumour effects as well as an effect on drug resistant cancer cells and is produced in North American ginseng only through a thermal conversion where Rb_1 and Rc are converted to other ginsenosides (Popovich & Kitts, 2004).

Yang et al. (2004) have explored the use of 50% ethanol with water as an extraction solvent assisted by microwaves for the extraction of ginsenosides from North American ginseng. The optimum conditions were a solvent to solid ratio at 40:1 mL per g of solvent, 100 – 140 mesh particle size and a 3-minute extraction time. The authors found no significant difference between the microwave-assisted extraction and a Soxhlet and ultrasound-assisted extraction in terms of the ginsenoside content obtained for Rb_1, Rc and Rd and could obtain the maximum quantity in a greatly reduced time period (3 minutes compared with 3 hours for Soxhlet and 1 hour for ultrasound-assisted). The solvent to solid volume was also lower when compared with both Soxhlet and ultrasound-assisted extraction (Yang et al., 2004). The authors only obtained standards for these 3 ginsenosides, so information about possible degradation and loss of other ginsenosides was not available. Kwon, Lee et al. (2003) have also explored the use of MAP™ using aqueous ethanol as the extraction solvent for *Panax ginseng* and obtained consistent yields compared to conventional extraction (Kwon, Lee, Bélanger, & Paré, 2003).

Wu et al. (2001) have used ultrasound-assisted extraction of both *Panax ginseng* and *Panax quinquefolius*. They obtained the best results when using water-saturated butanol as the solvent in a 75 mL per g of ginseng solvent to solid ratio at 25°C and a 2-hour extraction time. The results for this case were consistent with an 8 hour Soxhlet using water-saturated butanol with the same solvent to solid ratio (Wu et al., 2001). Using methanol and water with 10% methanol resulted in similar yields after a 2-hour ultrasonic extraction vs. an 8 hour Soxhlet extraction. Both indirect and direct sonication were tested, with direct sonication being found to be more effective for removing ginsenosides from the plant materials. The results for this study are shown in Table 2.3.2.

Table 2.3.2 – Comparison of Ultrasound-assisted and Soxhlet extraction of Various Ginseng Species
(Wu et al., 2001)

The yields (wt.%) of total saponin extracted from various ginseng samples by different extraction methods[a]

Extracting solvent	Extraction method/time						
	Ultrasound bath		Ultrasonic probe		Soxhlet extractor		
	1 h	2 h	1 h	2 h	1 h	2 h	8 h
American ginseng							
MeOH	3.95	4.00	4.25	4.58	2.00	2.75	4.40
Water-saturated BuOH	4.10	4.30	4.32	4.75	2.10	2.95	4.61
Water with 10% MeOH	4.00	4.20	4.20	4.65	2.10	2.90	4.52
Chinese ginseng							
MeOH	2.10	2.30	2.15	2.31	1.20	1.58	2.28
Water-saturated BuOH	2.20	2.42	2.25	2.45	1.22	1.62	2.46
Water with 10% MeOH	2.15	2.38	2.20	2.40	1.15	1.70	2.35
Korean red ginseng							
MeOH	2.95	3.20	2.90	3.22	1.68	2.25	3.30
Water-saturated BuOH	3.00	3.35	3.12	3.45	1.75	2.60	3.30
Water with 10% MeOH	3.13	3.25	3.20	3.30	1.60	2.00	3.25
Ginseng cells							
MeOH	2.00	2.11	2.05	2.20	1.35	1.45	2.00
Water-saturated BuOH	2.10	2.20	2.15	2.30	1.45	1.65	2.10
Water with 10% MeOH	2.10	2.20	2.10	2.22	1.40	1.50	2.15

[Note: MeOH – methanol, BuOH – n-butanol.]
[a] Each point is the mean of three to four replicates with a standard deviation no more than 10% of the mean.

Pressurized liquid extraction (or assisted solvent extraction) is another potential method for ginsenoside extraction from ginseng. In this technique, a liquid solvent is put under a pressure higher than atmospheric pressure in order to allow for liquid conditions at higher temperatures, which allow for faster extraction kinetics. The technique was developed for use in environmental analysis and remediation and has been recently adapted for

pharmaceuticals and natural products, such as taxol (Choi, Chan, Leung, & Huie, 2003). The technique can potentially reduce extraction time and necessary solvent volume, making it potentially an attractive alternative to more conventional techniques. Choi et al. (2003) studied the use of pressurized liquid extraction for ginsenosides using water and methanol with and without a non-aqueous surfactant, Triton X-100. The authors found that a 10-minute pressurized liquid extraction with water as a solvent at 1500 psig and 90°C was equivalent to a 2-hour ultrasonic extraction at 50°C and that increasing temperature could increase the extraction efficiency to over 110% of ultrasonic extraction. The authors, however, used a 200 mL: 1 g solvent to solid ratio and did not study the effect of decreasing this ratio on extraction efficiency (Choi et al., 2003).

In the case of ultrasound-assisted extraction, the disadvantage is the need for a high solvent to solid ratio and a relatively large time scale (1 or 2 hours). Microwave-assisted extraction is much quicker and required a lower solvent to solid ratio for ginsenoside extraction from ginseng, however, it also leads to the same thermal conversion of ginsenosides present in conventional solvent extraction. Pressurized liquid extraction has the advantage of providing Soxhlet type results in a shorter time frame but the solvent to solid ratio may not be reduced. All of the techniques listed, Soxhlet, ultrasound-assisted extraction and microwave-assisted extraction require a separate purification step to obtain individual ginsenoside compounds. Using a supercritical fluid technique can ideally remove this step by using the tunable properties of the fluid (changing density by changes in pressure and temperature). Supercritical fluid extraction will also ideally use a smaller

solvent to solid ratio then other conventional solvent techniques and a lower time scale than Soxhlet (comparable or shorter than ultrasound-assisted).

To date, a small amount of open literature regarding supercritical fluid extraction of ginseng exists and it has focused almost exclusively on *Panax ginseng* (Korean ginseng). Wang et al. (2001) explored the extraction of the root hair of Panax ginseng using supercritical carbon dioxide and supercritical carbon dioxide aqueous ethanol as a modifier (Wang et al., 2001). In this work, ginseng root was obtained, ground to pass through a 140-mesh screen (105 μm diameter) and dried in a vacuum desiccator. The extraction system consisted of a 300 mL extraction vessel and two 1.4 L absorbing vessels for pressurized liquid phase trapping.

For experiments, the system was run either in batch mode with the desired co-solvent (if any) spiked directly on the ginseng prior to extraction or dynamically with co-solvent sequentially added to the system during an extraction. During this study, mole fractions ranging between 0 and 6% aqueous ethanol as a co-solvent were studied at 31.2 MPa and temperatures of 35° and 60°C respectively (Wang et al., 2001). Aqueous ethanol was chosen as a co-solvent likely due to its benign nature, being non-toxic. However, as a co-solvent for studying the dynamics of a CO_2 + co-solvent system, this is not necessarily the ideal choice due to low solubility in CO_2 due to the presence of water.

The authors found that for 4-hour extractions, the ideal method for extracting ginsenosides was to use a batch mode with co-solvent spiked directly on the ginseng,

while sequential addition gave a higher amount of overall ginseng oil extracted. This indicates that for these conditions, the ginsenosides in *Panax ginseng* are mass transfer/desorption limited for extraction while the overall oil tends to be solubility limited. Only approximately 55% of the total ginsenoside content present in the root hair could be extracted under the conditions studied by these authors, indicating that room for further optimization existed (Wang et al., 2001).

Experiments using pure CO_2 as a solvent were found to provide negligible quantities of ginsenosides, which is to be expected due to the polar nature and high molecular weight of the ginsenosides. Another interesting fact of these experiments is that a 4-hour extraction time was needed by the authors to achieve this quantity of ginsenosides, which is longer than usual for supercritical extractions (particularly those for particles less than 0.5 mm in diameter). This is an indication that there remains a significant solute-matrix interaction under the studied experimental conditions rather than just simple internal mass transfer resistance. These results could possibly be improved by increasing the amount of modifier used during extractions. The authors also provided no information on collection efficiency, making it difficult to determine the efficiency with which extracts were collected vs. total amount of material extracted. However, with two pressurized liquid trapping vessels in series (with aqueous ethanol as trapping liquid) the trapping efficiency is likely to be approximately 100%.

In terms of patent literature, the US Patent and Trademark Office has patents related to extraction of plants of the *Panax* genus and other ginseng plants using supercritical

fluids, both in terms of ginsenoside removal and for removal of pesticides. Inada et al. (1991) patented a technique for producing edible compositions from plants using fluids at sub and supercritical conditions. This technique was capable of extracting 1000 g of coarsely ground ginseng using supercritical CO_2 with ethanol as a modifier at 3115 psig and 39°C, obtaining 23 g of extract which contained various saponins. No information was given relating the composition of the extract in terms of saponins content or to the type of ginseng (Asian, North American, etc.) being extracted (Inada et al., 1991).

Kim et al. (1998) described a method for heat treating ginseng extracts in order to produce higher quantities of Rg_3 and Rg_5 relative to normally obtained extracts and listed supercritical fluids as a possible solvent for extraction (Kim, Park, Lee, Park, & Kim, 1998). The authors gave no information relating to the methodology involved in supercritical fluid extraction and appeared to include the technique as one of many possible methods for obtaining ginsenosides. Similarly, another group filed a patent application related to heat-treatment, acid-treatment or bio-conversion to obtain higher ratios of the ginsenosides Rk_2 and Rh_3 as well as Rg_3 and Rg_5 and mentioned supercritical fluid extraction as a potential method for solvent extraction to obtain raw extracts (Kim et al., 2003). This group also provided no information relating to supercritical extraction methodology.

Martin et al. (2004) have a patent related to preparing bioactive substances from natural sources using supercritical fluid extraction and/or fluorocarbon solvent extraction (Martin, Ashraf-Khorassani, & Taylor, 2004). This patent covered the extraction of

bioactive compounds from *Pfaffia paniculata* (Brazilian ginseng), including nortriterpenoid saponins. The authors described a method to use supercritical fluid chromatography to separate components of interest. No mention was given to the extraction of ginsenosides (triterpenoid saponins) from this natural product.

Schutz and Vollbrecht (1992) filed a patent related to using pure supercritical carbon dioxide for the extraction of pesticides from ginseng plants that have been moistened by adding water which acts as a modifier. Extractions were run at a pressure between 200 to 350 bar and a temperature greater than between 60 and 90°C. Runs used between 10 to 100 kg of CO_2 per kg of root to be purified, reducing pesticide content by more than 99.7% by weight. Ginsenosides were not extracted in any significant quantity in this technique according to the claims of the inventors (Schutz & Vollbrecht, 1992).

In addition to this work with root hair extraction and the existing patent literature, other work has been done regarding pesticide removal from ginseng plants using supercritical fluid techniques. Quan et al. (2004) studied the removal of 9 organochlorine pesticides from *Panax ginseng*. They found that even pesticide removal required 10 wt% $EtOH/H_20$ as a modifier for supercritical fluid extraction. Since the authors were only interested in an analytical technique to detect pesticide level in ginseng, they reported no information on the extraction of ginsenosides and other medicinal components along with pesticides (Quan et al., 2004).

In spite of the lack of open literature on supercritical fluid extraction of ginseng itself, there is a large amount of literature devoted to extraction of other natural products using either neat supercritical carbon dioxide or with modifiers. Zancan et al. (2002) studied the effect of oleoresin from ginger using neat CO_2, CO_2 + ethanol and CO_2 + isopropanol at 1.2 wt% (Zancan et al., 2002). The studied system was found to be suitable for extraction without co-solvent and found to be solubility limited (matrix modification of surface was not required). Trapping using a solid-phase adsorbent was found to be effective (Zancan et al., 2002). The particle size distribution approximately varied from 350 to 1200 microns with a uniform distribution by mass (Zancan et al., 2002).

Yin et al. (2003) studied the extraction of seed oil from *Hippophae rhamnoides* L. seeds using supercritical CO_2. The system was found to have an optimum at 20 MPa, 35 to 40°C, a residence time of 24 to 40 seconds and an extraction time of 4 to 5 hours. The seed oil extraction was desorption/mass transfer limited in this case. Milling of the seeds reduced the time required for extraction of the easily accessible solute. Trapping was accomplished on a glass collection vessel without temperature control (Yin, Sun, Ding, & Liang, 2003).

Grigonis et al. (2005) examined the extraction of the antioxidants 5,8-dihydroxycoumarin and 5-hydroxy-8-O-β-D-glucopyranosyl-benzopyranone from sweet grass (*Hierochloë odorata*) using CO_2 and CO_2 + ethanol. Extractions with pure CO_2 yielded negligible amounts of both antioxidants compared with Soxhlet extractions under the studied experimental conditions. CO_2 + ethanol was found to be effective for extracting both

antioxidants in direct spiking experiments, with higher modifier percentages giving higher total yields and higher antioxidant extracts. The selectivity of SFE decreased above a weight fraction of 20% ethanol in the fluid phase, meaning that the extraction could no longer selectively extract the two antioxidants only. A two-stage extraction process with CO_2 + ethanol at 35 MPa and 40°C followed by pure CO_2 at 25 MPa and 40°C was found to be effective for both recovery and selectivity. Ethanol was used as the trapping solvent as well as a modifier (Grigonis, Venskutonis, Sivik, Sandahl, & Eskilsson, 2005).

Mannila et al. (2003) extracted bioactive components from St. John's Wort (hyperforin) and *Ginkgo biloba* (ginkolides) using supercritical fluid extraction. For the extraction of St. John's wort, pure CO_2 was found to be more effective since modifiers cause partial extraction of polar compounds without increasing the extraction of hyperforin and adhyperforin. For ginkolides, a polar organic modifer was needed. A mixture of ethanol/acetic acid (9:1 v/v) was used in place of methanol for toxicity concerns. The highest extraction efficiency was achieved with two stages, a static stage with 3 mL solvent/g solid and dynamic stage which used 2 mL solvent/g solid. The pressure chosen was 350 atm, the temperature 100°C and a 40 minute extraction period (20 minutes static and 20 minutes dynamic). Experimental results indicated a significant decrease in extraction time due to the modifier effect. Boiling ethanol extraction required 40 mL/g for similar recovery on a much larger time scale and was less selective than supercritical fluid extraction (Mannila et al., 2003).

Ellington et al. (2003) studied the extraction of colchicine and related alkaloids using supercritical carbon dioxide with methanol as a modifier (Ellington, Bastida, Viladomat, & Codina, 2003). They found that optimal results could be obtained with a 25-minute static/30 minute dynamic stage using 3% methanol as a modifier, obtaining over 97% recovery for all alkaloids compared with conventional solvent extraction using a greatly reduced organic solvent volume and extraction time using ODS as a trapping method (Ellington et al., 2003).

Overall, there is a small volume of available literature devoted to extraction of ginsenosides using supercritical techniques when compared with other extraction methods. Supercritical fluid extraction processes have been applied successfully to a number of natural products to date, taking advantage of fast extraction times, reduction in solvent volume, potential selectivity and fractionation capacity. To this end, further research on using supercritical fluids for extraction of ginseng components will help to determine if this technique has applications in sample analysis or in larger scale production processes.

3. RESEARCH OBJECTIVES

The purpose of this study was to investigate the effect of various operating variables on the extraction of ginsenosides, as well as on overall ginseng oil yield from North American ginseng using supercritical carbon dioxide with modifiers. The ultimate goal of the project was to determine if experimental conditions existed using supercritical fluid techniques that could reproduce the yield of ginsenosides present in conventional solvent extraction techniques. The root of North American ginseng was obtained for the purposes of this study, and the following objectives were set:

1. Determine ginsenoside content and total ginseng oil yield present by using conventional solvent extraction techniques (i.e. Soxhlet using methanol).

2. Preliminary study of experimental conditions to determine a set of conditions under which ginsenoside and overall extraction yields could be determined.

3. Using the previously determined conditions, test various trapping methods for determination of the most effective trapping scheme to use for supercritical extraction for the bulk of studies using CO_2 + methanol as a test system.

4. Perform preliminary investigation into extraction of ginsenosides from the root of North American ginseng using supercritical CO_2 + modifiers in order to gain an understanding of which variables effect the extraction in the greatest manner.

5. Determine a rough optimum set of conditions under which ginsenoside extraction is maximized (i.e. as close as possible to conventional extraction techniques).

6. Determine which modifiers are most effective for extraction of ginsenosides.

7. Determine sufficient information about the system characteristics, such as trapping efficiency, effect of recovery stages and effect of extraction types in order to be able to recommend future operating conditions for larger scale extractions.

4. EXPERIMENTAL METHODOLOGY

4.1 Materials

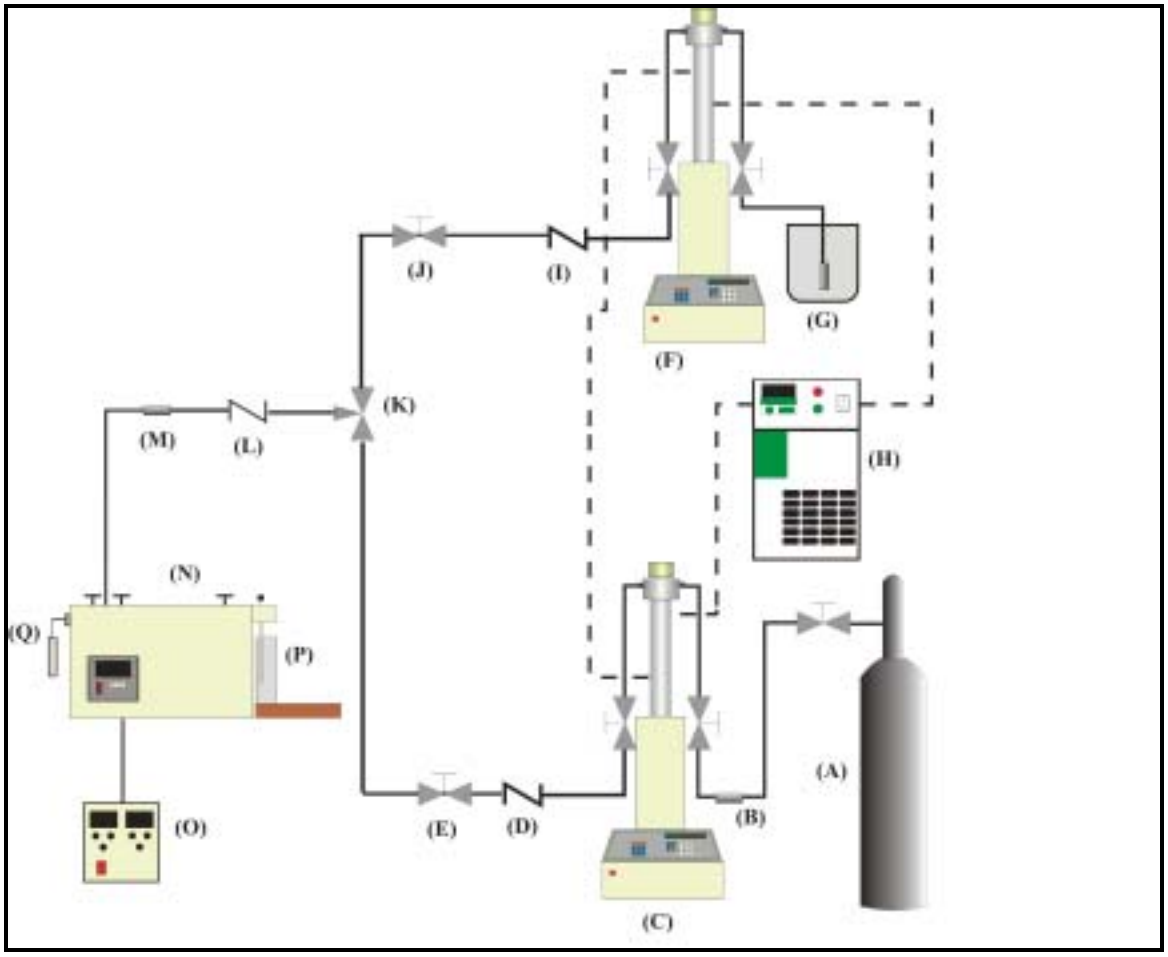
Carbon Dioxide (99.99% purity) was purchased from BOC Canada Ltd. of London, Ontario, Canada and further purified by passage through columns containing molecular sieves (Aldrich Canada) and copper (II) oxide supported by alumina (Aldrich Canada) to remove water and oxygen, respectively. Ground, dried, root of North American ginseng was obtained from Agriculture and Agri-Food Canada. The sample was a mixture of various collected ginseng roots, ground together to insure consistency. The volume weighted mean diameter of the powder was approximately 550 μm, as determined by analysis using a Malvern Mastersizer, described in section 4.3.

HPLC grade methanol and DMSO were obtained from Aldrich Canada, anhydrous ethanol and aqueous ethanol were obtained from Commercial Alcohols Ltd. and glacial acetic acid (99.7% purity) was obtained from EMD Chemicals Inc. for use in the experiments. Sand (50+70 mesh) and Amberchrom GC161M poly(styrene divinyl benzene) were obtained from Alrich Canada. Ginsenoside standards for HPLC of Rb_1, Rb_2, Rc, Rd, Re and Rg_1 were provided by Dr. Mark Bernards, Department of Biology, University of Western Ontario and were originally obtained from the INDOFINE Chemical Company (Somerville, New Jersey, USA).

4.2 Experimental Setup

For conventional solvent extractions, a Soxhlet apparatus was obtained from VWR Canada, Ltd. with a 250 mL solvent flask. Whatman cellulose thimbles were also obtained from VWR for use in the extractions. For supercritical extractions, an Isco SFX 2-10 extraction unit was used (Teledyne Isco, Lincoln, Nebraska, USA) along with a restrictor to control flow rate out of the system (both a capillary and a heated variable volume restrictor were used in experiments). Two Isco D series syringe pumps were used to provide carbon dioxide and modifier, a 260D and 100 DX respectively. Temperature of the fluid in the pumps was controlled using a NESLAB RTE-101 Chiller/Circulator with water as the cooling liquid. A mixing three-way union from Valco was used to mix fluids together for experiments requiring a dynamic flow of carbon dioxide and modifier. Various trapping methods were implemented to collect extracts, with specific details outlined in Section 4.7. A schematic of the supercritical extraction system used for experiments is shown in Figure 4.2.1.

