Acetyl Rb1 Ginsenoside from North American Ginseng: Extraction and Application

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Chemical and Biochemical Engineering

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ACETYL RB₁ GINSENOSIDE FROM NORTH AMERICAN GINSENG: EXTRACTION AND APPLICATION

(Thesis format: Integrated Article)

by

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Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

North American ginseng is a unique medicinal plant which is believed to show several biological activities including: anti-stress, anti-angiogenic, immunosuppressive, and antioxidant activity. Components previously isolated from North American ginseng include ginsenosides, polycarbohydrates, peptides, polyacetylenic alcohols, and fatty acids. The biological and pharmacological effects of ginseng are mainly related to the ginsenoside components, making their extraction and characterization of interest in order to identify them, and study their biological activities.

This thesis focused on the extraction of ginsenosides from North American ginseng by an ultrasonication method with methanol and DMSO as solvents and their aqueous mixtures. Quantitative analysis of individual ginsenosides from the extracts was measured by HPLC, which demonstrated that ultrasonication significantly enhanced the extraction efficiency, with the best efficiency found using 80% solvent (methanol, and DMSO) and 20% water. Immunosuppressive activity of these ginseng extracts was tested in LPS-induced macrophage cells showing that the 80% DMSO and 80% methanol extracts gave significant potency toward immunosuppressive activity in a dose-dependent manner. Moreover, significant quantities of 6"'-O-acetylginsenoside Rb1 were obtained using DMSO as the extraction solvent during ultrasonication, and identified using MS, FTIR, and 1D (1H and 13C) and 2D (gCOSY, gHSQC, and gHMBC) NMR. Also, subsequent bioassay experiments confirmed that acetyl ginsenoside Rb1 demonstrated additional immunosuppressive activity towards inhibiting the production of nitric oxide (NO) and tumor necrosis factor (TNF)-α in LPS-induced macrophage cells in a dose-dependent manner using murine macrophages. In addition, acetyl ginsenoside Rb1 gave significant anti-angiogenic activity and exhibited enhanced potency towards inhibiting tube-like structure formation of endothelial cells.

Supercritical fluid chromatography (SFC) using supercritical carbon dioxide which is considered as a “green” separation method and believed as a promising technique for separation, isolation, and identification of herbal and medicinal plants, was used to
separate and isolate ginseng extracts obtained by supercritical CO\textsubscript{2} extraction (SFE). The effect of temperature and pressure on the separation of ginsenosides was studied with methanol being added to the CO\textsubscript{2} mobile phase. Acidic, basic, and ionic additives were introduced to the mobile phase, respectively, to study their effect on the separation of ginsenosides. The best separation condition was obtained by adding 0.05\% v/v trifluoroacetic acid in methanol. A high-concentration component in the extracts from the supercritical fluid extraction of North American ginseng was isolated by SFC and identified as sucrose using NMR, HPLC, and ESI-MS.

Because of it's unique biological activities, development of a suitable delivery system for acetyl ginsenoside Rb\textsubscript{1} (ac-Rb\textsubscript{1}) was investigated for the first time in this research. PLGA microspheres were used to encapsulate ac-Rb\textsubscript{1}, examining both a double emulsion and a microfluidic technique. The size and morphology of the ac-Rb\textsubscript{1} loaded PLGA microspheres were characterized by SEM and ZEISS light microscopy, showing unimodal 50-65 µm size diameters, respectively using the microfluidic technique. Also, another delivery system of PLGA in gelatin hydrogel was prepared in order to achieve a localized delivery method, overcoming drawbacks such as PLGA removal by macrophages and a high initial burst effect from gelatin hydrogel that can damage tissues around the injection site. The ac-Rb\textsubscript{1} loaded microspheres were incorporated into the gelatin hydrogels to form a new delivery system examining glutaraldehyde crosslinking concentrations from 10-100µl. FTIR, DSC and TGA confirmed the formation and chemical stability of the gelatin encapsulated composites. Release profiles were studied and quantified by UV-Vis spectrophotometry with the results showing that the release of ac-Rb\textsubscript{1} from the unimodal microspheres prepared by the microfluidic technique showed a lower initial burst effect than those from the double emulsion method. The burst effect was followed by a slow release profile which can be used for long term drug delivery applications to maintain the ginsenoside concentration for an extended time period. It should be mentioned that although the large burst effect could release a therapeutic agent relatively fast, it can also damage tissues around the treatment site. Hence, a combination delivery system was developed using cross-linked gelatin. The release of ac-Rb\textsubscript{1} from the cross-linked gelatin encapsulated microspheres was effected by the pH of the releasing medium as well as the crosslinker concentration. Then, the \textit{in vitro} cumulative release
data of the core and core-composite systems was analyzed using empirical equations in Matlab. The results showed that the *in vitro* release kinetics data followed Fickian diffusion with the best fit observed using the Weibull model, for all investigated cases. Moreover, the released ac-Rb₁ from delivery systems showed a significant immunosuppressive effect on LPS-induced macrophages indicating the novel delivery systems for ac-Rb₁ have potential for next-generation biomedical agents in drug-release devices.