Figure 4.2.1 – Supercritical Extraction System Schematic



A – CO_2 Cylinder

B – Filter

C – Isco 260D Syringe Pump

D – CO2 Check Valve

E – CO2 Gate Valve

F – Isco 100DX Syringe Pump

G – Modifier Vessel with In-line Filter

H – Chiller/Circulator

I – Modifier Check Valve

J – Modifier Gate Valve

K – Valco Mixing Tee

L – Check Valve

M - Filter

N – Isco SFX 2-10 Unit

O – Restrictor Temp. Controller

P – Trapping Vessel

Q – Vent Valve

The Isco syringe pumps operate by allowing for a liquid to be drawn into the pump and then compressed to the desired pressure by reducing the system volume by lowering a piston. The pump controller allows for measurement of system volume, pressure and flow rate and can be run in several modes, including constant pressure (where the pump will run such that the pressure in the pump is kept at a constant value), constant flow (where the pump runs at a constant flow rate) and modifier (where the two pumps can be run in constant pressure mode simultaneously with a constant volume gradient from the secondary pump). Calibration of the pumps was performed periodically to insure that the atmospheric pressure in the room was taken as the zero (i.e. the pressure read by the pump was the gauge pressure of the system). The accuracy of the flow rate meter was ± 0.5% of the flow rate reading for the 260D pump and ± 0.3% for the 100DX. The pressure accuracy was ± 0.5% of the reading at constant temperature. The volume displacement was of an accuracy of 0.65 nl for the 100DX and 16.63 nl for the 260D. The maximum operating pressure of the pumps was 7,500 psig with a maximum volume of 266 mL in the 260D and 100 mL in the 100DX respectively.

Using the chiller/circulator allowed for control of temperature in the pumps and equilibration was allowed to occur over a period of 3 to 4 hours to determine the volume of liquid in the pump accurately. The two pumps were connected, as mentioned, using a Valco mixing tee which allowed for CO_2 + co-solvent experiments to be run dynamically. An inline filter (Upchurch, 5 micron) was used for the modifier line in order to prevent contamination of modifier when re-filling the pump.

The extraction unit chosen for this system was an Isco SFX 2-10 unit from Teledyne Isco. The extractor consists of an extraction chamber where samples can be placed, inlet, extract and vent valves for fluid flow as well as a temperature controller to maintain the temperature in the system from ambient to 150°C. The maximum operating pressure of the system is approximately 10,000 psig. The extraction chamber has a volume of 20 mL at ambient conditions. Samples are placed in extraction vials with a volume of 10 mL, either constructed of stainless steel or PEEK, containing filter frits of 0.5 micron. The extractor unit also contains 2 micron frits at the inlet and exit to the unit. The extraction vials can be snapped into place on the extraction chamber cap and the cap is then screwed into the top of the extraction chamber to seal the system. The vent has a relief valve that will rupture under a pressure of 15,000 psig for safety considerations. The flow rate out of the system is controlled using an Isco heated adjustable restrictor. Pictures of the equipment used for supercritical extractions are provided in Figures 4.2.2 (pump system), 4.2.3 (extractor system) and 4.2.4 (extraction vial clamping to chamber cap).

Figure 4.2.2 – Dual Pump System Used for Supercritical Extractions

Figure 4.2.3 – Isco SFX 2-10 Extraction System

Figure 4.2.4 – Extraction Vial Clamping to Chamber Cap

Left – Extraction Vial used in experiments

Right – Vial Clamped to Chamber Cap for Isco SFX 2-10 Extractor

During the collection of extracts, the rapid depressurization of fluid causes a drop in both temperature (Joule-Thomson effect) and density. This can lead to deposition of extracted materials along the restrictor length, which plugs the extraction. In order to overcome this difficulty, heated restrictors are used. Two restrictors were used for this study; an Isco capillary restrictor (rated to 0.5 mL/min in the liquid state for CO_2 at 5000 psig and a 60°C temperature in the extractor) and an Isco heated adjustable restrictor. The capillary

restrictor gives a flow rate set by the pressure, temperature and sample present in the extractor. Heating was provided by an air convection heater, with a range of ambient to 120°C. The adjustable restrictor allows for control of flow rate by adjusting the restriction knob within ± 20%, with a temperature range of ambient to 150°C and a temperature accuracy of ± 15°C. The adjustable restrictor temperature controller allows for control of both the tip of restriction temperature, and the temperature along the restriction length. The restrictor tip was placed directly in the trapping vessel to allow extracted components to be collected. The trapping methods studied in this project are discussed later in Section 4.7.

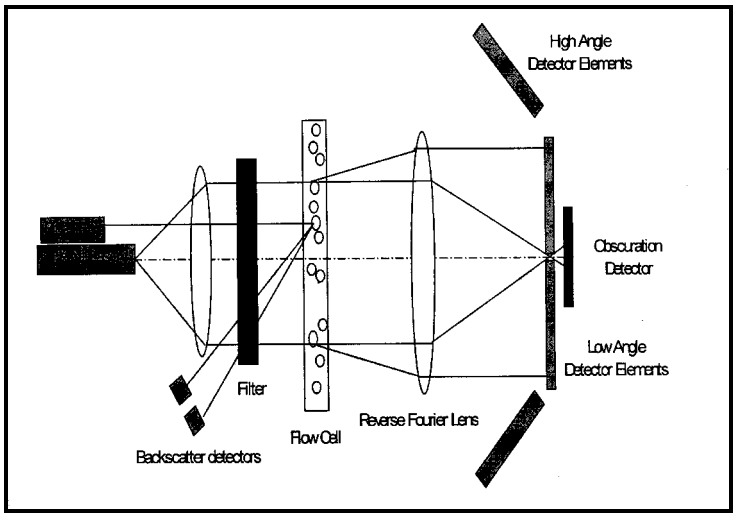
4.3 Particle Size Analysis

The ginseng powder obtained was previously dried and ground to a relatively small size. For supercritical extractions, particularly from plant materials, internal diffusion is frequently a limiting factor, so operating with a small particle size can have an important role in determining extraction efficiency. As well, the particle size distribution will determine if the sample is unimodal or not, which is important since using a non-unimodal distribution will increase the chance of channeling and poor extractions. In order to determine the particle size distribution, analysis by the Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK) was used.

The Malvern Mastersizer 2000 works on the principle of laser diffraction. The system contains an optical unit, computer and a unit for sample dispersion. The sample dispersion unit allows for dispersion of the solid sample in a liquid for analysis by the

optical unit, where size analysis is performed by laser diffraction. In the sample dispersion unit, agitation is continuously provided to circulate the sample ultrasonically. The sample is passed through a cell that allows it to be analyzed by a collimated beam of laser light. Two lasers (Neon-Helium and red light) and a short wavelength blue light source are used to determine particle size. Any solid particles passing through the cell will scatter the light, with the angle being a function of the size, shape, refractive index and wavelength of incident light. The scattered light is incident on a Fourier transform lens and focused on a photo diode array which detects the intensity of light. The scattered light pattern formed at the detector is the total pattern formed by each particle that is sampled and using deconvolution on the pattern, resulting in the size information of each individual particle sampled. This can then be used to generate a particle size distribution. A schematic of the laser diffraction principle used in the Malvern is provided in Figure 4.3.1.

Figure 4.3.1 – Schematic of Laser Diffraction Principle used by Malvern
(Bakhbakhi, 2004)



4.4 HPLC Analysis of Ginsenosides

In order to quantify the amount and type of ginsenosides present in various extracts, high performance liquid chromatography (HPLC) was employed. Chromatography is a general technique which separates a mixture into individual components. These components are then sent to a detection system which can characterize each component. HPLC is a chromatographic technique that involves passing a sample containing various components (analytes) in a high pressure solvent (mobile phase) through a column packed with sorbent (stationary phase). As the sample passes through the column, different components will interact between the mobile and stationary phases at different rates. The difference is primarily due to difference in polarities between the components being analyzed. The components with the highest affinity for the mobile phase, or least affinity for the stationary phase, will exit the column faster.

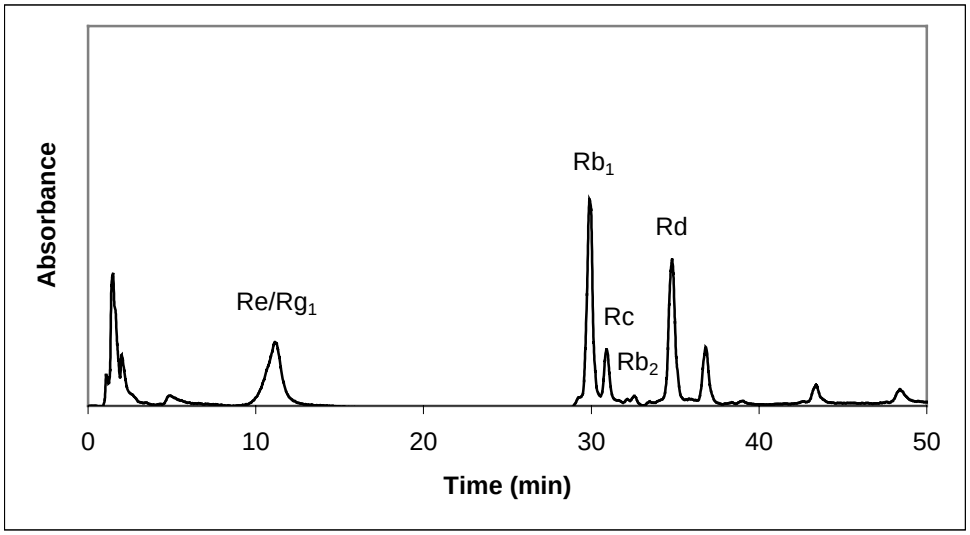
Mixtures of analytes can be analyzed by changing the polarities of the stationary and mobile phase. The stationary phase is typically bonded to a support phase, which is usually porous beads. The pore sizes in the beads can be varied to allow analytes of certain sizes to pass through at different rates. The dimensions of the column can also be altered to allow different sample quantities to be analyzed. Changes in the polarity of the mobile phase can also affect the quality of separation, by means of using a gradient mobile phase. As analytes exit the column, they can be detected using numerous means, such as refractive index, electrochemical potential or ultraviolet-absorbance changes in the mobile phase. The amount of analyte leaving the column will determine the intensity

of the signal produced by the detector, allowing for concentration curves to be constructed using standard solutions. The time that each analyte takes to appear as a peak is called the retention time, which also can be determined using standard solutions. Using the retention time and peak intensity will allow for the determination of the concentration of the analyte being examined.

The standards for ginsenosides Rb_1, Rb_2, Rc, Rd, Re and Rg_1 were used to construct individual calibration curves for ginsenoside content. A Microsorb ® C-18 (5 μm, 150 mm x 4.6 mm) column obtained from Varian Canada, Inc. was used for resolution of ginsenosides. For analysis, 20 microlitres of extract dissolved in methanol was injected into the column and eluted with a 10 minute isocratic mixture of 21.5% v/v acetonitrile in water, followed by a gradient to 50% v/v acetonitrile over 50 minutes at a flow rate of 1.0 mL min^{-1}. The UV detector for the system was set at 203 nm. Extracts from supercritical extractions were concentrated in the following manner: 1.0 mL of dissolved extract was taken and placed in a microfuge tube and the solvent was evaporated using heat (60°C) and nitrogen. The samples were redissolved in 100 μL of methanol and centrifuged for 5 minutes at 19,800 x g. The supernatant was transferred to a HPLC vial and placed into the system for analysis. For Soxhlet extractions, the concentration step was unnecessary and 1.0 mL of dissolved extract was centrifuged at 19,800 x g for 5 minutes before the supernatant was placed in a HPLC vial for analysis. The standard mixture of ginsenosides yields an HPLC chromatogram shown in Figure 4.4.1. The calibration curves obtained were linear with an $R^2 > 0.96$ for all cases. The difference between Re and Rg_1 could not be resolved using this system (same retention time) and so the

concentration of these two ginsenosides was lumped into a single calibration. The calibration curves were similar for ginsenosides with identical numbers of sugars attached to the parent carbon chain and are shown in Appendix A.

Figure 4.4.1 – Standard Mixture of Ginsenosides Chromatogram



Re/Rg_1 – 10.7 min, Rb_1 – 29.6 min, Rc – 30.7 min, Rb_2 – 32.3 min, Rd – 34.5 min

4.5 LC/MS Analysis for Identification of Unknown Components

Liquid chromatography/Mass spectrometry is an extension of basic HPLC. In this setup, a liquid chromatography system is coupled to a mass spectrometer. By splitting the analyte flow from the column between the conventional UV detector of the HPLC and between a mass spectrometer, the molecular mass of various ions can be determined. This is useful in two ways: 1) confirming the validity of stock solution calibration, and 2)

determining molecular mass of unknown compounds with retention times in areas of interest.

During the course of experiments, it was found that supercritical extractions were yielding an unknown peak in the ginsenoside region on HPLC. In order to identify this peak, liquid chromatography/mass spectrometry was used with the gracious assistance of the Biological Mass Spectrometry Laboratory at the University of Western Ontario. The system was an Agilent 1100 HPLC with a DAD detector and a Micromass Triple Quad T mass spectrometer operating in negative ion mode (desolvation temperature of 200°C). The HPLC was run using the same procedure as listed in Section 4.3 and samples were prepared in a similar manner for concentration purposes. Sodium Iodide (NaI) was used as an internal standard to calibrate the mass spectrometer and mass spectra between 200 and 1100 m/z were recorded with a 20 V cone voltage. The eluent from the HPLC column was split, with 1/20 (50 μL min^{-1}) mixed with 1% aqueous NH_3 (10 μL min^{-1}) before being passed to the electrospray ionization source of the mass spectrometer. The mass accuracy of the system was better than 0.05 Da.

4.6 Soxhlet Extraction Experiments for Total Ginsenoside Content

In order to quantify the ginsenoside content of the obtained batch of ginseng, Soxhlet extraction experiments using methanol were used in order to determine total ginsenoside content as well as the fraction of extractable ginseng components. This type of extraction has been established in the literature as capable of determining the total ginsenoside

content in a ginseng sample (Court et al., 1996; Cui, Song, Zhou, Liu, & Liu, 2000; Yang et al., 2004). The theory of operation for Soxhlet extractions was provided in Section 2.3.

For Soxhlet runs, approximately 5 g of powder was run with a 150 mL solvent volume (30 mL: 1 g solvent to solid ratio) for a 20-hour extraction period, in order to demalonylate any malonyl-ginsenosides and determine the total ginsenoside content. Court et al. (1996) found that a minimum 20-hour Soxhlet extraction with methanol was required to demalonylate the malonyl ginsenosides. This level of solvent volume and extraction time is sufficient to essentially extract the entire quantity of ginsenosides present in ginseng. The solvent used in this study was methanol, which has been shown to be able to extract ginsenosides effectively from ginseng. Weighing of the powder before and after extraction allowed for determination of total extracts obtained and HPLC analysis of the collected liquid allowed for determination of the ginsenoside content. Due to the high concentration of ginsenosides present in the collected liquid, concentrating the samples was not required before proceeding with the HPLC analysis.

4.7 Trapping Efficiency Testing

Trapping is one of the largest problems associated with supercritical fluid extraction and, as mentioned in the literature review, is also one of the least understood problems. As such, numerous trapping methods were tested using a model CO_2 + methanol system in order to determine the one most suitable for use in experimentation in this system.

Trapping efficiency was gauged from both an overall recovery and ginsenoside recovery viewpoint. Extracts collected in the various traps were tested for ginsenosides and the extracted material was re-extracted using the Soxhlet technique to determine residual ginsenoside content and complete the mass balance based on the total ginsenoside content determined from preliminary Soxhlet extractions of pure ginseng. The following trapping methods were tested for use: 1) inert solid phase trap, 2) adsorbent solid phase trap, and 3) liquid phase trap. Extractions with other modifiers were carried out as well to determine the suitability of the trapping methods for these solvents.

4.7.1 Inert Solid Phase Trap

Two inert solid phase trapping systems were investigated, glass wool and glass beads. These systems were relatively simple to setup, although unlikely to work for the full range of experimental conditions due to the planned use of modifier, which has been shown to reduce trapping efficiency for inert solid traps. The trap consisted of a weighing a set mass of glass wool or glass beads and placing them in a vial of known mass and volume (40 mL). The decompressed gas was then passed directly through the solid phase trap and analytes were able to deposit on the system. The restrictor was placed 2 cm above the bottom of the vial. Temperature control was not implemented for this trap due to the presence of modifier, which causes solid phase traps to become less effective at lower temperatures. Total mass recovered was determined by weighing the solid trap before and after extraction, allowing for runs with modifier to dry to constant weight.

4.7.2 Adsorbent Solid Phase Trap

Due to the fact that modifiers were likely required for trapping, the use of a polymeric adsorbent solid phase trap was also investigated. Chaudot et al. (1998) found that trapping on a styrene divinylbenzene copolymer resin was able to quantitatively recover extracts in CO_2 + methanol extractions at up to 10% v/v of methanol. The resins studied in that work had a high specific surface area (965 m^2/g and 1100 m^2/g) as well as small pore sizes (27 and 85 nm). In order to test this type of system for trapping of ginsenosides in supercritical fluid extraction with modifier, the resin Amberchrom GC161M was obtained from Aldrich. This poly(styrene divinylbenzene) resin has a surface area of 900 m^2/g, a particle size distribution ranging between 50 and 100 μm, and a pore size of 15 nm. The resin was dissolved in solvent and was filtered using a Buchner funnel with Whatman filter paper (10 μm diameter) for use in trapping experiments.

4.7.3 Liquid Phase Trapping

HPLC grade methanol, anhydrous ethanol, and dichloromethane were compared as a trap solvents in a liquid phase trap. Two trapping geometries were investigated, one a two-stage trap consisting of two connected 250 mL Pyrex vacuum flasks, each containing 100 mL of solvent, and the other a 50 mL Pyrex vessel with a slightly sloping increase in diameter with height. Temperature control using both an ice bath and water at room temperature was implemented for the two 250 mL Pyrex vacuum flask. The restrictor tip was well immersed in the liquid phase for both systems.

For runs where overall collection was determined, the solvent reservoir was transferred to another beaker and the solvent evaporated in a vacuum oven (under a vacuum pressure of 75 kPa) and slight heating (35°C). After the solvent was fully evaporated, the sample was weighed, allowing for the total amount of material to be determined from the difference in the empty beaker weight and the weight of the beaker + collected material.

4.8 Pure CO_2 Extraction Experiments

To confirm the established literature reports that pure CO_2 has very poor solubility for the bulk of the constituents of ginseng, including most notably the ginsenosides, extractions using pure supercritical carbon dioxide were carried out. Several pressures and temperatures were selected to determine what effect (if any) that changing fluid density had on extraction efficiency. Extractions were run using the Isco SFX 2-10 system with the 260D syringe pump to provide CO_2 at the desired pressure. The restrictor used for the system was the Isco capillary restrictor with convective air heating. Extracts were trapped on an inert solid phase trap (glass wool) system since modifier was not being used in these experiments. The trap was washed with methanol to recover extracts for analysis. Various methods for placing ginseng in the system were investigated, in order to obtain conditions where fluid channeling or compression of the solid did not occur.

Extractions were run in the following manner:

1. The solids were loaded into the extraction vial in the desired manner for the experiment being performed.
2. The extractor temperature was set to the desired temperature.

3. After temperature equilibration, the extraction vial was placed in the extractor.

4. CO_2 was pressurized to the desired pressure in the syringe pump and the pump valve was opened to allow the connecting lines to fill with CO_2 at the desired pressure and equilibrate. The pumps were run in constant pressure mode to insure that the system pressure would be the desired pressure.

5. The volume of CO_2 in the pump before and after opening the inlet valve was recorded in order to determine the volume of CO_2 that filled the chamber, which allowed for determination of the mass of CO_2 used, since pressure and temperature of carbon dioxide were known.

6. The heating element on the restrictor being used was activated.

7. The system was allowed to equilibrate for 1 – 2 minutes before the extract valve was opened and fluid allowed to flow out of the restrictor as decompressed gas into the trapping vessel at the desired flow rate.

8. The extraction was run at the desired pressure, temperature and with the desired flow rate for a set period of time before the extractor inlet valve was closed and the system vented.

9. The extraction vial was weighed before and after extractions to determine total mass lost (total ginseng extract). The solid phase trap was weighed before and after in order to determine total material collected. Extracts (if any) were recovered by washing the solid phase trap with methanol.

4.9 Static CO_2 + Modifier Extraction Experiments

The presence of modifier has been demonstrated to have a profound effect on extraction efficiency using supercritical carbon dioxide, particularly in the case of natural product extraction. The extraction of both ginsenosides and root hair oil from the root hair of *Panax ginseng* was found to be greatly increased when using modifiers (aqueous ethanol) (Wang et al., 2001). Using static CO_2 + modifier extractions with a pure CO_2 recovery stage was then tested for extraction of materials. The modifiers selected in this study were methanol, aqueous ethanol/acetic acid (9:1 v/v), DMSO, propylene carbonate and PEG 200.

Methanol was selected as a modifier due to its high solubility in CO_2, its ability to disrupt plant-solute complexes as well as solvent properties, since methanol is capable of dissolving ginsenosides and other ginseng components. Although methanol is toxic and

therefore not likely suitable for a commercial process involving ginsenoside extraction and purification, it was nevertheless an excellent modifier with which to test the system dynamics and determine trapping efficiencies and other useful information.

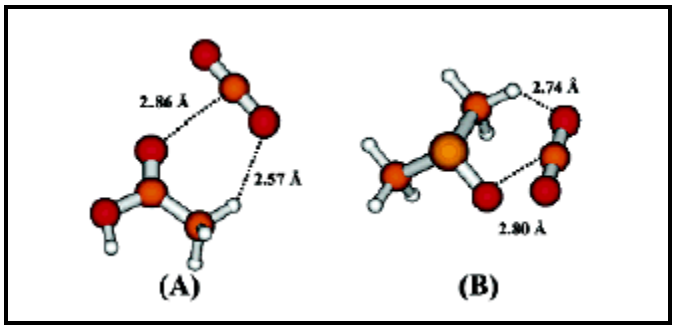
After the methanol experiments were performed, less toxic modifiers were selected. Aqueous ethanol was used as a modifier in experiments with ginseng root hair oil, however, the solubility of aqueous ethanol in CO_2 is limited by the presence of water, which has very low solubility in CO_2. In order to overcome this limitation, a 9:1 v/v mixture of aqueous ethanol and acetic acid was used. Acetic acid has been shown to have a high interaction with CO_2 (Raveendran & Wallen, 2003). Hydrogen atoms in carbonyl compounds which are attached to a carbonyl carbon or an α-carbon are mildly acidic, due to the electron withdrawal by the carbonyl group (Raveendran & Wallen, 2003).

The partial positive charges carried on these atoms allow for C-H bonds on the carbonyl group to work for weak C-H$^{\cdots}$O hydrogen bonds with the oxygen atoms of CO_2, while the carbonyl oxygen can act as a Lewis base for interactions with the electron deficient carbon atom of CO_2. These types of interactions form a complex, which can CO_2-philize systems (Raveendran & Wallen, 2003). Ideally, the use of acetic acid with aqueous ethanol would allow an increase in the amount of water which is soluble in CO_2. In addition, the use of acetic acid would increase the hydrogen bonding and acidity of aqueous ethanol, further increasing any potential solvent-matrix interactions as well as possibly increasing the solubility of desired components. A 9:1 v/v mixture of anhydrous

ethanol and acetic acid was used by Mannila et al. (2003) for extraction of ginkgolides for *Ginkgo biloba* and found to be effective (Mannila et al., 2003).

Dimethyl sulfoxide (DMSO) was also chosen as a modifier for study in this work. DMSO is a non-toxic solvent which is frequently used in the pharmaceutical industry. Similar to acetic acid, DMSO can exhibit Lewis acid – base interactions with carbon dioxide. In this case, the sulfonyl group of DMSO acts as the Lewis base and allows for CO_2-phillic interactions through cooperative C-H···O interactions (Raveendran & Wallen, 2002). DMSO was chosen as a modifier for experimentation in this system due to its high solubility in CO_2, its potential ability to act as a surface modifier and its application as a non-toxic solvent. The acetic acid – carbon dioxide and DMSO – carbon dioxide complexes, as predicted by the work of Raveendran and Wallen (2002) are shown in Figure 4.9.1.

Figure 4.9.1 – CO_2 – Carbonyl and CO_2 – Sulfonyl Complexes
(Raveendran & Wallen, 2002)



(A) is acetic acid – carbon dioxide complex, (B) is DMSO – carbon dioxide complex

The other modifiers chosen to analyze in this system were propylene carbonate and poly(ethylene glycol) with a molecular weight of 200 (PEG 200). These solvents are both "green" and are used in the pharmaceutical industry, although the suitability for use in a supercritical CO_2 extraction process was undetermined at the time as they are not commonly used modifiers for CO_2 in extraction.

In static extractions + modifier, the procedure was similar as described for pure CO_2 extractions with the following exceptions:

1. After adding the mixture of solids to the system, a known volume of modifier being studied was spiked directly onto the system. Mass was recorded before and after this step, allowing for an exact determination of the mass of modifier used in each experiment.

2. The system was left under pressure in static (batch) conditions for a set period of time (ranging from 15 minutes to 2 hours) before the extract valve was opened and extracts were collected. Pure CO_2 was provided by the pump in the recovery phase.

3. For solid phase trapping, the amount of recovered material was determined in the same manner as described in the previous section. For liquid phase trapping, the trap solvent + extract was transferred to a vial of known mass and the solvent was slowly evaporated and the mass of the remaining vial + extract taken to determine the total mass recovered.

The mole fraction of modifier in the supercritical fluid was determined in the following manner:

Equation 4.9.1

$$n_{modifier} = \frac{m_{modifier}}{MM_{modifier}}$$

Equation 4.9.2

$$m_{CO2} = \rho_{CO2} V_{CO2}$$

Equation 4.9.3

$$n_{CO2} = \frac{m_{CO2}}{MM_{CO2}}$$

Equation 4.9.4

$$y_{modifier} = \frac{n_{modifier}}{(n_{CO2} + n_{modifier})}$$

The density of carbon dioxide was determined at the appropriate temperature and pressure using the data available from the National Institute of Standards and Technology (NIST) for pure carbon dioxide. Densities are calculated based on the Span-Wagner equation of state developed for carbon dioxide. This data has been found to be accurate to within 0.03 – 0.05% for density for pressures up to 30 MPa and temperatures up to 523 K (NIST, 2003).

4.10 Dynamic CO_2 + Modifier Extraction Experiments

Although direct spiking of modifier on *Panax ginseng* was shown to be more effective for extraction of ginsenosides by Wang et al. (2001), the effect of a single dynamic stage extraction using CO_2 + modifier was considered. For this type of extraction, methanol alone was considered in comparing this approach with static extractions with modifier. Samples were prepared similarly to pure CO_2 extractions. For dynamic runs with modifier, a second pump was required. Both pumps were run in modifier mode, which allows for constant pressure operation with a constant volume gradient of modifier to be added by the second syringe pump.

4.11 Static + Dynamic CO_2 + Modifier Extraction Experiments

After completion of the static CO_2 + modifier and dynamic CO_2 + modifier, it was desired to observe the effect of adding a dynamic CO_2 + modifier stage after the static extraction as compared with a recovery stage where pressure is maintained by adding pure CO_2. The result on overall extraction, ginsenoside extraction, as well as ginsenoside recovery, were observed. The experimental methodology to run these types of experiments, was to first perform a static CO_2 + modifier extraction, and then run both pumps in modifier mode at the desired modifier percentage for the desired dynamic extraction time.

5. RESULTS AND DISCUSSION

5.1 Particle Size Analysis Results

The ground ginseng root powder obtained from Agriculture Canada was analyzed to determine the particle size distribution using the Malvern Mastersizer 2000 (theory described in Section 4.3). The refractive index of ginseng powder was taken as that of talc for the Mastersizer runs. The following particle size distributions were obtained with randomly collected batches of ginseng.

Figure 5.1.1 – Particle Size Distribution for Ginseng (Run 1)

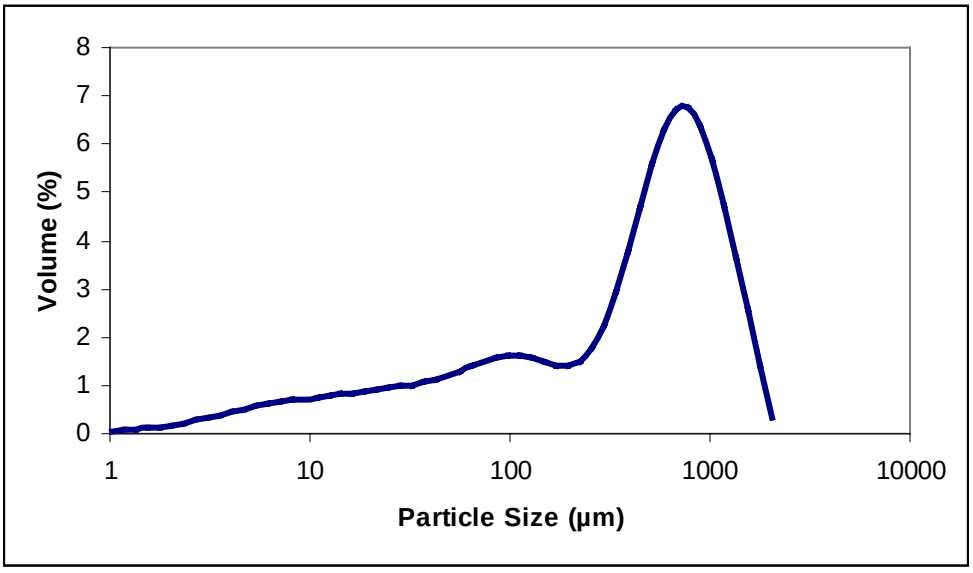


Figure 5.1.2 – Particle Size Distribution for Ginseng (Run 2)

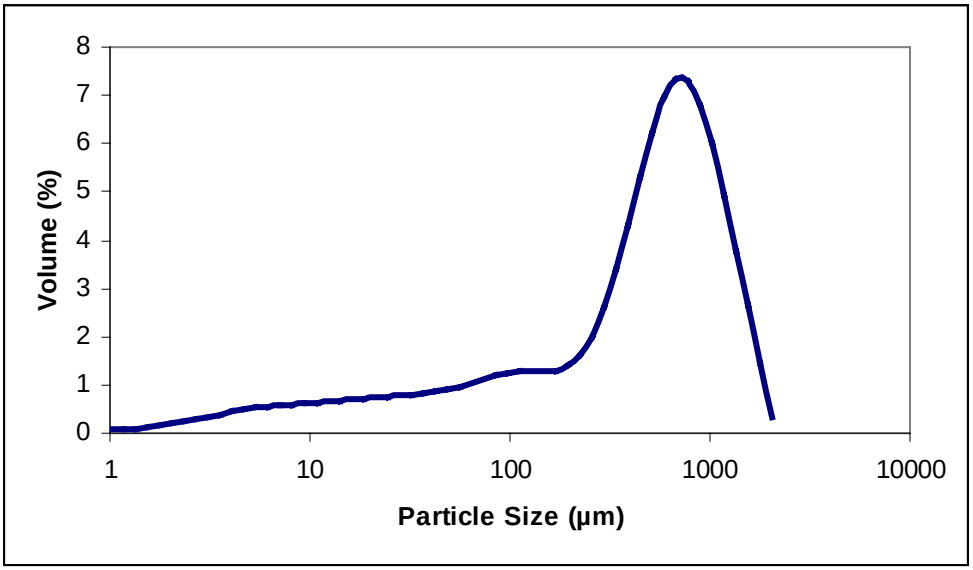


Table 5.1.1 – Particle Size Information from Malvern Mastersizer 2000

Volume Weighted Mean Diameter (% RSD)	552.2 µm (4.3 %)
$d_{0.1}$ (% RSD)	20.4 µm (19.3 %)
$d_{0.5}$ (% RSD)	500.8 µm (5.8 %)
$d_{0.9}$ (% RSD)	1187.9 µm (0.7 %)

The volume weighted mean diameter, as well as $d_{0.5}$ and $d_{0.9}$ have relatively low error associated with the measurement. In the case of $d_{0.1}$, the fines distribution is more subject to error from sampling, which explains the larger variation. 50% (by volume) of the particles are less than 500.8 µm and 90% are smaller than just under 1.2 mm. Based on these Mastersizer results, the particles were deemed to be already sufficiently small to use for supercritical fluid extraction tests without further grinding, which could lead to mechanical stability problems, as the volume weighted mean was 552.2 µm which is in the range of typical particle sizes for supercritical fluid extraction (100 to 1000 µm). Based on the variation in particle sizes due to the presence of larger particles as well as

fines, there would appear to be a need for a dispersant to be mixed with the ginseng in order to obtain a more uniform voidage and avoid channeling as well as potential compression of solid samples during extraction.

5.2 Soxhlet Extraction Experiments for Total Ginsenoside Content

For Soxhlet extractions for determination of total ginsenoside content per gram of ginseng 5 g of ginseng powder was loaded into a cellulose thimble and extracted for 20 hours with a solvent to solid volume of 30 mL solvent : 1 g of ginseng. Court et al. (1996) indicated that an extraction time of 20 hours was required for full conversion of malonyl ginsenosides into their neutral counterparts. The results for overall yield and ginsenoside content are given in Table 5.2.1 along with the percentage of each individual ginsenoside in the total ginsenoside extracted quantity and relative standard error estimates calculated from experimental repeats using methanol. Four Soxhlet extractions were performed for the purpose of determining reproducibility. Representative chromatograms of the Soxhlet are shown in Figures 5.2.1 and 5.2.2, without sample concentration.

Table 5.2.1 – Total Extract and Ginsenoside Yields for Methanol Soxhlet

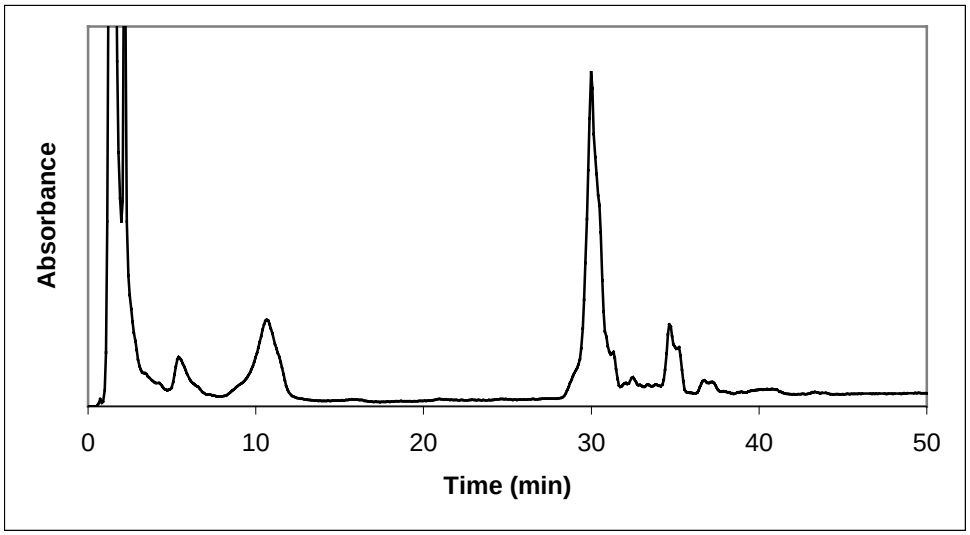
Quantity	mg /g ginseng (% RSD)	Percent Composition (% RSD)
Total Extract Yield	408.86 (5.95)	N/A
Rb_1	44.96 (3.96)	59.54 (1.52)
Rb_2	1.60 (43.69)	2.14 (45.88)
Rc	4.81 (5.93)	6.37 (5.95)
Rd	7.64 (3.58)	10.12 (3.58)
Rg_1/Re	16.51 (12.56)	21.84 (10.32)
Total Ginsenoside Yield	75.52 (3.80)	N/A

The results for MeOH Soxhlet were reproducible and consistent with existing literature both in terms of quantity of total extract obtained, total ginsenoside content present, and the composition of ginsenosides in the extract. The exception is for ginsenoside Rb_2, which had the highest variation of any ginsenoside analyzed. The amount of Rb_2 varied between 1 and 2 mg/g for all experiments undertaken. This variation is likely due to the small amount of Rb_2 present in the root, which made it difficult to accurately quantify. According to existing literature, the roots of *Panax quinquefolius* contain primarily Rb_1 and Re, which was the result observed for the standard extractions performed in this study, in which 81% of the ginsenosides were comprised by Rb_1, Re and Rg_1 (Assinewe

et al., 2003). Although the individual amounts of Re and Rg_1 could not be determined, likely it is mostly Re as it is more common in North American ginseng.

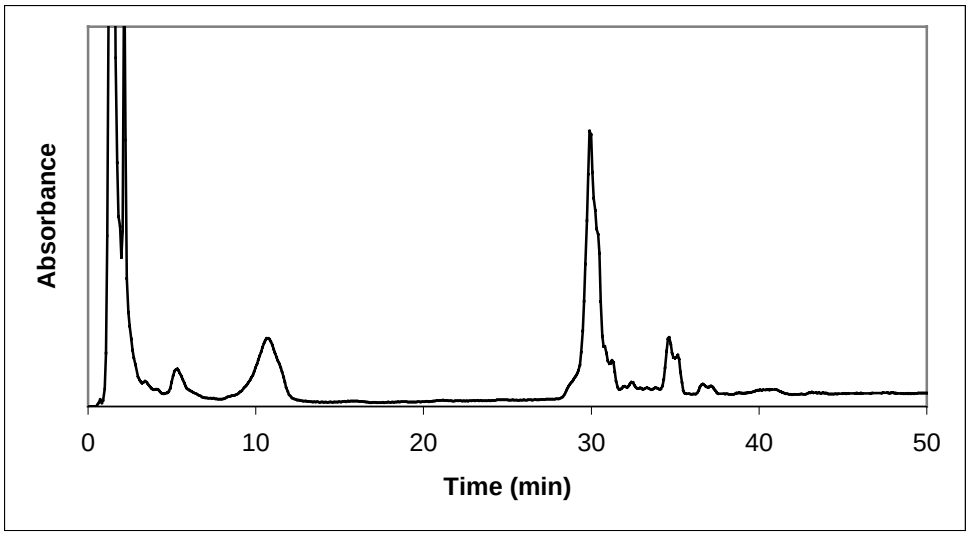
The total ginsenosides obtained was found to be 7.55% on a dry weight basis (g ginsenosides/g dry root), which is consistent with existing literature stating that the ginsenoside content can range from between 3 to over 7% on a dry weight basis (Nicol et al., 2002). From these results, it was determined that the batch of ginseng obtained from Agriculture and Agri-Food Canada was of sufficient quality to be suitable for extraction experiments with supercritical fluid extraction. In addition, with standardized extracts the results from supercritical fluid extraction could be compared in terms of overall extracts obtained, total ginsenosides obtained, as well as composition of ginsenosides in the extracts, for the various supercritical fluid extractions performed.

Figure 5.2.1 – 20 Hour Methanol Soxhlet Chromatogram (Run 1)



Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Rb_2 (32.3 min), Rd (34.5 min)

Figure 5.2.2 – 20 Hour Methanol Soxhlet Chromatogram (Run 2)



Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Rb_2 (32.3 min), Rd (34.5 min)

5.3 Trapping Efficiency Experiments

Trapping efficiencies were determined for a number of systems, as described in Section 4.7. Inert solid phase trapping, solid phase trapping using a polymeric adsorbent, and liquid phase trapping using methanol, anhydrous ethanol and dichloromethane, were all tested using neat and modified CO_2 to determine the suitability for use when recovering extracts from supercritical fluid extraction. The results for the tests are discussed in the following sections.

5.3.1 Inert Solid Phase Trapping

Inert solid phase trapping using glass wool and glass beads was attempted for both pure and CO_2 + methanol experiments. In all cases examined, this type of trap was unsuitable for recovering material. The percentage of total extracts recovered was found to be less than 5% (wt/wt) for cases with modifier and closer to 50% for cases using neat CO_2. This can be explained in the following manner; for experiments without modifier, using an inert solid phase trap requires cryogenic cooling which was not implemented in this case (McDaniel, Long, & Taylor, 1998). For experiments with a modifier, higher temperatures are required in order to boil off solvent to prevent solute loss due to aerosol formation, however, this cannot overcome the effect of using large quantities of modifier. Based on these results, for our analytical SFE system an inert solid phase trap was deemed to be unfeasible.

5.3.2 Adsorbent Solid Phase Trapping

Due to the previously mentioned limitations found with the inert solid phase trap, a polymeric adsorbent (styrene divinyl benzene) was tested as a trapping material. This type of trap was found to be effective for collecting materials at up to 10% v/v CO_2 + methanol, after which it had to be used in tandem in a two stage trapping system, where the polymeric adsorbent trap was followed by a liquid phase trap (Chaudot et al., 1998). Amberchrom GC161M was chosen as the polymeric adsorbent, due to its high specific surface area (900 m^2/g) and other favourable trapping properties.

Although this type of trap has been shown to be effective for up to 10 v/v % methanol in CO_2 for other solutes, such as decanoic acid, naphthalene, acetophenone and N,N-dimethylaniline, it proved to be unfeasible in this study for a number of reasons. Firstly, the mechanical stability of the system was difficult to maintain. When flowing extractions through the trap, the decompressed gas would cause the particles to swirl around in the trap and deposit on the walls, reducing contact and trapping efficiency.

As a result of this deposition on the walls, in terms of overall trapping efficiency, the trap did not perform particularly well (approximately 60% overall collection for 0 – 10 % methanol in CO_2). Additionally, it was desired to determine the effect of modifier concentrations in excess of 10% v/v, making the use of a single stage trap unfeasible and requiring the use of a secondary liquid trap. This would greatly increase the complexity of recovering extracts, requiring rinse stages for the solid phase and concentration for the liquid. In addition, the cost of obtaining PS-DVB with a high specific surface area was prohibitive to scale up, as the only resins available were pre-dissolved in liquid and were not sold as bulk solids, reducing the amount of material available from a single batch of resin, and increasing time to prepare the trapping medium through filtration. As a result, the use of solid phase trapping was rejected in order to implement a more effective, simple method using liquid trapping.

5.3.3. Liquid Phase Trapping

Several solvents were considered for liquid phase trapping, with and without temperature control from an ice or water bath surrounding the trapping vessel. Dichloromethane is a

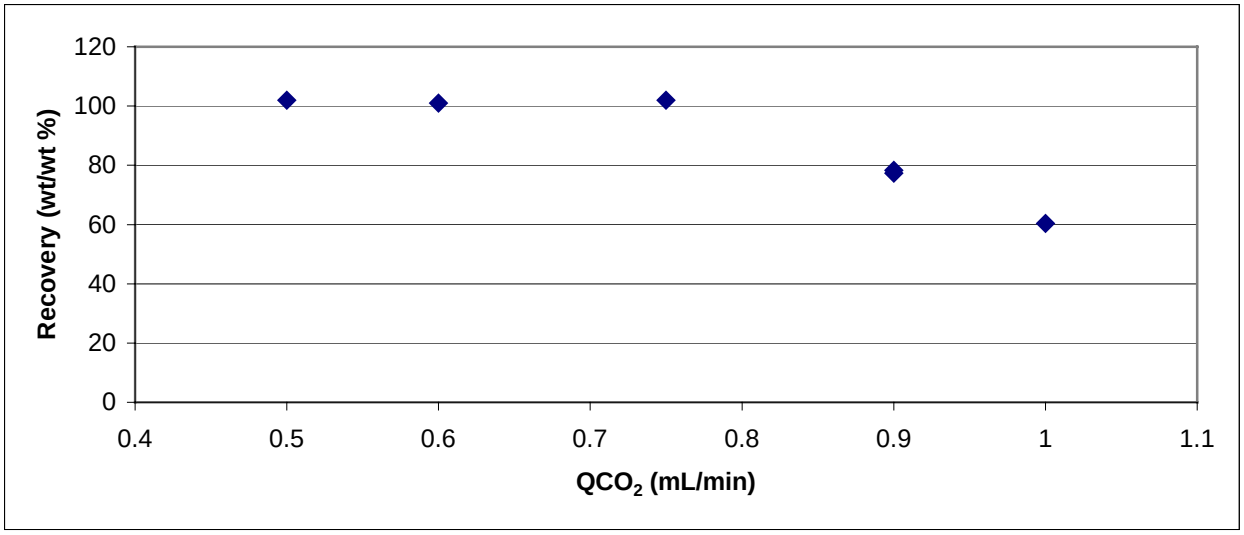
commonly used trapping solvent with high polarity, however, it proved to be too volatile and required frequent make-up during the extraction process as the bubbling of decompressed gas caused the solvent to evaporate, even at relatively low flow rates. In addition, the toxicity of dichloromethane made it undesirable for use as a trapping solvent when compared with the relatively less toxic solvents such as methanol or ethanol. Methanol was next considered, as it has been shown to have good solubility for ginseng components and although it is still somewhat toxic, is not as dangerous to work with as dichloromethane or as volatile.

Extractions performed using CO_2 + modifier with methanol as the trapping solvent found that using an ice bath greatly reduced the trapping efficiency, likely due to the drop in solvation power being greater than the effect of reducing aerosol formation by cooling the gas (~25% recovery of total extracted material). Use of a room temperature bath had no discernable effect on trapping under the conditions studied. Overall, methanol was a somewhat effective trapping solvent (~70% total recovery of extracted material) as long as the gas flow rate was below 0.6 mL/min in the liquid state, as measured by the syringe pumps.

As a next step, anhydrous ethanol was tested as a trapping solvent. Anhydrous ethanol has good solubility for ginseng components, and in addition, has a higher viscosity than methanol, which should increase residence time, decrease bubble size, and thereby theoretically provide more effective trapping at higher decompressed gas flow rates. In addition, anhydrous ethanol is a more environmentally friendly and less toxic solvent

than either methanol or dichloromethane. Tests were run using CO_2 at 5000 psig, 110°C, and a methanol percentage of 15% in the fluid phase at the start of the recovery stage, with a solvent to solid ratio of 1.8. The flow rate of decompressed gas through the system was found to be the dominant effect on trapping efficiency compared with controlling temperature of the system by placing the trapping vessel in a room temperature water bath. A plot of total recovery at these conditions vs. flow rate in the liquid state is given in Figure 5.3.1.

Figure 5.3.1 – Total Recovery of Extract vs. Q_{CO2} for an Anhydrous Ethanol Trap



As can be seen from this plot, as long as flow rate was less than 0.75 mL/min throughout the extraction, the recovery efficiency for total extracts obtained was approximately 100% (calculated as slightly higher due to uncertainty present in mass determination from the scale). This result was repeatable whether two-stage liquid trapping in two 250-mL Pyrex vessels each with 100 mL of ethanol, or in the single stage 45 mL collection vessel, indicating that restrictor depth in the trapping solvent, as well as residence time and

mixing/bubbling effects, were important to maintain effective trapping. As a result, the 45 mL collection vessel was more efficient, providing similar trapping to the larger system using a reduced solvent volume and making sample recovery easier. This result was also confirmed by existing literature that narrower trapping vessels can provide more effective trapping (Lang & Wai, 2001).

The effect of CO_2 flow rate on the recovery of extracts is not entirely accounted for by the decompression of gas into the trapping solvent. At higher flow rates, a larger amount of neat CO_2 is entering into the system per unit time. If this rate is high enough, there can be a loss of solubility in the extraction unit as the amount of material exiting the unit at a given time will not be sufficiently large to allow the new, lower modifier concentration fluid in the extraction unit to continue to dissolve the remaining material in solution. This type of effect is very noticeable, as it leads to deposition of solids along the extraction vessel walls and on the extraction vial. It is in these cases that the trapping efficiency begins to drop off from the approximately 100% recovery obtained at lower flow rates.

The alternative extraction method, to allow extracts to exit the extractor without adding pure CO_2 was found to be impractical for a few reasons. Firstly, without using the pump system the flow rate of fluid out of the system could not be determined with the current setup. Secondly, the exact same deposition problem was observed for the runs performed with this technique as fluid exited to the point where the pressure dropped enough to reduce the solubility of components in the extraction vessel.

As a result, extractions were chosen to be run at a flow rate less than 0.75 mL/min of pure CO_2, wherever possible, in order to maximize collection efficiency. For dynamic extractions with CO_2 + modifier or static + dynamic extractions, the flow rate was also kept at less than or equal to 0.75 mL/min of CO_2 + modifier. In the cases with modifier in the dynamic stage, the solubility drop effect was not expected due to the continual presence of modifier, and the trapping efficiency was fully controlled by the flow rate of decompressed gas and modifier through the trapping solvent.

In order to determine the trapping efficiency for ginsenosides, a mass balance was performed by comparing the total ginsenoside content (as determined by methanol Soxhlet) with the amount of ginsenosides recovered in the trap, and the amount of ginsenosides recovered by extracting the solid sample after supercritical fluid extraction. In this way, trapping efficiency for ginsenosides in the system can be approximated. These types of balances were used for trapping with anhydrous ethanol, as it proved to be the most effective liquid trapping solvent studied in this work. The trapping efficiency obtained for ginsenosides is discussed in Sections 5.4, 5.5, 5.6 and 5.7 on a case-by-case basis. Based on the results from the preliminary trapping experiments, anhydrous ethanol was used as the trapping solvent in a 45 mL trapping vessel, with no temperature control.

5.4 Pure CO_2 Extractions

To confirm existing literature that pure CO_2 extractions of ginseng yielded negligible amounts of ginsenosides, as well as total ginseng extracts, extractions were carried out using pure CO_2. Before experiments could be run to determine extraction quantities using pure CO_2, the problem of channeling/sample compression was examined. When pressurizing the system, the dead volume of the extraction vial must be filled in order to prevent a compression of the sample into an impermeable plug. In addition, without the use of a dispersant, channeling of fluid flow through the solid can occur.

Two different types of dispersants were tested, sand (50+70 mesh) and HyFlo (diatomaceous earth). HyFlo has the additional benefit of being a drying agent, to remove any residual moisture that could be present in the sample. The results of the various mixing methods tested are shown in Table 5.4.1. Samples were prepared and placed in the system, with the pressure ranging from 3000 to 5000 psig.

Table 5.4.1 - Mixing Scheme Results

Scheme	Powder Quality After Experiment
Pure Ginseng	- Very hard material - Hard to break apart
50 - 50 Ginseng/Sand (wt/wt)	- Very hard material - Hard to break apart
25 - 75 Ginseng/Sand (wt/wt)	- Hard material - Hard to break apart
5 - 95 Ginseng/Sand (wt/wt) with sand placed in bottom of extraction vial (20% of extraction vial volume)	- Hard material - Hard to break apart
5 - 95 Ginseng/Sand (wt/wt) with sand placed in bottom of extraction vial (~25% of extraction vial volume) and sand used to fill remaining empty volume of vial	- Free flowing powder
50 - 50 Ginseng/HyFlo (wt/wt) with HyFlo placed in bottom of extraction vial (20% of extraction vial volume) and HyFlo used to fill remaining empty volume of vial	- Breaks under applied force and becomes free flowing powder

From these results, a 5:95 wt/wt ginseng to sand mixture with sand at the bottom of the extraction vial, and on top of the ginseng-sand mixture to fill the remaining dead volume, was chosen. A 50:50 wt/wt Ginseng-HyFlo was also capable of preventing significant compression, however, HyFlo is more expensive than sand and the sample was thoroughly dried previous to experimentation, therefore a drying agent was not required. The solids loading scheme used in these experiments is shown in Figure 5.4.1. The dispersant – sample mixing results were also confirmed for the case of CO_2 + modifier, as discussed in the next section.

Several conditions using pure CO_2 for extraction of ginseng were tested using the optimum solids loading method, and the amount of total material extracted determined and shown in Table 5.4.2.

Figure 5.4.1 – Mixing Scheme for Dispersant and Ginseng

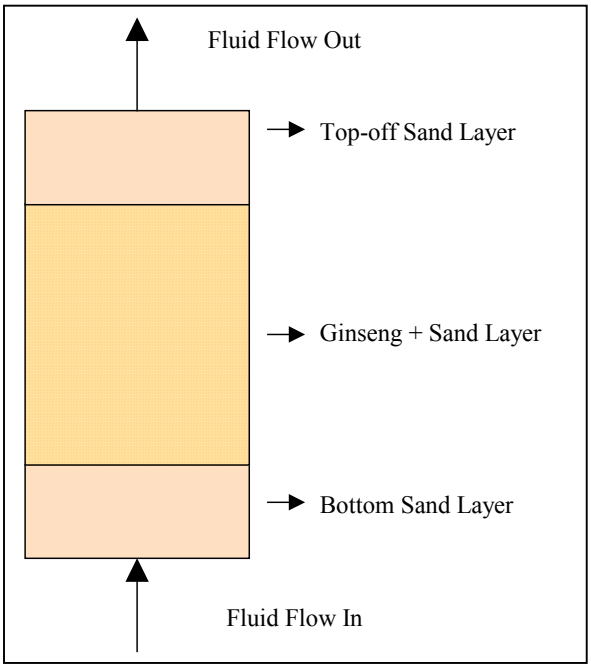


Table 5.4.2 – Extract Yields for Pure CO_2 Experiments

Pressure (Psig)	Temperature (°C)	Extraction Time (min)	Extract Yield ($mg_{extract}/g_{ginseng}$)
3000	50	15	Negligible
3000	70	15	Negligible
4000	70	15	Negligible
5000	60	15	Negligible
5000	70	15	2.5
5000	90	15	5

Solid phase trapping using glass wool was used for these experiments to collect extracted materials. The restrictor used was a capillary restrictor and incapable of simultaneous

control of both pressure and flow rate through the system. For several of the runs, negligible quantities of overall extract were obtained. This corresponds to extract quantities less than 0.5 mg/g of ginseng, which were of an order of magnitude of the error of the scale (± 0.1 mg). Negligible quantities of ginsenosides were extracted in these experiments.

As previously mentioned, existing literature has confirmed the low solubility of ginseng components in pure supercritical CO_2, particularly ginsenosides. Use of the capillary restrictor with convection heating proved unfeasible, as the restrictor would plug due to insufficient heating from the heater. Restrictor plugging during extraction was not a significant concern when using the heated variable volume restrictor, most likely due to the more efficient heating method used (conduction) as well as the small amount of solids extracted. The temperature of the restrictor tip was kept at 125°C and the temperature of the restrictor length was kept at the operating temperature of the system for the case of the variable volume restrictor. Pure CO_2 experiments were not entirely without merit from determining optimal experimental conditions, however, as they were used effectively to find a solids loading procedure which eliminated channeling and the formation of an impermeable solid plug.

5.5 Static CO_2 + Modifer Extractions

For CO_2 + modifier extractions, modifiers were studied at different conditions in order to attempt to find conditions where supercritical extraction could approach more conventional techniques. A set of preliminary runs were performed with CO_2 + methanol to determine which variables were most important for ginsenoside and overall extract amounts. These results are shown in Table 5.5.1. Liquid phase trapping was used for these experiments due to the presence of modifier.

Table 5.5.1 – Preliminary Static CO_2 + Methanol Extractions

Pressure (Psig)	Temp. (°C)	Mol %	Total Extract Yield (mg/g)	Rb_1 (mg/g)	Re/Rg_1 (mg/g)	Rc (mg/g)	Rd (mg/g)	Rb_2 (mg/g)	Total Ginsenoside Yield (mg/g)
5000	80	9	105	N/A	N/A	N/A	N/A	N/A	Negligible
5000	110	8	174	0.075	0.264	0	0	0	0.339
5000	120	7	189	0.078	0.346	0.009	0.082	0	0.515
7000	80	7	115	N/A	N/A	N/A	N/A	N/A	Negligible

* All extractions performed with capillary restrictor for a 1 hour static + 30 minute recovery period with pure CO_2.

From these results, it appears that the result of Wang et al. (2001) that extraction of ginsenosides from *Panax ginseng* appears to be mass transfer/desorption limited vs. solubility limited holds for *Panax quinquefolius* as well. The solids after extraction were found to be free flowing, with no case producing the impermeable solids plug, indicating that the solids loading procedure which worked for pure CO_2 extractions was also effective at preventing solid compression in the case of using CO_2 + modifier, and that solids compression was not the reason for negligible ginsenoside results. Although high amounts of overall extract could be obtained (~50% of methanol Soxhlet), negligible quantities of ginsenosides were obtained when compared to the total ginsenoside content

present in ginseng. This result may be useful in developing a multi-stage extraction process where non-ginsenoside components are removed in a preliminary extraction stage before ginsenosides are extracted. However, this may not be advantageous over a techique which can extract both ginsenosides and other components and then fractionate selectively based on phase equilibria.

Wang et al. (2001) found that grinding particles to 100 μm did not eliminate the mass transfer/desorption resistance under the conditions they studied, as they were unable to extract more than 55% of the total ginsenosides in a 4-hour extraction period. This is an indication that the extraction of ginsenosides from Korean ginseng root hair was desorption-limited, which may be the case for North American ginseng. Consequently, it is important to examine another crucial variable, the amount of modifier per gram of ginseng being extracted. This quantity is directly related to the ability of a modifier to modify the solid matrix sufficiently to allow for meaningful extractions of ginsenosides, which is of critical importance in cases where desorption-limited extraction is occurring. With this in mind, the amount of modifier used in extractions was increased in order to study the effect of increasing modifier quantities on ginsenoside and overall extraction yields.

One of the drawbacks of increasing modifier concentration in the system is that a higher temperature is required in order to insure that the system temperature is above the critical temperature of the CO_2 + modifier mixture, and therefore in the supercritical region. As an example, the critical mixture properties of CO_2 + methanol are shown in Figure 5.5.1

(critical pressure) and Figure 5.5.2 (critical temperature) as a function of methanol mole fraction in the mixture, based on the data of Yeo et al. (2000). Methanol exhibits the highest solubility in CO_2 of all modifiers studied, meaning that the effect of mole fraction on the critical properties should be the least pronounced of all modifiers studied. This in turn means that the effect on mole fraction modifier in the supercritical phase will be a minimum for methanol, and as such higher temperatures and pressures and larger increases with mole fraction will be observed for the cases of DMSO and aqueous ethanol/acetic acid in supercritical CO_2.

Figure 5.5.1 – Mixture Critical Pressure vs. y_{MeOH} for CO_2 + Methanol Mixture
(adapted from (Yeo, Park, Kim, & Kim, 2000))

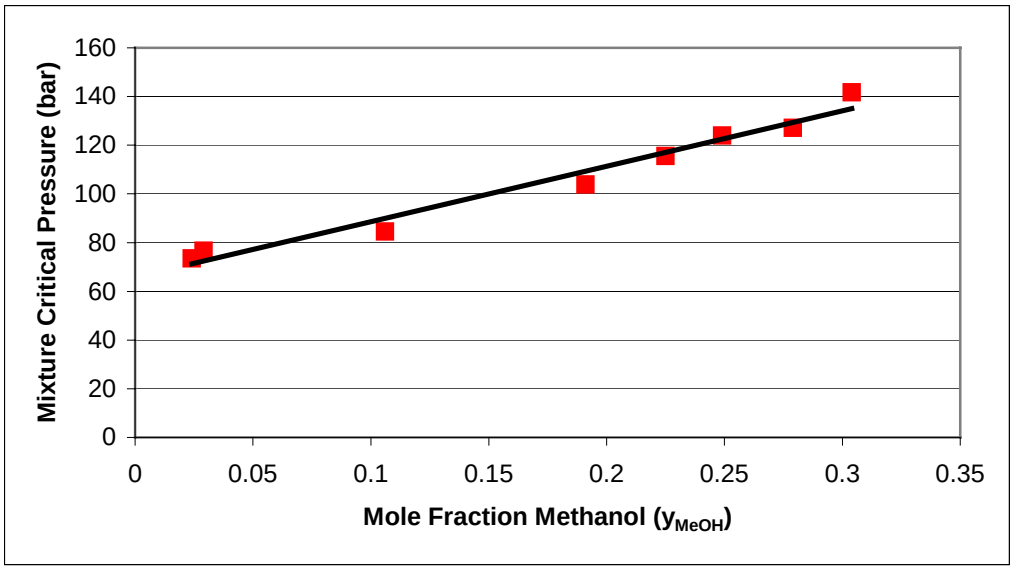
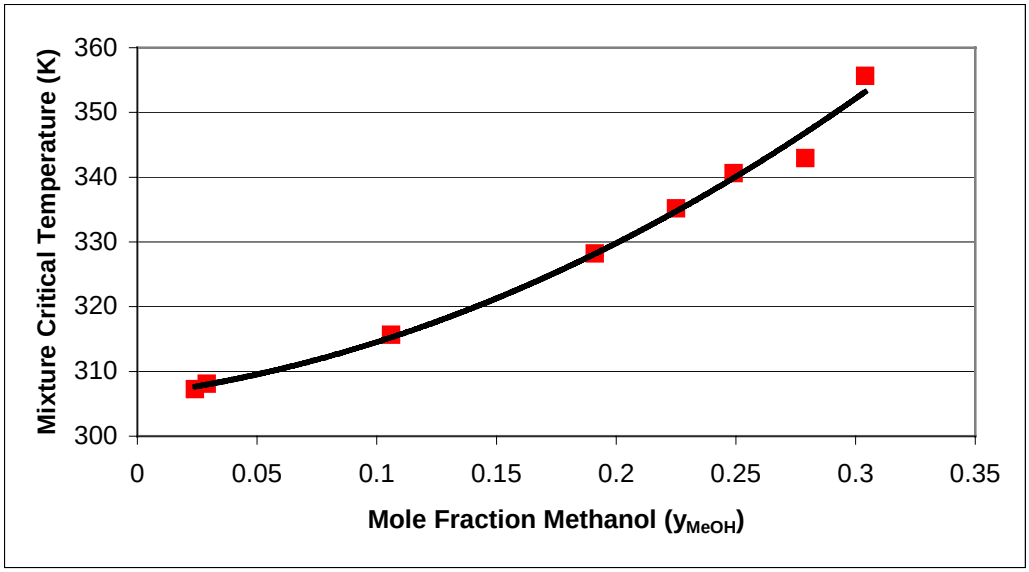


Figure 5.5.2 – Mixture Critical Temperature vs. y_{MeOH} for CO_2 + Methanol Mixture (adapted from (Yeo et al., 2000))



As can be observed from these figures, the critical properties are a strong function of mole fraction of modifier even for the case of methanol, in particular the critical temperature, which shows nearly quadratic dependence on mole fraction for the mixture critical temperature. Since methanol has the highest solubility in supercritical carbon dioxide, and still exhibits such a large increase in critical properties with mole fraction, an even more pronounced effect would occur for aqueous ethanol/acetic acid or DMSO.

Care then must be exercised in order to insure that the operating conditions are sufficiently high to obtain a single supercritical fluid phase, as operating at a temperature or pressure lower than the mixture critical properties will result in two-phases being formed, which eliminates the benefits of using a supercritical technique. Similarly, for the other modifiers used in this system, high system temperatures are required in order to insure single supercritical phase operation during extractions. Two-phase operations are

easily identified after extractions, as the higher density liquid phase will sink to the bottom of the extractor and deposit on the walls of the extraction unit, leaving a noticeable quantity of liquid at the bottom of the extractor which can be visually observed after extractions are complete.

Although higher system temperatures are required to accommodate higher modifier amounts, this can lead to faster extractions due to an increase in the diffusivity and a decrease in viscosity. In order to provide sufficient solvation at higher temperatures, the pressure of the system must be above the crossover pressure (defined in Section 2.1) in order to insure that there is either an increase or no change in the solubility of components in the supercritical fluid, rather than a decrease with increasing temperature. As the ginsenosides are among the lower volatility components of ginseng extract, higher fluid density is also desirable in order to dissolve ginsenosides. Selectivity of the extraction for ginsenosides will either decrease unavoidably or stay the same with an increase in modifier percentage, depending on the partition coefficients of solutes in the system.

Consequently, a new set of extractions was performed. Temperature was kept at 110°C and pressure at 5000 psig for the bulk of runs. The temperature was sufficiently high to allow large modifier percentages to be used (27 mole % methanol, 14.5 % aqueous ethanol/acetic acid and 11 mole % DMSO) as well as higher mass modifier per mass solid ratios. Simulation using the Peng-Robinson Equation of State with the Wong-Sandler mixing rules in ASPEN indicated that the operating temperature was higher than

the critical temperature at all chosen conditions. The pressure was high enough to be above the mixture critical pressure, as well as crossover pressure (tentatively identified as approximately 350 atm for plant components in general) (Mukhopadhyay, 2000). Extractions were run for a set time period in static mode with direct modifier spiking on the solids with a 30-minute recovery phase where pure CO_2 was passed into the system (at a flow rate of 0.65 mL/min in the liquid state) and into a liquid trapping vessel. Controlling the flow rate of CO_2 out of the system was found to be important for trapping both in terms of the dynamics of the trap as well as preventing deposition of extracted materials in the extraction vessel due to sudden loss of solvation power. The experimental conditions studied are shown in Table 5.5.2, with the results in terms of overall extract and ginsenosides shown in Table 5.5.3. At least 2 experiments were run for the bulk of conditions listed in these tables, with exceptions for extractions that yielded lower ginsenoside amounts.

Table 5.5.2 – Static CO_2 + Modifier Extractions: Conditions

Run No.	Modifier	Pressure (Psig)	Temperature (°C)	Time (min)	$m_{modifier}/m_{ginseng}$ (g/g)	Modifier Mole %
1	MeOH	5000	110	60	1.3	14
2	MeOH	5000	110	60	3.1	24
3	MeOH	5000	110	60	4.1	27
4	DMSO	5000	110	60	1.2	5.7
5	DMSO	5000	110	60	3.2	10
6	DMSO	4000	100	60	3.9	10.4
7	DMSO	5000	110	120	3.2	10
8	DMSO	4000	100	120	3.2	11
9	EtOH(aq)/Acetic Acid	5000	110	60	1.1	8.5
10	EtOH(aq)/Acetic Acid	5000	110	60	1.8	8.5
11	EtOH(aq)/Acetic Acid	5000	110	60	2.6	11.5
12	EtOH(Aq)/Acetic Acid	5000	110	60	3.1	14.5

Table 5.5.3 – Static CO_2 + Modifier Extractions: Results

Run No.	Rb_1 (mg/g)	Rb_2 (mg/g)	Rc (mg/g)	Rd (mg/g)	Re/Rg_1 (mg/g)	Mono-*O*-Acetyl Rb_1s (mg/g)	Total Ginsenoside Recovered (mg/g)	Total Extract Yield (mg/g)
1	6.45	0.07	1.06	1.59	6.47	0.62	16.28	171
2	23.00	0.24	1.53	2.63	13.67	1.6	42.68	308
3	20.59	0.74	3.01	3.86	10.7	1.6	40.48	283
4	0.54	0	0.81	0.16	0.328	0	1.20	7
5	14.89	0.45	1.71	2.74	8.83	6.92	35.53	261
6	13.5	0.40	1.66	3.78	9.03	6.40	34.73	360
7	15.3	0.54	1.75	2.92	9.10	7.84	37.42	262
8	17.96	0.12	1.41	2.12	12.68	8.4	42.79	191
9	0.26	0	0.12	0.39	1.54	0	2.30	69
10	0.824	0	0.46	1.01	3.33	0.2	5.83	84.0
11	9.23	0.37	1.75	2.42	7.24	0.3	21.33	120
12	21.45	0.34	1.94	2.97	12.10	1.10	39.90	257

* All extractions used a steady-state Q_{CO2} of approximately 0.65 mL/min

From the results shown in Table 5.5.3, it is clear that increasing the amount of modifier present per gram of solid has a profound effect on the ability of the supercritical fluid technique to extract ginsenosides. The total ginsenosides recovered accounted for approximately 57% of the ginsenosides obtained during a 20-hour methanol Soxhlet for

the most effective conditions for each type of modifier. However, the ginsenosides recovered was in fact not equal to the total ginsenosides extracted. At higher modifier percentages (> 2 mg modifier/g ginseng), larger amounts of solid material were extracted into the fluid phase and during the recovery stage the restrictor opening would plug if the valve was not opened to a level around 2 mL/min for between 1 to 2 minutes at the start of recovery. Plugging would prevent quantifiable recovery of ginsenosides, and also result in deposition of solid materials over the extraction vessel during venting. The variation in overall yield obtained was similar for all extraction runs observed and was found to be on average a relative standard deviation of 18.1%.

This higher flow rate was required to prevent solids deposition on the restrictor reaching a point where the restrictor would plug and no materials could be collected. This flow rate was established to be incompatible with effective trapping by the previously performed trapping experiments. The time for which this higher flow rate was used was relatively short compared with the total recovery time, however, and in order to determine the effect on ginsenoside recovery, a 20-hour methanol Soxhlet was employed to re-extract the samples after supercritical fluid extraction in order to determine total remaining ginsenoside content after supercritical extractions. Trapping efficiency for CO_2 + DMSO extractions was closer to 65%, while for methanol and aqueous ethanol/acetic acid it was closer to 80%. This may be due to DMSO having lower solubility in ethanol, than either methanol or aqueous ethanol/acetic acid.

As a result, the amount of ginsenosides actually being extracted during the process is higher than the recovery rate and that although the higher flow rate operation is only for a short period of time (1 – 2 minutes), there is a significant loss of ginsenoside compounds during this period of operation. This recovery problem may be eliminated by the use of modifier in a dynamic recovery/extraction stage, as the modifier will have sufficient density at the lower pressures found in the restrictor to force solid materials through, preventing plugging. In addition to this problem, another difficulty was observed which was that the chaotic nature of the restriction could lead to sudden spikes in flow rates from 2 mL/min to 10 mL/min due to the sudden removal of a plug of solids during extraction, which would lead to a sudden loss of solvation in the extraction vessel and a deposition of materials along the extraction vessel and extraction vial.

This deposition would not only result in a wasted experiment but would require extensive cleaning of the unit to insure that future runs were not contaminated by extracted ginsenosides and other ginseng components being flushed out during a new recovery stage. The cleaning procedure utilized was to wash the extraction vessel with methanol under heat (40°C) for 10 minutes (repeated 4 – 5X), rinse with acetone, and then heat the extraction vessel to 60°C, place a 5 mL volume of methanol in the extraction vessel and pressurize the system at 5000 psig and flow material out at 2 - 5 mL/min for 15 minutes. After this rinse, the system was depressurized and then heated to 110°C and pressurized to 5000 psig to allow pure CO_2 to flow through the system at 2 – 5 mL/min for another 15 minutes to remove any wash methanol in the restrictor. In contrast, cleaning after successful runs required only the addition of 5 mL of MeOH, heating to 60°C,

pressurizing with CO_2 at 5000 psig and allowing flow of 2 – 5 mL/min for 15 minutes, followed by a pure CO_2 flush stage.

Based on the trapping efficiency found from the Soxhlet extractions performed, the amount of ginsenosides extracted in a static CO_2 + modifier extraction was actually found to be closer to 58 mg/g for CO_2 + DMSO and 52 mg/g for CO_2 + MeOH and CO_2 + EtOH(aq)/acetic acid, indicating that the extractions were somewhat similar for overall ginsenoside yield, if not for composition of individual ginsenosides. These values correspond to approximately 70-77% of the total ginsenoside yield from methanol Soxhlet, in a much shorter extraction time. This result is an improvement from the work of Wang et al. (2001) who could only obtain 55% of the ginsenoside content in a 4-hour extraction, although with a lower amount of modifier and at a lower temperature (Wang et al., 2001). The recovery percentage obtained with these types of extractions, however, is low enough to warrant investigation of other extraction methods to attempt to improve collection efficiency of ginsenosides. Comparison of individual ginsenoside amounts with Soxhlet is difficult to obtain due to the variable trapping of ginsenosides and the fact that the exact profile cannot be generated by completing the mass balance since methanol Soxhlet will provide the total amount of ginsenosides remaining, rather than the same individual component compositions.

During supercritical extractions at higher modifier percentages, there was an unknown peak found in the ginsenoside region. This peak did not correspond to the standard peaks of the six ginsenosides considered (Rb_1, Rb_2, Rc, Rd, Re and Rg_1) nor was it present in

the 20-hour methanol Soxhlet extractions performed. This indicates that the peak is a thermally sensitive ginsenoside which may be converted due to the heating and longer extraction time of Soxhlet. CO_2 + DMSO extractions contained very high percentages of this unknown in terms of the total ginsenosides obtained, with 18 - 20% of ginsenosides recovered being this unknown for all runs over 10 mol % DMSO in the fluid phase. As this peak clearly represents a component of interest for CO_2 + DMSO extractions, liquid chromatography/tandem mass spectrometry was used to identify the peaks in terms of molecular weight. The unknown peak was identified as two mono-*O*-acetylated ginsenoside Rb_1 compounds based on LC-MS (described in Section 5.8).

In addition to a higher percentage of these acetylated ginsenosides, there was also a different composition to supercritical extractions vs. a 20-hour methanol Soxhlet and for supercritical extractions using different modifiers. For DMSO, at a modifier percentage higher than 10 mol % there was a very similar composition for ginsenosides obtained between runs, even if the collection efficiency, extraction time, pressure and temperature varied from run to run. The average of these compositions, considering all runs with > 10 mol% DMSO as equivalent and with the relative standard deviation shown, is reported in Table 5.5.4.

Table 5.5.4 – % Composition of Ginsenosides in CO_2 + DMSO Extracts (>10 mol% DMSO) vs. 20 Hour Methanol Soxhlet

Ginsenoside	CO_2 + DMSO Percentage Composition (% RSD)	MeOH Soxhlet Percentage Composition (% RSD)
Rb_1	41.57 (3.13)	59.54 (1.52)
Rb_2	1.32 (19.03)	2.14 (45.88)
Rc	4.78 (5.89)	6.37 (5.95)
Rd	7.74 (1.08)	10.12 (3.58)
Re/Rg_1	24.64 (3.21)	21.84 (10.32)
Mono-O-acetyl ginsenoside Rb_1s	19.94 (4.72)	0

From Table 5.5.4, it becomes clear that the only ginsenoside which showed large variation for the CO_2 + DMSO runs over 10 mol % was Rb_2, which is likely due to the small amount of Rb_2 present among the ginsenosides (lowest percent composition out of total ginsenoside content). Similarly for methanol Soxhlet, there was low variation in component composition, except in the case of Rb_2. Comparing with methanol Soxhlet, there are clear differences in the composition of Rb_1 and the acetylated compounds, which was not detected in analysis of methanol Soxhlet runs. This difference was confirmed statistically by comparing the mean of composition for each ginsenoside with the mean from Soxhlet, assuming unequal variances between Soxhlet and supercritical fluid extraction based on performed F-tests for the variance. The mean difference tested

was set at zero as the null hypothesis and a two-tailed t-test was performed to determine the probability that the null hypothesis is true. The alternate hypothesis was that the compositions were not equal. MS Excel was used to perform the analysis. The results from this test are shown in Table 5.5.5.

Table 5.5.5 – Results for t-test Comparing Compositions Obtained in Static CO_2 + DMSO Extraction (>10 mol% DMSO) vs. MeOH Soxhlet Extraction

	Rb_1	**Rb_2**	**Rc**	**Rd**	**Re/Rg_1**	**Mono-*O*-Acetyl ginsenoside Rb_1s**
p-value	9.22E-05	0.282	5.51E-03	5.57E-03	0.153	7.41E-4

As illustrated in Table 5.5.5, there is a very small probability that the mean values for composition of the ginsenosides Rb_1, Rc, Rd and the acetylated ginsenosides in the extracts are equal for CO_2 + DMSO vs. MeOH Soxhlet extraction. The probability that Rb_2 and Re/Rg_1 are equal is also low, but falls outside even a 90% confidence interval. This is likely due to larger variation in the composition of these two ginsenosides, particularly for MeOH Soxhlet. The t-test indicates that with over 99% confidence for Rb_1, Rc, Rd and the acetylated ginsenosides that there is a difference between a 20-hour methanol Soxhlet and CO_2 + DMSO extraction for ginsenoside composition for each ginsenoside studied in this work.

The data in Table 5.5.4 is an indication that the acetylated compounds may be thermally converted into other ginsenosides in methanol Soxhlet, which explains the large

difference in composition between the DMSO and the methanol Soxhlet runs. The CO_2 + DMSO runs contain approximately 20% by weight of the acetylated components, while having an Rb_1 composition approximately 20% less than methanol Soxhlet, indicating that the likely product of thermal conversion of the acetylated component is Rb_1.

The composition for CO_2 + methanol extractions are similar for the runs at 27 mol %, although there is a larger variance for the compositions, particularly in the Re/Rg_1 percentage composition. However, there is a much lower fraction of the acetylated components and a higher amount of Rb_1 when compared with DMSO, which indicates that DMSO may provide thermal stability to these acetylated ginsenosides that methanol does not provide. The compositions for CO_2 + MeOH extractions vs. MeOH Soxhlet are shown in Table 5.5.6. For CO_2 + aqueous ethanol, the relative standard deviation was fairly large for all ginsenosides at 14.5 mol % (> 10% for all ginsenosides). As such, these runs would need to be replicated in a situation where the problems currently associated with static extractions with modifier are eliminated in order to determine if this variation is natural with the co-solvent, or the product of the trapping inefficiencies and flow rate spikes. CO_2 + EtOH(aq)/Acetic Acid comparisons were not performed due to this higher variance.

Table 5.5.6 – Percentage Composition of Ginsenosides in CO_2 + MeOH Extracts at 27 mol % vs. 20 Hour Methanol Soxhlet

Ginsenoside	CO_2 + MeOH Percentage Composition (% RSD)	MeOH Soxhlet Percentage Composition (% RSD)
Rb_1	53.05 (11.2)	59.54 (1.52)
Rb_2	1.48 (9.73)	2.14 (45.88)
Rc	9.46 (0.53)	6.37 (5.95)
Rd	8.27 (9.86)	10.12 (3.58)
Re/Rg_1	24.46 (19.02)	21.84 (10.32)
Mono-*O*-acetyl ginsenoside Rb_1s	3.26 (6.75)	0

As in the case of CO_2 + DMSO, t-tests were performed to determine the probability that the null hypothesis (equal mean percent compositions of ginsenosides between extraction methods) was true, with the results shown in Table 5.5.7.

Table 5.5.7 - Results for t-test Comparing Compositions Obtained in Static CO_2 + Methanol Extraction vs. MeOH Soxhlet Extraction

	Rb_1	Rb_2	Rc	Rd	Re/Rg_1	Mono-*O*-acetyl ginsenoside Rb_1s
p-value	0.108	0.505	0.303	0.222	0.301	0.0127

Based on the calculated p-values in Table 5.5.7, the composition of Rb_1 is different between CO_2 + MeOH supercritical fluid extraction and MeOH Soxhlet with approximately 90% confidence and the acetylated compounds with 99% confidence. Interestingly, the probabilities that Rb_2, Rc, Rd and Re/Rg_1 are the same between the two runs falls between the null and alternate hypothesis (values are equal vs. values are not equal) as it is not in a 90% confidence interval for either the null or the alternative hypothesis to be true. This means that it cannot be determined if significant differences exist in the composition of these ginsenosides between static CO_2 + MeOH and MeOH Soxhlet extractions, although indications are that there are differences as the p-values tend to fall on the lower end, making it more unlikely that the null hypothesis is true. Overall, the results for Rb_1 and the acetylated compounds illustrate the differences in ginsenoside composition between CO_2 + MeOH and MeOH Soxhlet. Comparisons between the ginsenoside composition of CO_2 + DMSO vs. CO_2 + MeOH also show interesting results, as illustrated in Table 5.5.8.

Table 5.5.8 – t-test Comparison of Static SFE with CO_2 + DMSO and CO_2 + MeOH

	Rb_1	**Rb_2**	**Rc**	**Rd**	**Re/Rg_1**	**Mono-*O*-acetyl ginsenoside Rb_1s**
p-value	0.033	0.233	0.120	0.249	0.997	3.2E-05

From Table 5.5.8, it can be shown that the composition of Rb_1 as well as the unknown vary significantly between CO_2 + DMSO and CO_2 + MeOH extraction with over 95% confidence for both ginsenosides. Interestingly, once again there are no real clear trends

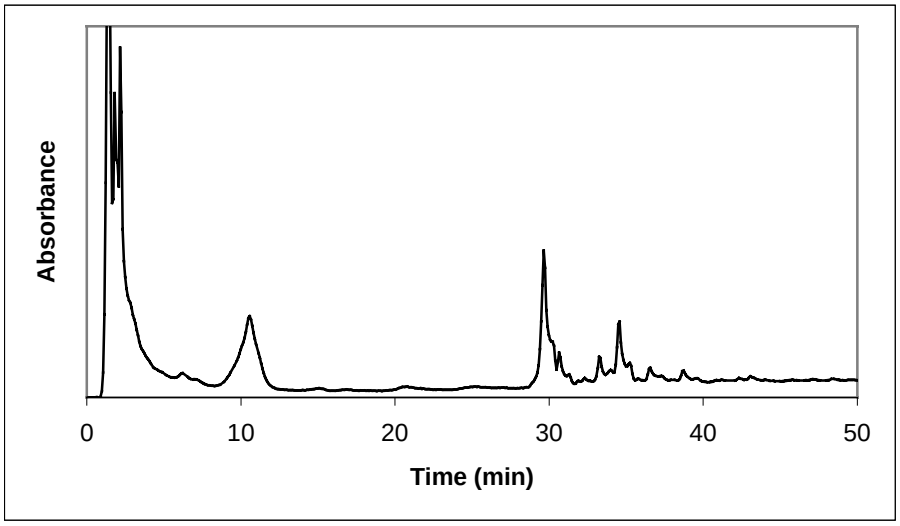
composition for Rb_2, Rc and Rd, however Re/Rg1 is deemed identical between the extraction methods with over 99% confidence. As a result, the amount of Rb_1 and the acetylated ginsenosides obtained from CO_2 + DMSO and CO_2 + MeOH are clearly different, Re/Rg_1 appears similar between the extraction methods at the conditions chosen for extraction, and no significant conclusion can be reached on whether or not the composition of Rb_2, Rc and Rd are different between the runs (although indications are that they are different due to lower probability for null hypothesis to be true). Further experimental repeats should be attempted to ascertain if significant differences can be observed for the composition of Rb_2, Rc and Rd. The differences in composition for Rb_1 and the acetylated ginsenosides between CO_2 + MeOH and CO_2 + DMSO indicate that significant differences do exist between using these two modifiers in supercritical fluid extraction.

Also of interest in the supercritical extractions is the fraction that ginsenosides represent of the total extracted components. For MeOH Soxhlet, this fraction of ginsenosides was 18.5% with a relative standard deviation of 6.5%. In comparison, when accounting for trapping efficiencies and the variation in overall extract, supercritical extractions contained 19.5% for CO_2 + MeOH (4.1 MeOH g/g, 27 mol%) and 26% for CO_2 + DMSO (3.2 g DMSO/g ginseng, 10 mol%) ginsenosides out of the total extract obtained, with larger relative standard deviations (closer to 14% in both cases) due to the larger variation in total extracts obtained in the static supercritical extractions. With such large variation, it is difficult to tell if there is a significant difference between the fraction of ginsenosides in the total extract for the static case vs. the fraction extracted in Soxhlet.

Although there is evidence for supercritical extractions avoiding the thermal conversions of a 20-hour methanol Soxhlet, based on the higher composition of the acetylated ginsenosides and lower amounts of Rb_1, there is also the case of the malonyl ginsenosides. Malonyl ginsenosides are present in ginseng but are thermally converted during the 20-hour Soxhlet extraction into their neutral ginsenoside counterparts. During the supercritical extractions, no significant quantities of an unknown peak in the ginsenoside region were detected other than the previously mentioned peak which corresponded to the acetylated ginsenosides. As such, it appears that supercritical extraction with CO_2 + modifier does not thermally convert neutral ginsenosides to the degree that Soxhlet can but in fact does convert the malonyl ginsenosides. This is not an entirely unexpected result as Ren and Chen (1999) have shown that malonyl ginsenosides can have rate constants for thermal conversion 3 to 60 times that for neutral ginsenosides and the temperature of operation was between 100 and 110°C (Ren & Chen, 1999).

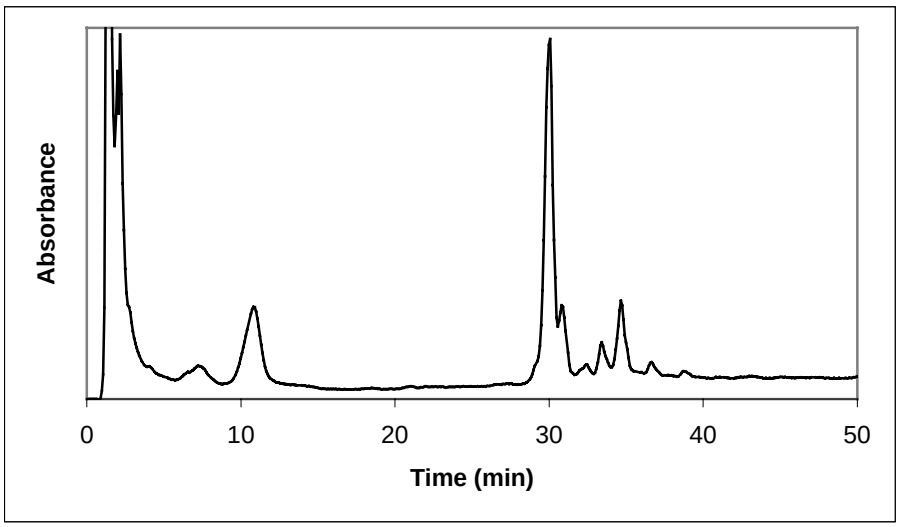
Representative chromatograms of various supercritical extractions are shown in Figures 5.5.3 through 5.5.8, also clearly illustrating the presence of the unknown in the case of DMSO compared with the other supercritical extractions. In addition, the effect of increasing the amount of modifier on ginsenoside yield is shown by comparison of chromatograms of runs with increasing $m_{modifier}/m_{ginseng}$.

Figure 5.5.3 – HPLC Chromatogram of Static CO_2 + Methanol SFE (Run 1)
(5000 psig, 110°C, 60 minute extraction time, 1.3 g MeOH/g ginseng and 14 mol % MeOH in SCF phase)



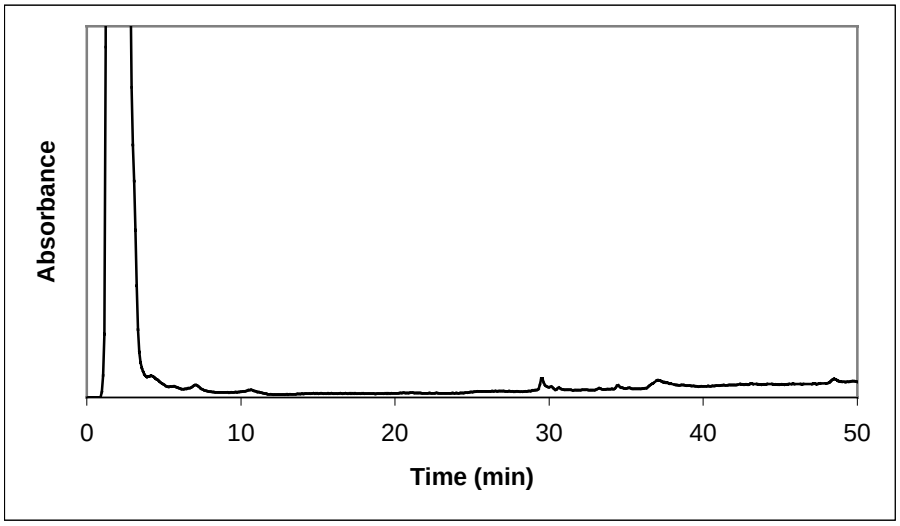
Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Rb_2 (32.3 min), Unknown (33.3 min), Rd (34.5 min)

Figure 5.5.4 – HPLC Chromatogram of Static CO_2 + Methanol SFE (Run 3)
(5000 psig, 110°C, 60 minute extraction time, 4.1 g MeOH/g ginseng and 27 mol % MeOH in SCF phase)



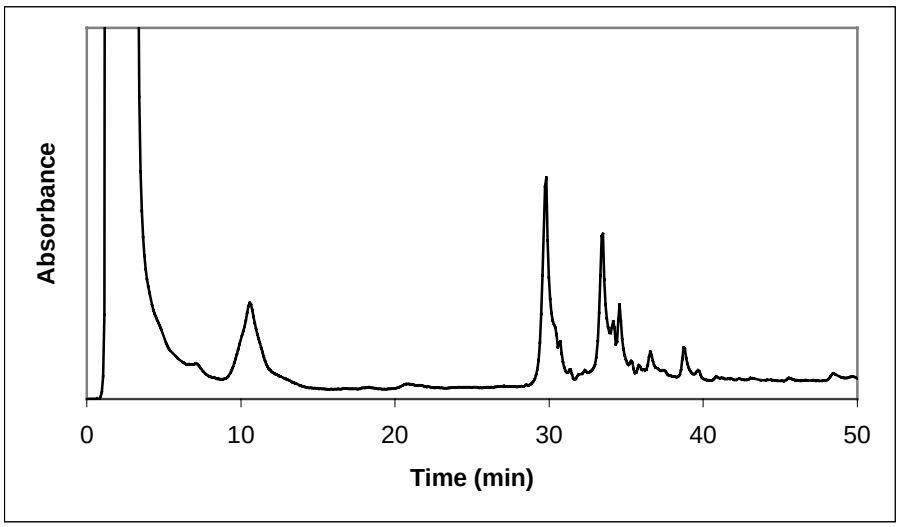
Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Rb_2 (32.3 min), Unknown (33.3 min), Rd (34.5 min)

Figure 5.5.5 – HPLC Chromatogram of Static CO_2 + DMSO SFE (Run 4)
(5000 psig, 110°C, 60 minute extraction time, 1.2 g DMSO/g ginseng, 5.7 mol% DMSO in SCF phase)



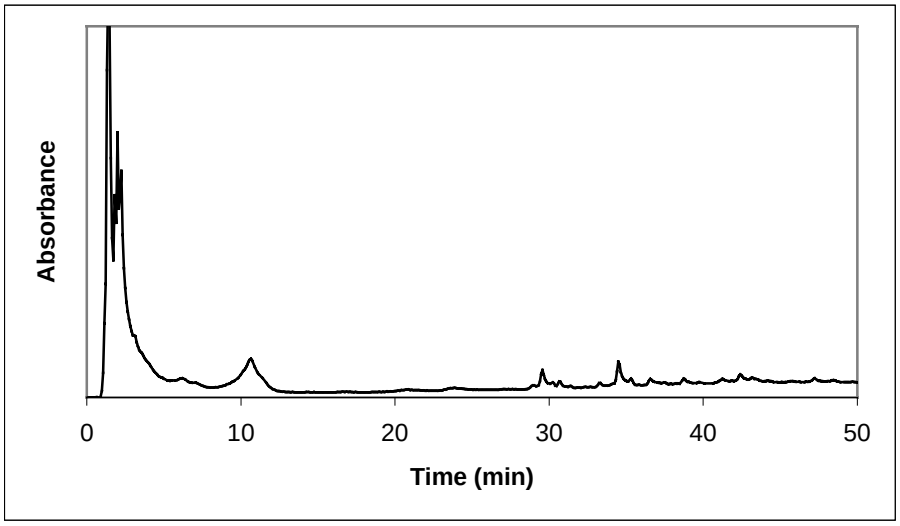
Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min)
Rd (34.5 min)

Figure 5.5.6 – HPLC Chromatogram of Static CO_2 + DMSO (Run 5)
(5000 psig, 110°C, 60 minute extraction time, 3.2 g DMSO/g ginseng, 10 mol% DMSO in SCF phase)



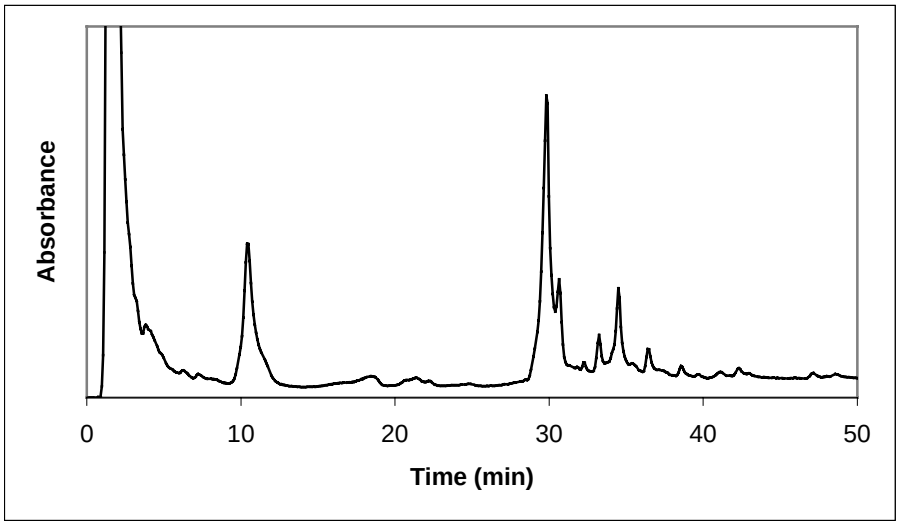
Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Rb_2 (32.3 min), Unknown (33.3 min), Rd (34.5 min)

Figure 5.5.7 – HPLC Chromatogram of Static CO_2 + $EtOH_{(aq)}$/Ac. Acid SFE (Run 9)
(5000 psig, 110°C, 60 minute extraction time, 1.1 g EtOH(aq):Acetic Acid/ginseng, 8.5 mol % in SCF phase)



Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Unknown (33.3 min), Rd (34.5 min)

Figure 5.5.8 – HPLC Chromatogram of CO_2 + $EtOH_{(aq)}$/Ac. Acid SFE (Run 12)
(5000 psig, 110°C, 60 minute extraction time, 3.1 g EtOH(aq):Acetic Acid/ginseng, 14.5 mol% in SCF phase)



Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Rb_2 (32.3 min), Unknown (33.3 min), Rd (34.5 min)

The use of propylene carbonate and poly(ethylene glycol) (MW 200) was also examined. These solvents are so-called "green", environmentally friendly solvents. During extractions with both of these solvents, it was found that there was a liquid layer left behind for virtually all modifier concentrations (even down to 3-5 mol%). Based on this result, it appeared that the operating condition of 5000 psig and 110°C was not sufficient to insure single-phase operation for both of these modifiers in a binary mixture with CO_2, at the modifier conditions studied. To confirm this, available literature was consulted and the solubility of PEG 200 in CO_2 as well as propylene carbonate in CO_2 was very low for a supercritical phase, although high for a liquid phase which dissolved CO_2 (Lopes, Gourgouillon, Pereira, Ramos, & Nunes da Ponte, 2000; Williams, Mas, & Rubin, 2002). Consequently, these solvents were not appropriate to use as modifiers of carbon dioxide for supercritical extraction of ginsenosides from ginseng, as higher modifier percentages are required in order to overcome solute-matrix effects and those conditions could not be met by using PEG 200 or propylene carbonate as modifiers.

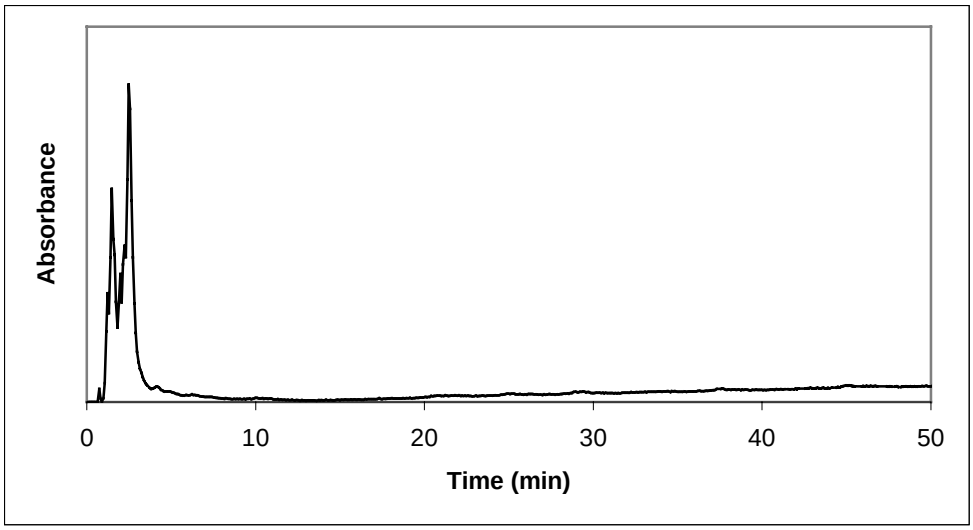
5.6 Dynamic CO_2 + Modifier Extractions

Although the results of the previous sections demonstrated how the extraction of ginsenosides was a mass transfer/desorption limited process, it was decided to study the effect of using a single, dynamic extraction stage using CO_2 + modifier. A dynamic stage preceding a static extraction could potentially remove the bulk of the ginseng extract without significantly affecting the ginsenoside content, allowing for a static extraction to follow, removing primarily ginsenosides. The modifier chosen was methanol, as it was

shown in the previous section to have high solubility in CO_2 and the ability to disrupt the solute-matrix complex.

A run at 5000 psig, 110°C and 27 mol% methanol as a modifier, with a flow rate of 0.5 mL/min using anhydrous ethanol as a trap solvent, was attempted for a 1 hour extraction. Analysis of the extract showed no detectable amount of ginsenosides, even after concentration of the collected material. An HPLC chromatogram for the dynamic CO_2 + methanol condition studied is given in Figure 5.6.1, and the lack of discernible peaks clearly illustrates the absence of detectable ginsenosides, even when concentrating the extract. The total extract obtained from the dynamic run was 31.9 mg extract / g solid, which was significantly less than static-only extractions of the same length of time or methanol Soxhlet. Consequently, a dynamic-only stage for extraction appears to be untenable for developing a supercritical extraction process for ginsenosides from ginseng, as it was unable even to extract meaningful quantities of any ginseng component. This result emphasizes the desorption-limited nature of the extraction and the need for modifier to be in direct contact with the solid matrix in order to disrupt the solute-matrix complex.

Figure 5.6.1 – Chromatogram for Dynamic CO_2 + Methanol Extraction



Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Rb_2 (32.3 min), Unknown (33.3 min), Rd (34.5 min)

Although a dynamic CO_2 + methanol extraction stage on its own was unable to provide any useful quantities of ginsenosides, the use of such a stage in a combined extraction process may prove to be useful. Using a CO_2 + modifier stage will theoretically improve trapping by reducing the loss of solvation power due to addition of higher flow rates of pure CO_2. This could eliminate the need for higher gas flow rates at the start of recovery and may potentially enhance extraction if the static stage is solubility limited, as well as desorption limited at the time that the dynamic stage is introduced.

5.7 Static + Dynamic CO_2 + Modifier Extractions

Following static CO_2 + modifier and dynamic CO_2 + modifier, the effect of combining the two extraction stages together was examined to determine if there was any effect on either the quantity of material extracted, the recovery efficiency, or both. Methanol was once again used as a test modifier, as it was in the dynamic CO_2 + modifier extraction case. The effect of extraction time was also examined to determine if shorter static extractions, followed by dynamic extraction with modifier, could approach the efficiency of longer static-only extractions or methanol Soxhlet extractions. The experiments considered are shown in Table 5.7.1 with the results in Table 5.7.2. At least 2 runs were performed at each condition studied. Representative chromatograms of the extractions are shown in Figures 5.7.1 – 5.7.3

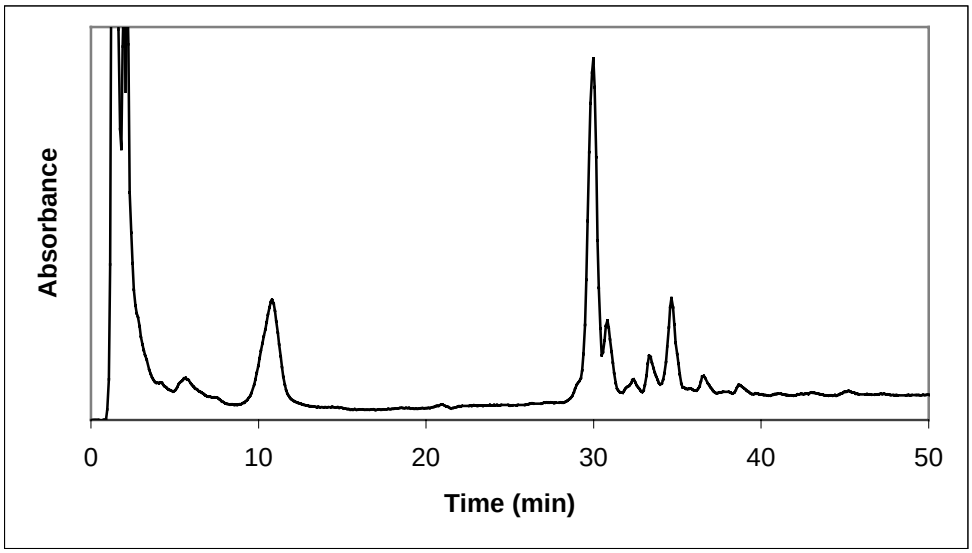
Table 5.7.1 – Static + Dynamic CO_2 + Methanol Extraction: Conditions

Run	Mol % Modifier	$m_{modifier}/m_{solid}$ (static) (g/g)	Time – Static (min)	Time – Dynamic (min)	Q_{CO2} (mL/min)	V_{MeOH}/V_{CO2}
1	27	4.1	60	30	0.8	0.332
2	27	4.1	60	30	0.3	0.332
3	27	4.1	15	15	0.5	0.332

Table 5.7.2 – Static + Dynamic CO_2 + Methanol Extraction: Results

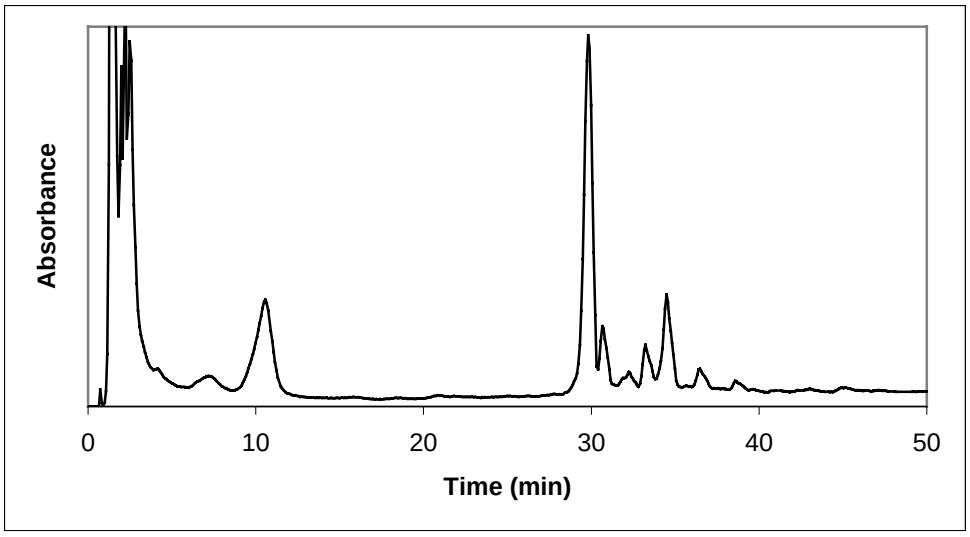
Run	Rb_1 (mg/g) (% RSD)	Rb_2 (mg/g) (% RSD)	Rc (mg/g) (% RSD)	Rd (mg/g) (% RSD)	Re/Rg_1 (mg/g) (% RSD)	Mono-*O*-acetyl ginsenoside Rb_1s (mg/g) (% RSD)	Total Ginsenoside (mg/g) (% RSD)	Total Extract Recovered (mg/g) (% RSD)
1	35.3 (2.11)	1.00 (15.88)	6.12 (2.27)	5.88 (4.11)	17.44 (7.90)	2.14 (6.08)	67.88 (3.71)	297.3 (13.18)
2	36.06 (4.39)	1.14 (39.3)	4.69 (36.1)	6.19 (12.3)	16.48 (0.39)	2.37 (21.1)	65.37 (2.24)	336 (12.2)
3	27.42 (3.75)	0.78 (5.99)	5.03 (4.30)	4.38 (5.20)	15.22 (16.93)	1.13 (9.11)	53.96 (7.78)	310.4 (4.83)

Figure 5.7.1 – HPLC Chromatogram of a Static + Dynamic Run 1 Type Extraction



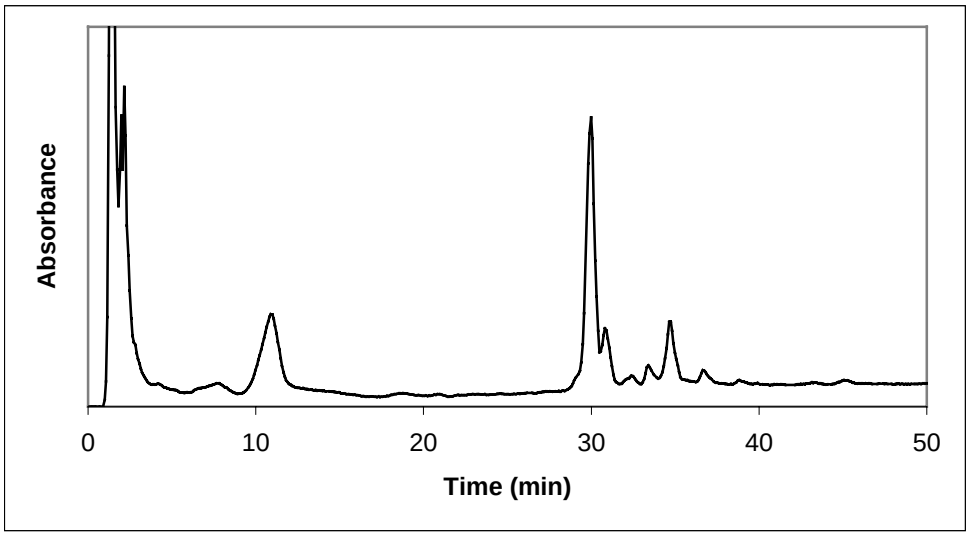
Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Rb_2 (32.3 min), Unknown (33.3 min), Rd (34.5 min)

Figure 5.7.2 – HPLC Chromatogram of a Static + Dynamic Run 2 Type Extraction



Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Rb_2 (32.3 min), Unknown (33.3 min), Rd (34.5 min)

Figure 5.7.3 – HPLC Chromatogram of a Static + Dynamic Run 3 Type Extraction



Retention Times: Re/Rg_1 (10.7 min), Rb_1 (29.7 min), Rc (30.7 min), Rb_2 (32.3 min), Unknown (33.3 min), Rd (34.5 min)

Methanol Soxhlet extraction was used in order to determine the remaining ginsenoside content in the extracted powder and to compare with the established total ginsenoside content obtained in the Soxhlet extraction experiments. Based on these results, the trapping efficiency was determined to be approximately 100% for all cases studied in static + dynamic extraction with anhydrous ethanol as the trapping solvent, when factoring in for the variance involved in determining the ginsenoside content extracted in both supercritical and Soxhlet stages. This is a significant improvement from the static extraction case, where the pure CO_2 recovery stage tended to lead to approximately a 60-70% recovery of ginsenosides during an extraction process, due in large part to the need to keep the restriction valve open past 2 mL/min for the first few minutes in order to avoid plugging difficulties in the system. In addition, the variation in total extract yield obtained was less than that observed in the static-only supercritical fluid case.

The trapping efficiency was independent of flow rate of the system for the flow rates studied in these runs (0.3, 0.5 and 0.8 mL/min). This result was not unexpected as there is no sudden loss of solvation power due to addition of pure CO_2, and the flow rate of fluid in the liquid state was kept below 1 mL/min in all cases, which has been shown to be a cut-off value for effective trapping. In addition, the need to keep the restriction open past 2mL/min for the first few minutes of the recovery stage was eliminated with the use of modifier, which also contributed to effective trapping of essentially all extracts in these cases. The amount of total ginsenosides obtained using supercritical fluid extraction is compared with the amount obtained using methanol Soxhlet in Table 5.7.3.

Table 5.7.3 – Comparison of Total Ginsenosides Extracted between Supercritical Extraction with Static and Dynamic Stages vs. Methanol Soxhlet

Experimental Procedure	Total Ginsenosides (% RSD)	Percentage of Soxhlet
Run 1	67.88 (3.71)	89.9 (5.31)
Run 2	66.93 (2.24)	88.63 (4.41)
Run 3	53.96 (7.78)	71.45 (8.66)
20 Hour Methanol Soxhlet (24 g MeOH: g ginseng)	75.52 (3.80)	100

Based on these results, supercritical fluid extraction using a 4.1 g solvent/ g ginseng ratio with a 1 hour static extraction and a 30 minute dynamic recovery period can obtain between approximately 89 to 90% (with a RSD% of 4.4 – 5.3%) of the ginsenosides extracted in a 20-hour methanol Soxhlet extraction using a 30 mL MeOH: 1 g ginseng ratio (24 g MeOH: 1 g ginseng ratio), although there is moderate variation for these results. A t-test comparing the total ginsenoside yield between Run 1 and 2 vs. 20 Hour Methanol Soxhlet showed with approximately 99% confidence that the value for total ginsenosides obtained was higher for MeOH Soxhlet compared to static + dynamic CO_2 + MeOH extraction. The total ginsenosides obtained for runs changing the flow rate was similar within experimental variation for these conditions, indicating that the dynamic aspect of the extraction was not changed by increasing the flow rate. This indicates that the extraction as well as recovery of ginsenosides was insensitive to flow rate given a minimum dynamic run time, as flow rates of 0.8 mL/min and 0.3 mL/min over a 30 minute dynamic extraction period yielded identical results (within variation) for

ginsenosides extracted/recovered. Further work into the minimum time required at each flow rate should be investigated in order to minimize the amount of modifier used in extractions.

Run 3 was capable of extracting 71.5% of the ginsenosides (8.66% RSD) that a 20-hour methanol Soxhlet obtained using a 15 minute static extraction period followed by a 15 minute dynamic period. These results indicate that a supercritical technique is capable of quickly extracting a large fraction of components, although not able to completely extract ginsenosides at the operating conditions chosen in this work. The effect of dynamic flow rate and time should be investigated further to confirm that it does not play a significant role in extraction of ginsenosides beyond a baseline recovery time, where all extracted material has had sufficient time to be collected in the trapping vessel.

Of the runs performed, Run 2 had the highest variation in terms of the individual ginsenosides Rb_2 and Rc, as well as the acetylated components. This is potentially due to lower fluid flow rate used in these runs, which could have resulted in producing runs where full recovery of all ginsenosides was not obtained, although further experimentation would be required to confirm this result. In terms of composition of extracts, all of the run types produced fairly similar results within experimental variation. The mean compositions of ginsenosides for each run type are shown in Table 5.7.4 along with the relative standard deviation for that ginsenoside.

Table 5.7.4 – Mean Compositions for Different Run Types in Static + Dynamic CO_2 + Methanol Extraction

Ginsenoside	Run 1 Composition (% RSD)	Run 2 Composition (% RSD)	Run 3 Composition (% RSD)
Rb_1	52.02 (1.60)	53.87 (2.15)	50.89 (4.04)
Rb_2	1.48 (12.21)	1.71 (41.3)	1.45 (1.80)
Rc	9.02 (5.98)	6.97 (33.97)	9.33 (3.49)
Rd	8.67 (0.40)	9.27 (14.49)	8.13 (2.59)
Re/Rg_1	25.68 (4.20)	24.63 (2.63)	28.12 (9.21)
Mono-*O*-acetyl ginsenoside Rb_1s	3.15 (2.37)	3.55 (23.31)	2.09 (1.33)

From Table 5.7.4, it is clear that the only ginsenosides which show large variation within the treatment group are Rb_2 and the acetylated ginsenoside(s) and that is primarily under Run 2 conditions. The variation for these components is likely higher due to the small fraction these ginsenosides represent of the total ginsenoside yield, as the relative standard deviation percentages for the mg/g values show a more uniform distribution and smaller error. In addition, although there is larger uncertainty associated with the acetylated ginsenosides, it represents 2.96% of the recovered extracts for static + dynamic extraction with a relative standard deviation of 28.17%. This is in contrast with a 20-hour methanol Soxhlet extraction, which yielded no detectable amount of these acetylated ginsenosides.

In addition, comparing the composition of extracts between the static + dynamic runs vs. a static CO_2 + methanol extraction at the same modifier percentage and mass modifier per mass of ginseng shows a very similar result for ginsenoside composition, as illustrated in Table 5.7.5, with comparisons to a 20-hour methanol Soxhlet extraction as well.

Table 5.7.5 – Comparison of Static + Dynamic CO_2 + Methanol Extraction Composition vs. Static CO_2 + Methanol Extraction and Methanol Soxhlet Extraction Compositions

Ginsenoside	Mean of Run 1, 2, 3 Composition (% RSD)	Static CO_2 + Methanol SFE (27 mol%, 4.1 g MeOH/g ginseng) (% RSD)	Methanol Soxhlet (% RSD)
Rb_1	52.26 (3.35)	53.05 (11.2)	59.54 (1.52)
Rb_2	1.54 (22.77)	1.48 (9.73)	2.14 (45.88)
Rc	9.07 (4.65)	9.46 (0.53)	6.37 (5.95)
Rd	8.69 (9.13)	8.27 (9.86)	10.12 (3.58)
Re/Rg_1	26.14 (7.86)	24.46 (19.02)	21.84 (10.32)
Mono-*O*-acetyl ginsenoside Rb_1s	2.93 (26.24)	3.26 (6.75)	0

From examination of Table 5.7.5, the similarity between the runs in static vs. static + dynamic mode in terms of ginsenoside composition are readily apparent. This is not overly surprising as the dynamic stage will still only be capable of extracting components

with solubility in CO_2 + methanol and the time under heating was the same for all cases except for Run 3. In addition, the differences between supercritical extraction and a 20-hour methanol Soxhlet are also made apparent, as there is a lower quantity of Rc and the acetylated ginsenosides and a higher amount of Rb_1 in the Soxhlet extractions vs. supercritical. A statistical comparison of the static + dynamic CO_2 + Methanol extractions vs. the static CO_2 + methanol extraction for composition results in a set of p values for the two-tailed t-test (unequal variances between sets assumed) with the null hypothesis being that the mean values are equal (difference in means is negligible) and the p-value being the probability that the null hypothesis is true.

Table 5.7.6 – Results for t-test Comparing Compositions Obtained in Static CO_2 + Methanol Extraction vs. Static + Dynamic CO_2 + Methanol Extraction

	Rb_1	**Rb_2**	**Rc**	**Rd**	**Re/Rg_1**	**Mono-*O*-acetyl ginsenoside Rb_1s**
p-value	0.993	0.807	0.926	0.930	0.822	0.567

From Table 5.7.6 there is a clear indication that the composition of extracts does not vary significantly for CO_2 + methanol extractions whether performed in a static extraction or in a static + dynamic extraction at 27 mol% and 4.1 g MeOH/g ginseng and 1 hour static extraction time in both cases, as the probability that the mean of each sample set is equal is high for all ginsenosides tested, in particular Rb_1. The acetylated ginsenosides have the lowest probability of the null hypothesis being true, likely associated with the lower amounts of these compounds in the extract and variation based on trapping in the static

case, making it difficult to determine if there is a true difference or not between the static and the static + dynamic runs for the unknown component. Similar to the case comparing static CO_2 + MeOH to CO_2 + DMSO, static + dynamic CO_2 + MeOH vs. CO_2 + DMSO resulted in significantly different compositions, particularly in the case of Rb_1 and the acetylated ginsenosides (> 99% confidence).

In terms of total ginsenosides extracted, however, there was a difference between static and static + dynamic extractions. The minimum ginsenosides extracted for Runs 1 and 2 was 66 mg/g extracted/recovered versus a maximum of 58 mg/g in the static only case. Even when accounting for experimental variation, this result indicates that there may be a slight increase in ginsenosides extracted when using a dynamic stage, although there is a maximum effect to this result, since cases with higher flow rates vs. lower flow rates with the same modifier percentage, resulted in the same yield of ginsenosides. This result indicates that the static case may reach a solubility limit and that a dynamic stage of extraction is required after to remove the remaining freed ginsenosides from the ginseng, but that there remains a fraction of the ginsenosides which remain bound to the solid surface, although further experimentation would be required to confirm this.

Comparison of static + dynamic CO_2 + methanol vs. Soxhlet extraction for ginsenoside compositions using the same two-tailed t-test used earlier resulted in the p-values shown in Table 5.7.7.

Table 5.7.7 – Results for t-test Comparing Compositions Obtained in Static + Dynamic CO_2 + Methanol Extraction vs. MeOH Soxhlet

	Rb_1	**Rb_2**	**Rc**	**Rd**	**Re/Rg_1**	**Mono-*O*-acetyl ginsenoside Rb_1s**
p-value	0.001	0.446	0.174	0.068	0.112	0.001

When comparing static + dynamic CO_2 + MeOH extraction vs. MeOH Soxhlet, there is a significant drop in the p-value for most ginsenosides. In fact, the amount of Rb_1 and the acetylated ginsenosides are not equal between Soxhlet and these supercritical extractions with greater than 99% confidence. In addition, the values for Rd and Re/Rg_1 are quite low indicating that there is in fact a difference between the runs. Further experimentation is required to determine if the values can be taken as different with greater than 90% confidence for all ginsenosides. Taken together, the supercritical extractions were clearly producing extracts with varying composition compared with methanol Soxhlet.

In terms of selectivity of the extraction, an ANOVA of the different run types showed that with 90% confidence there was no difference among the different static + dynamic run conditions for selectivity. More runs are required to confirm that this is the case, as the fraction of ginsenosides obtained in the shorter extraction time case may in fact be shown to be lower than longer extraction times with further repeated experiments. Since the ANOVA provided no convincing reason to do otherwise, the selectivity for the group of runs was taken as a group and the average was found to be 20.1% with a relative standard deviation of 14.1%, which places it in the same range as a methanol Soxhlet.

This is advantageous in the sense that even at higher modifier percentages in the supercritical fluid there was not a drop in selectivity of extraction compared to Soxhlet and disadvantageous in the sense that ideally supercritical fluid extractions have greater selectivity than conventional extraction techniques.

Comparing the amounts of ginsenosides obtained in the static + dynamic CO_2 + MeOH extraction vs. conventional extraction is feasible since the trapping efficiency was approximately 100% in all cases. The results for this are shown in Table 5.7.8, with the relative amount as well as the relative standard deviation (in percentage). From this table, it can be shown that Re/Rg_1 and Rc are extracted quickly (comparing Run 1 and 2 with Run 3 relative amounts), while Rb_1 as well as Rb_2 and Rd are slower to extract and present in lower amounts than Soxhlet extracts.

Table 5.7.8 – Relative Extraction Amounts of Ginsenosides between MeOH Soxhlet and Static + Dynamic CO_2 + MeOH Extraction

Relative to MeOH Soxhlet	**Rb_1 (% RSD)**	**Rb_2 (% RSD)**	**Rc (% RSD)**	**Rd (% RSD)**	**Re/Rg_1 (% RSD)**	**Mono-*O*-acetyl ginsenoside Rb_1s (% RSD)**	**Total Ginsenosides (% RSD)**
Run 1	0.785 (4.49)	0.629 (46.48)	1.271 (6.35)	0.770 (6.68)	1.056 (14.84)	N/A	0.899 (5.31)
Run 2	0.802 (5.91)	0.715 (58.73)	0.974 (36.56)	0.811 (13.36)	0.998 (12.56)	N/A	0.886 (4.41)
Run 3	0.610 (5.45)	0.488 (44.09)	1.045 (7.33)	0.574 (7.40)	0.922 (21.08)	N/A	0.714 (8.66)

Longer extraction times may extract the potentially matrix-bound ginsenosides, although no significant difference was observed between the case of a 1 hour and 2 hour CO_2 + DMSO extraction at 10 mol% DMSO. As such, additional extraction stages may be required, such as performing a static + dynamic extraction, depressurizing the system and then re-spiking the solids with modifier and performing another static + dynamic extraction. Depressurization of the chamber with the solids present may result in the solid structure of the ginseng being broken up or expanded sufficiently to remove the bound fraction of ginsenosides. However, this technique may not be practical for larger scale extractions.

As with the case of static extractions, no significant quantities of malonyl ginsenosides were observed during the extraction processes for supercritical extraction, based on the complete ginsenoside balance obtained from supercritical extraction and Soxhlet of SFE samples. This is an indication that while the thermal conversion of the acetylated ginsenosides is impeded or slowed compared with conventional extraction in a supercritical fluid extraction, the malonyl ginsenosides are still thermally sensitive enough to be de-malonylated and converted into their neutral analogues. This lack of malonyl ginsenosides was present even for the shortest extraction run performed, 15 minutes static + 15 minutes dynamic. The temperature for extraction was significantly higher than that of methanol Soxhlet, which may be why the malonyl ginsenosides were essentially completely demalonylated. Further evidence that at least to some degree thermal conversions are still present in the system, is the presence of such a larger quantity of the acetylated ginsenosides in CO_2 + DMSO extractions and smaller quantity

of Rb_1, versus the other supercritical modifiers, as it appears that DMSO is potentially providing thermal stability to the acetylated ginsenosides and is preventing degradation into the ginsenoside Rb_1.

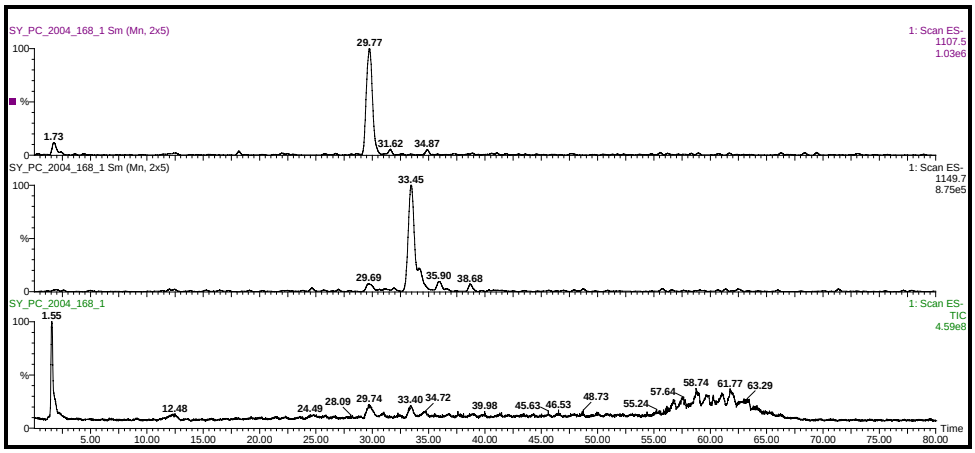
5.8 Identification of Unknown Ginsenoside(s) by LC/MS

As discussed in Sections 5.5 and 5.7, supercritical extractions using CO_2 + modifiers, particularly DMSO, yielded an unknown peak in the ginsenoside range. This unknown peak was at approximately 33.5 minutes and it did not correspond to any of the ginsenoside peaks generated by the standard curve. The concentration of this ginsenoside was not negligible in supercritical extractions as in many cases it constituted a significant fraction of the ginsenoside extract obtained (CO_2 + DMSO extractions), so in order to determine the nature of this component, liquid chromatography/tandem mass spectrometry was used to determine the molecular weight of this component.

The unknown peak was present in varying degrees in all supercritical fluid extractions with high enough modifier percentages, so extracts from aqueous ethanol/acetic acid and DMSO extractions were all tested under LC/MS to determine the molecular weight of the unknown peak, in order to verify that the same unknown component was being extracted for the various modifiers being used in this study. The LC/MS procedure used to determine the molecular weight of the unknown was previously given in Section 4.7. The results for these runs are shown in Figures 5.8.1 – 5.8.8, which show the HPLC result with the retention time of the unknown peak and the corresponding mass spectrum

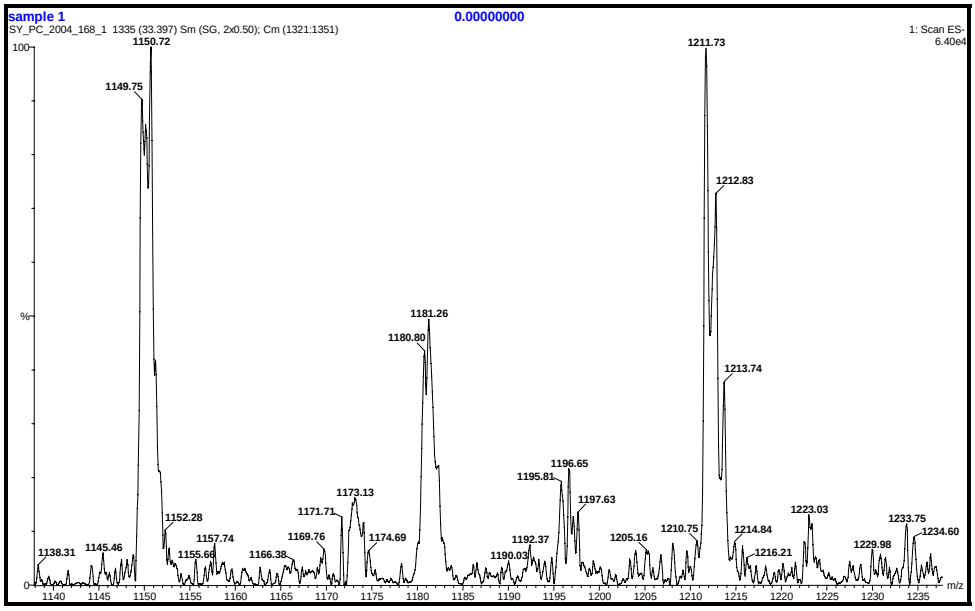
plot, with the m/z ratio to various components in the extract. The unknown component m/z value as well as HPLC retention time are shown in the text below each figure.

Figure 5.8.1 – HPLC for Static CO_2 + DMSO SFE (Run 6)
(4000 psig, 100°C, 10.4 mol%, 1 hour static)



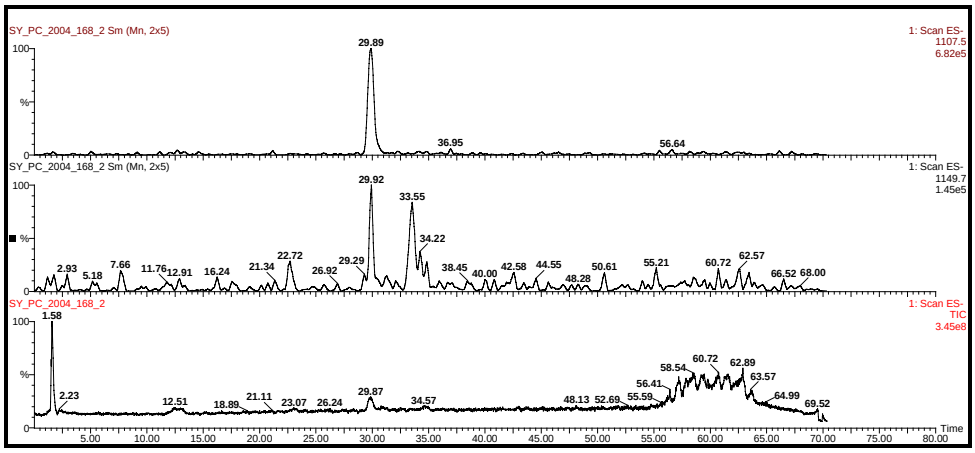
Unknown peak is at 33.45 minutes

Figure 5.8.2 – Mass Spectrum for Static CO_2 + DMSO SFE (Run 6)
(4000 psig, 100°C, 10.4 mol%, 1 hour static)



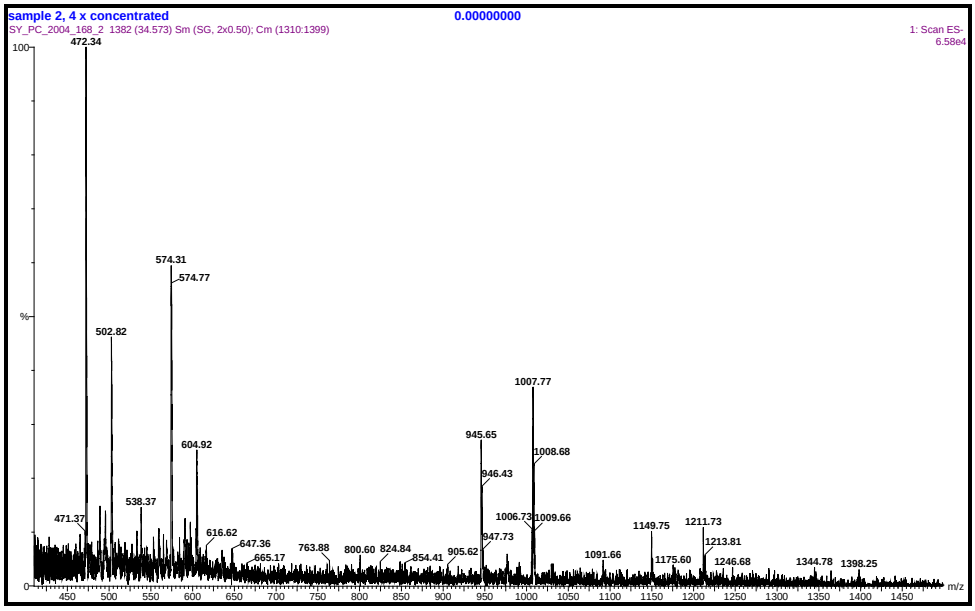
Unknown component(s) correspond to 1149.75 m/z with a clear overlap at 1150.72 (single component should appear as 1211.73, 1212.83 and 1213.74 peaks appear).

Figure 5.8.3 – HPLC for Static CO_2 + $EtOH_{(aq)}$/Ac. Acid SFE (Run 11)
(5000 psig, 110°C, 11.5 mol%, 1 hour static)



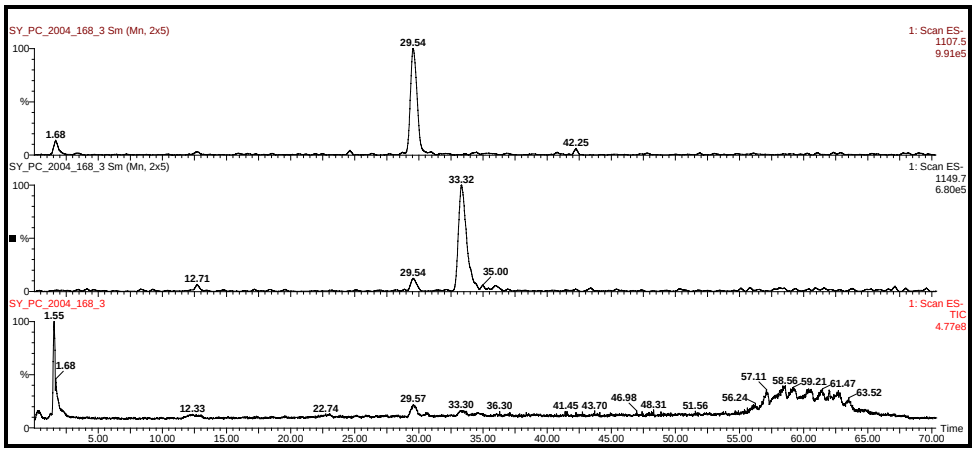
Unknown peak is present at 33.55 minutes

Figure 5.8.4 – Mass Spectrum for Static CO_2 + $EtOH_{(aq)}$/Ac. Acid SFE (Run 11)
(5000 psig, 110°C, 11.5 mol%, 1 hour static)



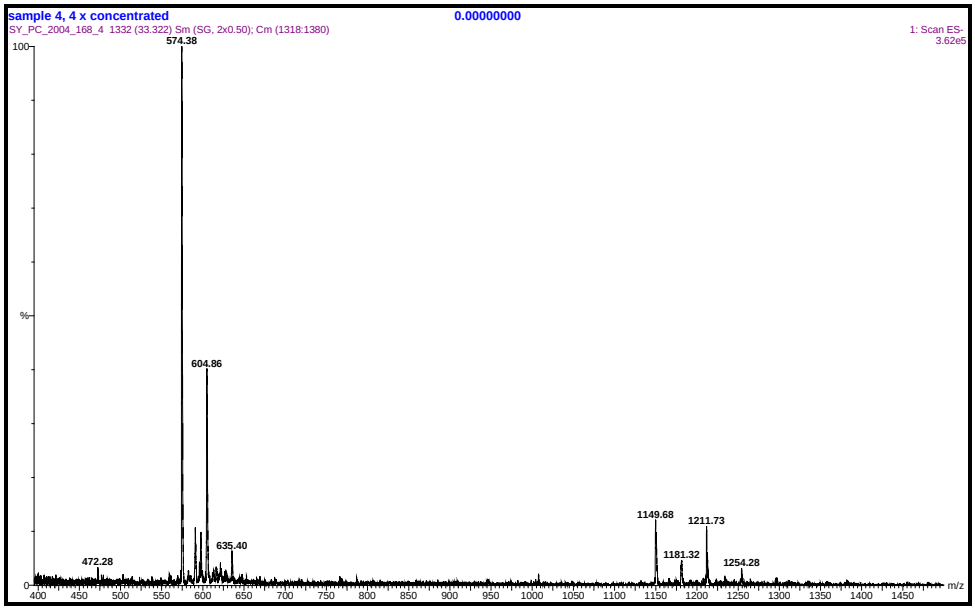
Unknown component has an m/z of 1149.75

Figure 5.8.5 – HPLC for Static CO_2 + DMSO SFE (Run 7)
(5000 psig, 110°C, 10 mol%, 2 hour static)



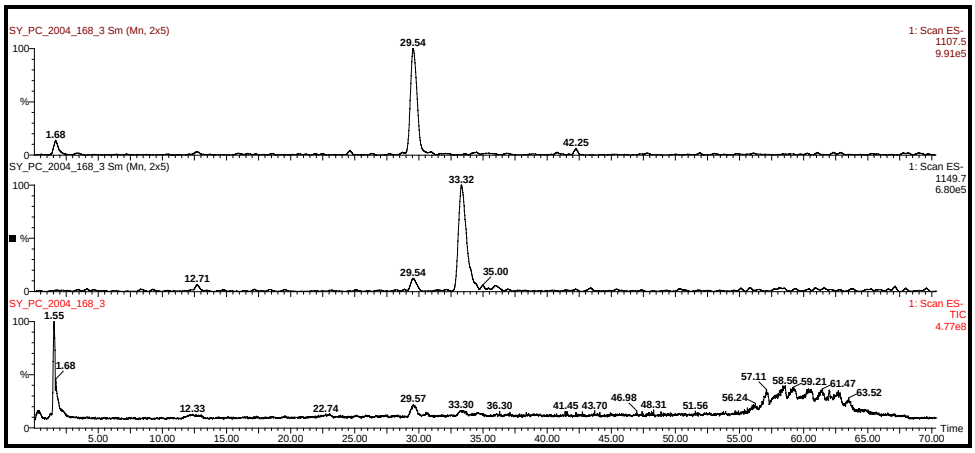
Unknown peak at 33.32 minutes

Figure 5.8.6 – Mass Spectrum for CO_2 + DMSO Extraction (Run 7)
(5000 psig, 110°C, 10 mol%, 2 hour static)



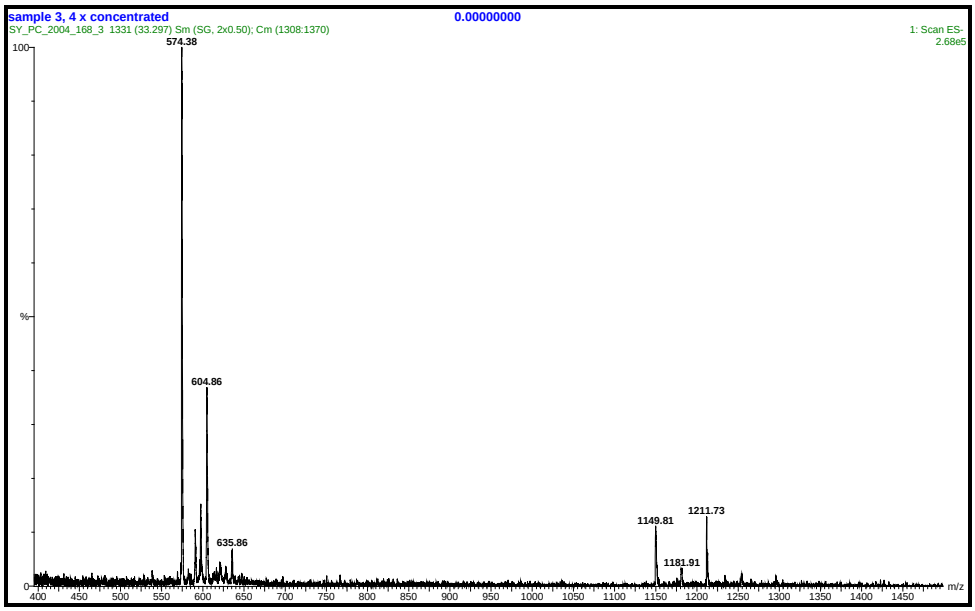
Unknown Peak has an m/z of 1149.68

Figure 5.8.7 – HPLC for Static CO_2 + DMSO SFE (Run 5)
(5000 psig, 110°C, 10 mol%, 1 hour static)



Unknown peak at 33.32 minutes

Figure 5.8.8 – Mass Spectrum for Static CO_2 + DMSO SFE (Run 5)
(5000 psig, 110°C, 10 mol%, 1 hour static)



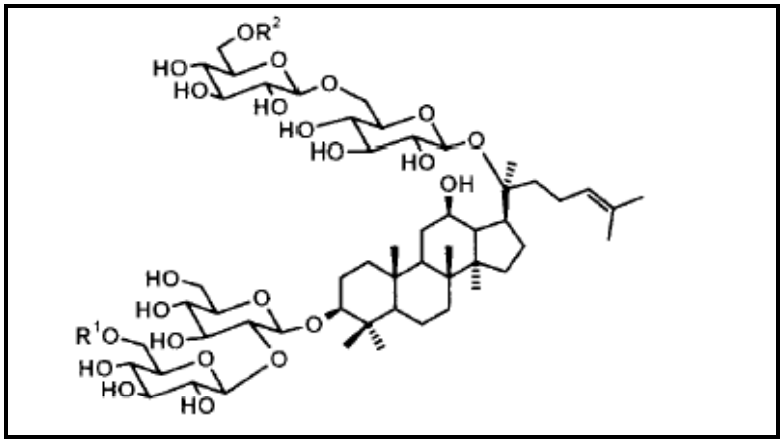
Unknown peak has an m/z of 1149.81

From all analyzed extracts, the retention time of the unknown peak was between 33.32 and 33.55 minutes. The mass spectra, as illustrated most clearly in Figure 5.8.2 shows that there are two overlapping compounds between an m/z of 1149.75 and 1150.72, as there two decreasing peaks followed closely by a larger peak, which is indicative of two components with similar molecular weight overlapping each other. In order to identify the potential components at this location, literature was consulted regarding LC/MS of ginsenosides from ginseng and other plants. Kite et al. (2003) analyzed various ginseng species with LC/MS, with the purpose of using malonyl ginsenoside content to authenticate different ginseng species (Kite et al., 2003). The authors report that they observed a ginsenoside at an m/z of 1149 operating in negative ion mode, which is identical to the unknown observed in our case. The authors identified this structure as quinquenoside R_1, although they did not give an indication about the potential identity of the other unknown compound.

Quinquenoside R_1 is an acetyl-ginsenoside, with the proper name given by mono-*O*-acetyl-ginsenoside-Rb_1. The acetyl group is located at the 6-hydroxyl group of the terminal glucosyl moiety of the b-sophorosyl group (Gebhardt et al., 2002). The structure of this ginsenoside is shown in Figure 5.8.9. This type of ginsenoside is present in very small quantities normally after conventional extraction, or is fully converted through a thermal process depending on the time of extraction (Court et al., 1996). This explains why the unknown compounds did not appear on the HPLC analysis of ginseng extract from a 20-hour methanol Soxhlet extraction, as this was sufficient time to thermally convert the mono-*O*-acetyl-Rb_1 compounds into Rb_1.

Gebhardt et al. (2002) discussed the use of enzymes to convert ginsenoside Rb_1 into more hydrophilic and lipophillic derivatives using biocatalysis with the enzymes *β*-1,4-Galactosyltransferase and *Candida Antarctica* Lipase B. The goal of the authors work was to increase the structural diversity and bioactivity of the ginsenoside derivatives by acetylating the compounds (Teng et al., 2004). Acetylated compounds are more lipophillic, which can potentially increase uptake into cells (Gebhardt et al., 2002). Lipase B was used to acetylate the ginsenoside Rb_1 by this group. The major compound for acetylated Rb_1 was a monoacetate with a [M-H]- ion at 1149, which is identical to the unknown peak observed in this work. The authors analyzed the NMR spectrum and determined that the isolated product was not just quinquenoside R_1 but in fact two monoacetate compounds (Gebhardt et al., 2002). The structures of both monoacetates are given in Figure 5.8.9.

Figure 5.8.9 – Structure of Acetylated Ginsenoside Rb_1 Compounds
(Gebhardt et al., 2002)



R^1 – H, R^2 – Acetyl
for quinquenoside R_1

R^1 = Acetyl, R^2 = H
for 6”-O-monoacetyl
ginsenoside Rb_1.

The production of a second monoacetate by enzymatic reaction of Rb_1 explains the second compound of virtually identical molecular weight which is present on the mass

spectrum of the supercritical extractions. Gebhardt et al. (2002) found that the major compound was quinquenoside R_1 and that complete separation of the two components was not possible (quinquenoside R_1 could be isolated on its own but the minor component always contained quinquenoside R_1) (Gebhardt et al., 2002). The acetylated ginsenosides are likely present in supercritical CO_2 extractions due to the high affinity of CO_2 for acetylated-compounds due to the cooperative Lewis acid – Lewis base interactions discussed earlier in Section 2.1 and 4.9 as well as the shorter extraction time which limits the time for thermal conversion of the acetyl-ginsenoside Rb_1 compounds into Rb_1.

In addition, Gebhardt et al. (2002) found that DMSO was able to provide good thermal stability to products at 45°C and higher temperatures, which is an explanation of why DMSO in particular was able to obtain such high quantities of the acetylated ginsenosides (Gebhardt et al., 2002). The fact that these acetylated ginsenosides convert into Rb_1 is an indication that the true value of the acetylated ginsenosides obtained in static CO_2 + methanol extractions and static + dynamic CO_2 + methanol extractions is not significantly different, since the p-value for the composition of Rb_1 indicates that with 99% confidence the values for Rb_1 are the same between static and static + dynamic extraction (illustrated in Table 5.7.6).

The ability of supercritical fluid extraction, particularly that with CO_2 + DMSO, to yield high amounts of these acetylated ginsenosides is a major advantage over enzymatic synthesis and other chemical methods, which require pure Rb_1 to be converted in a 4 hour

reaction producing mono- and di-acetyl ginsenosides based on Rb_1. The di-*O*-acetyl ginsenoside Rb_1 was the minor component of this enzymatic conversion but was not observed in this work to a significant degree. In contrast to this enzymatic technique, the supercritical technique allows for high quantities of this material to be obtained directly from extraction, with the potential for separation based on phase equilibria in a recovery stage. To determine if conventional Soxhlet extraction yields any significant quantity of these unknowns, and if they are merely thermally converted, a 12-hour Soxhlet with a 30:1 mL methanol/g ginseng ratio was performed, as this is comparable with existing literature techniques (Korean Ginseng & Tobacco Research Institute). This extraction technique was capable of extracting 0.35 mg/g of the acetate (representing approximately 1.6% of the total ginsenosides extracted). This value is still significantly lower than that obtained in CO_2 + DMSO extractions, even without factoring in trapping efficiency as the amount of the acetylated ginsenosides recovered ranged from 6.9 to 8.4 mg/g for extractions run with over 10 mol % DMSO in the supercritical phase (> 3.2 g DMSO/g ginseng).

When accounting for the lower trapping efficiency in this experiments, the amount of the acetylated ginsenosides is even greater so it is clear that supercritical extraction with CO_2 + DMSO provides large quantities of these compounds when compared with conventional solvent extraction techniques. Even for extractions with CO_2 + MeOH and CO_2 + EtOH(aq)/Acetic Acid, the amount of unknown recovered (or definitively extracted in the case of static + dynamic CO_2 + MeOH extractions) was still between approximately 1 to 2 mg/g, with smaller amounts obtained in the shorter extraction time

run. The results from the 12-hour methanol Soxhlet and 20-hour methanol Soxhlet when compared to the case of supercritical extraction, particularly in the case of CO_2 + DMSO, are an indication that the mono-*O*-acetyl ginsenoside Rb_1 compounds are more thermally sensitive and are thermally converted through heating during conventional methanol Soxhlet extraction into Rb_1, in particular for the 20-hour methanol Soxhlet extraction.

Lower temperature extraction processes, such as ultrasound-assisted extraction could potentially overcome this thermal conversion while microwave, Soxhlet or pressurized liquid extraction seem to be incompatible with obtaining larger quantities of the acetylated ginsenosides due to the higher temperature or long extraction times associated with these techniques. Supercritical fluid extraction using CO_2 with the various modifiers studied in this work were capable of extracting much larger amounts of these acetylated ginsenosides, as illustrated in Table 5.8.1. The cases involving CO_2 + MeOH and CO_2 + EtOH(aq)/Acetic Acid had larger variation in the amount of acetylated ginsenoside obtained during extractions compared with DMSO, likely due to the thermal stability imparted by DMSO on the acetylated ginsenosides. The difference in unknown composition between the CO_2 + MeOH runs and CO_2 + EtOH(aq)/Acetic Acid was not determined due to the large variation in the case of EtOH(aq)/Acetic Acid as a modifier.

Table 5.8.1 – Percentage Composition of Total Ginsenosides of Acetylated Ginsenosides in Supercritical Extractions

Run	Percentage Composition
CO_2 + DMSO (over 10 mol% DMSO)	19.94 (4.72)
CO_2 + MeOH (27 mol% MeOH runs of static and dynamic nature)	3.48 (16.82)
CO_2 + EtOH(aq)/Acetic Acid (14.5 mol% EtOH(aq)/Acetic Acid runs)	2.37 (38.57)

Gebhardt et al. (2002) did not provide any information on why DMSO in particular was able to provide thermal stability to the acetylated ginsenosides, compared to other solvents, only that it was able to provide thermal stability and was unique in solvents in allowing determination of the structure of both unknown acetylated ginsenosides using NMR (Gebhardt et al., 2002). The supercritical nature of the extraction may have imparted further stability, although this is unclear as stability was the effect of DMSO was observed at atmospheric pressure by Gebhardt et al. (2002). In any case, the individual components of the solubility parameter of DMSO vs. methanol and ethanol may be able to provide an insight into this stability, and are shown in Table 5.8.2. The solubility parameters used are of the expanded set proposed by Karger et al. (1976) to expand the classic 3D solubility parameter proposed by Hansen to include orientation as well as acid and base effects (Hansen, 1969; Karger, Snyder, & Eon, 1976).

Table 5.8.2 – Solvent Properties of DMSO vs. Other Solvents Used in This Process
(Adapted from (Karger et al., 1976))

	δ_T $(MPa)^{1/2}$	δ_d $(MPa)^{1/2}$	δ_o $(MPa)^{1/2}$	δ_{in} $(MPa)^{1/2}$	δ_a $(MPa)^{1/2}$	δ_b $(MPa)^{1/2}$
DMSO	24.55	17.19	12.48	4.30	0	10.64
Ethanol	25.99	13.91	6.96	1.02	14.12	14.12
Methanol	29.67	12.69	10.03	1.64	16.98	16.98

δ_T represents the total hildebrand solubility parameter (measure of cohesive energy density and therefore solvent strength), δ_d the dispersive forces, δ_o the orientation or permanent dipole forces, δ_{in} the induction forces, δ_a the acidity and δ_b the basicity.

As can be seen from Table 5.8.2, DMSO has a zero acidity component to the solubility parameter, which results from being able to act only as a proton acceptor for hydrogen bonding. In addition, DMSO has a higher dispersive forces value as well as a higher orientation and induction force value. Any combinations these forces could be the reason that DMSO was able to provide higher stability to the acetylated ginsenosides vs. other solvents used in this work. Future investigation is warranted into this aspect of DMSO extractions of ginsenosides, to see if perhaps the basic nature of the solvent plays a role in providing thermal stability.

6. CONCLUSIONS

In this work, the extraction of ginsenosides from the root of *Panax quinquefolius* (North American ginseng) using supercritical fluids was undertaken. The fluids used were solvent modified mixtures of CO_2 + modifier. The work done in this study has shown that supercritical fluid extraction is a promising technique to extract ginsenosides without thermal degradation of neutral ginsenosides, and was able to obtain ginsenoside compounds not typically obtained during conventional extraction techniques.

Although neat CO_2 was unable to extract significant quantities of ginsenosides or of other ginseng components, adding modifier was found to have a profound impact on the quantity of ginsenosides extracted. The different modifiers of carbon dioxide studied were found to provide different ginsenoside compositions when compared with each other, although fairly similar total ginsenoside extract yields. The composition also varied when compared with that of a 20-hour methanol Soxhlet, which was taken as the standard extraction. Supercritical extraction was found to produce significant quantities of unknown ginsenosides, which have been identified as mono-*O*-acetyl ginsenoside Rb_1s, in comparison with existing literature for conventional extraction techniques. CO_2 + DMSO in particular was capable of extracting larger quantities of these ginsenosides (close to 20% of ginsenosides in extract were acetylated ginsenosides), which is consistent with existing literature claiming that DMSO imparts thermal stability to these compounds preventing conversion into Rb_1 (Gebhardt et al., 2002).

The effect of the mass of modifier per mass of ginseng extracted was very pronounced on the amount of ginsenosides obtained, going from negligible levels at ratios less than 1 g solvent: g ginseng, to levels of approximately 70-77% of a 20-hour methanol Soxhlet at values between 3 and 4 g solvent: g ginseng for static extractions. The fraction of ginsenosides out of the total material extracted for supercritical extractions was similar to that obtained in methanol Soxhlet, with approximately 20% of the extract being ginsenosides. Higher temperature operation was required in order to insure single-phase operation during the extraction processes by maintaining operation above the mixture critical temperature. This was required to take advantage of the favourable viscosity and diffusivity in the supercritical state for effective mass transfer. Pressure was kept at 5000 psig for most runs in order to provide sufficient density to dissolve non-volatile ginsenosides, and insure that the pressure was greater than the mixture critical pressure.

In terms of system operation, it was discovered that operating with a single static CO_2 + modifier extraction and using a pure CO_2 recovery stage had a number of potential pitfalls. One, the recovery of extracts, in particular ginsenosides, was found to be limited by the need to maintain the restriction at levels higher than 1 mL/min for the first few minutes of extraction at higher modifier amounts in the supercritical fluid phase, which is incompatible with liquid phase trapping. Another pitfall was the potential for higher flow rates to cause a sudden loss of solubility of components in the supercritical fluid, which would lead to deposition of solid materials in the extractor vessel and a loss of collection efficiency. The trapping efficiency for static extractions varied from around 65% for the case of DMSO, to approximately 80% for other modifiers used in this work. Overall, the

composition of extracts was found to be fairly consistent, even though the trapping efficiency varied from run to run due to the chaotic nature of flow at the start of the recovery phase. No significant variation was observed in ginsenoside composition between static CO_2 + methanol extractions and static + dynamic CO_2 + methanol extractions at the same mol % and mass of modifier per mass of ginseng. The trapping efficiency for static + dynamic CO_2 + methanol extractions was approximately 100% and fewer operational difficulties were encountered in this mode of operation. Changing the extraction time from one to two hours had no noticeable effect on the ginsenoside yield at conditions studied, indicating that the extraction process is desorption-limited rather than mass transfer limited.

Thermal conversion of the acetylated ginsenosides was not observed for supercritical extraction to the degree that occurred in a 20-hour methanol Soxhlet extraction. No other significant peaks were observed for supercritical extractions and unaccounted for, which is an indication that although neutral ginsenosides are not being thermally converted, malonyl-ginsenosides are converted into their neutral counterparts during these extractions, likely due to the higher temperatures (>100°C). Based on the 20-hour methanol Soxhlet runs, the total ginsenoside content per gram of ginseng was determined, and by re-extracting supercritical samples with methanol Soxhlet the total amount of ginsenosides extracted could be compared to the total recovered, determining the trapping efficiency. In addition, in the case of static + dynamic CO_2 + methanol extraction there was close to 100% trapping, indicating that the two extraction methods account for

approximately 100% of the ginsenosides present. This is a further indication that no significant quantity of malonyl ginsenosides were present after supercritical extraction.

The static + dynamic CO_2 + methanol extractions were able to obtain approximately 89-90% of the ginsenosides extracted with methanol Soxhlet, with the flow rate of fluid in the dynamic stage found to have no appreciable effect on the quantity of ginsenosides extracted. The amount of ginsenosides obtained in static + dynamic CO_2 + methanol extractions is less than the total extracted in MeOH Soxhlet, although the fraction obtained relative to Soxhlet is closer within variation to approaching complete Soxhlet extraction. Nonetheless, there are indications that there may be a remaining bound fraction of ginsenosides due to a performed t-test between the total ginsenosides obtained in the static + dynamic CO_2 + methanol extractions vs. MeOH Soxhlet, which showed that with greater than 95% confidence the two values were not equal. This bound fraction may be extracted using longer extraction times (although tests using DMSO showed no significant change in yield), or potentially through further grinding of the material or other methods.

Overall, supercritical extraction remains a technique of interest when compared with conventional extraction, although the economics of a larger scale process will require further investigation and optimization with larger scale units. The selectivity advantage of supercritical fluid extraction was partially negated by the requirement of higher modifier percentages to extract ginsenosides. One potential extraction method would be to run at conditions which were capable of extracting larger amounts of material from

ginseng but not the ginsenosides and then re-extracting in order to obtain a ginsenoside-rich fraction. This approach may or may not be more advantageous than a single extraction method which extracts all components and then fractionates components of interest based on phase equilibria.

7. RECOMMENDED FUTURE WORK

Based on the experimental work carried out to date, the following recommendations are made to assist in future investigations with this system:

1. CO_2 + DMSO and CO_2 + EtOH(aq)/Acetic Acid should be run in a static + dynamic extraction mode as this will eliminate the trapping and operational difficulties observed in the static only case.

2. A potential technique to extract the remaining ginsenoside fraction is to extract the ginseng with multiple supercritical extraction stages, with depressurization between stages. Depressurizing the solid sample may expand and break up the plant material sufficiently to allow extraction of the remaining components of interest.

3. Grinding of the solids to a smaller particle size could potentially reduce internal mass transfer resistance and allow for faster extractions and higher extract amounts, although it was found to be ineffective for SFE of Korean ginseng root hair and can lead to mechanical stability problems during operation. In addition, there is a sizeable fraction of the current sample already of a sufficiently small size to make grinding redundant. As such, grinding should be considered as a last alternative to improve extraction efficiency.

4. Investigation of the multicomponent phase equilibrium involved in this process should be undertaken. This is a difficult problem to address, as the large number of components will make conventional thermodynamic modeling techniques ineffective, requiring a more empirical approach, such as neural network modeling, as well obtaining the individual phase equilibria data for components of interest is difficult given the large number of components. It may be possible to obtain experimental data using an on-line technique which can monitor when certain functional groups disappear from solution, such as ATR/FTIR. The suitability of this type of system to determine phase equilibria of ginsenosides is unknown at this time.

5. Investigation of extraction yields with a larger scale unit. A larger scale, 500 mL extraction vessel unit has been designed, containing a pressurized liquid trapping vessel. This unit will be able to handle a larger mass of ginseng or other natural products for experimentation. The unit will be heated by an oven, with pressure and flow rate control from a backpressure regulator. This system will also lend itself to automated control through the use of Labview software. Ideally, the system will be less susceptible to plugging as well as enjoy more robust trapping efficiencies over a wider range of conditions. In addition, optimization of process conditions on a larger scale unit will allow for a more accurate assessment of the economic feasibility of this process.

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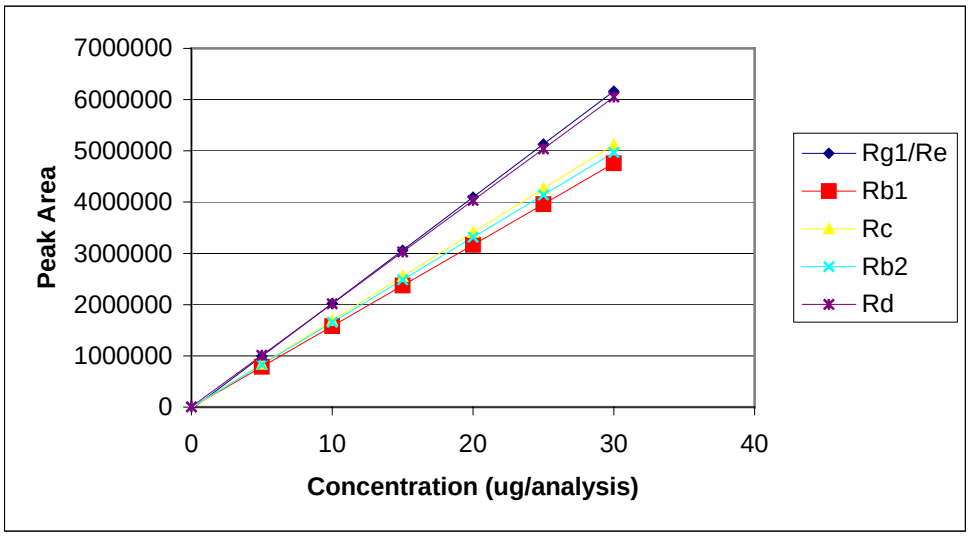
9. APPENDIX A – GINSENOSIDE CALIBRATION PLOTS

The ginsenoside calibration plots used in this study were graciously provided by Dr. Mark Bernards, Department of Biology, University of Western Ontario.

Ginsenoside Calibration Curves (Peak Area vs. Concentration)



The clustering of calibration curves for ginsenosides with similar number of sugar groups is clear, as Rc, Rb_2 and Rb_1 (4 sugar groups) have very similar calibration curves while the curves for Rg_1/Re and Rd are also very similar to one another (3 sugar groups).

10. CURRICULUM VITAE

Name:	Jeffery A. Wood
Post-Secondary: Education and Degrees	University of Western Ontario London, Ontario, Canada 1999 – 2003 B.E.Sc. University of Western Ontario London, Ontario, Canada 2003 – 2005 M.E.Sc.
Honours and: Awards	NSERC PGS A Scholarship, 2003 – 2005 Professional Engineers of Ontario Gold Medal in Engineering, 2003 Gold Medal in Chemical & Biochemical Engineering, 2003 Society of Chemical Industry Merit Award, 2003
Related Work: Experience	Teaching Assistant, University of Western Ontario, 2003 – 2005
Publications:	Wood, J. A.; Wan, W. K.; Bernards, M. A.; Charpentier, P. A.; "Extraction of Ginsenosides from Ginseng Using Supercritical Fluids". In preparation.
Conference: Presentations	Wood, J. A.; Wan, W. K.; Lui, E. M. K.; Charpentier, P. A.; "Extraction of Nutraceuticals from Ginseng Using Supercritical Carbon Dioxide". Poster Presentation, First Annual Conference on the Medicinal Use of Chinese Herbs, September 25, 2004, London, Ontario, Canada Wood, J. A.; Charpentier, P. A.; Wan, W. K.; "Supercritical Extraction of Ginseng". Poster Presentation, 53rd Canadian Chemical Engineering Conference, October 27th, 2003, Hamilton, Ontario, Canada