**Keywords**

Ginseng, Ultrasound extraction, DMSO, acetyl ginsenoside, SFC, Microsphere, Hydrogel, Microfluidic method.
Co-Authorship Statement

This dissertation is prepared in the integrated article format. Manuscripts that have been previously published, or submitted for publication, or finalized for submission form the body of this dissertation which are presented with some adjustments in Chapters 2 through 6.

Title: Isolation and Immunosuppressive effects of 6”-O-acetylginsenoside Rb₁ extracted from North American ginseng. Authors: Raziye Samimi, William Z. Xu, Edmund M.K Lui, Paul A. Charpentier. The experimental works were conducted by Raziye Samimi under the guidance of advisor Dr. Paul A. Charpentier. The biological works were done by Raziye Samimi under the guidance of Dr. Edmund M.K Lui. NMR measurement and interpretation were done by Dr. William Z. Xu. The draft of this manuscript was written by Raziye Samimi. Modifications were carried out under the close supervision of Dr. Paul A. Charpentier. The final version of this article was published by the Journal of Planta Medica 80 (2014) 1–8.

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Dedication

To people whose love and support make my life meaningful.

My husband, My parents and My sister
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List of Abbreviations, Symbols, Nomenclature

Abbreviations

AA Ammonium acetate
ABPR Automated back pressure regulator
Ac-Rb$_1$ Acetyl ginsenoside Rb$_1$
API Active pharmaceutical ingredient
CID Collision-induced dissociation
DCM Dichloromethane
DIPA Diisopropylamine
1D One dimensional
2D Two dimensional
DMSO Dimethyl sulfoxide
DSC Differential scanning calorimetry
ELISA Enzyme-linked immunosorbent assay
ELSD Evaporative light scattering detector
FDA Food and drug administration
FTIR Fourier transform infrared spectroscopy
GA Glutaraldehyde
HPLC High performance liquid chromatography
LPS Lipopolysaccharide
MS Mass spectroscopy
NMR Nuclear magnetic resonance
NO Nitric Oxide
PBS Phosphate buffered solution
PDMS Poly(dimethylsiloxane)
PLGA Poly(lactic-co-glycolic acid)
PVA Poly (vinyl alcohol)
scCO$_2$ Supercritical CO$_2$
SEM Scanning electron microscope
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFC</td>
<td>Supercritical fluid chromatography</td>
</tr>
<tr>
<td>SFE</td>
<td>Supercritical fluid extraction</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
</tbody>
</table>

**Symbols**

- **A**: Cross section area (m²)
- **b**: The slope of the regression line of Weibull model
- **Ca**: Capillary number
- **d**: Length (m)
- **Mₜ**: Cumulative amounts of drug released at time t (mg/L)
- **M_∞**: Cumulative amounts of drug released at time t_∞ (mg/L)
- **P**: Pressure (bar)
- **P_c**: Critical pressure (bar)
- **Q**: Flow rate (m³/s)
- **r²**: The correlation coefficient
- **Re**: Reynolds number
- **T_c**: Critical temperature (°C)
- **T_g**: Glass transition temperature (°C)
- **T**: Temperature (°C)
- **u**: Average velocity (m/s)
- **W_d**: Weight of dry gelatin hydrogel (mg)
- **W_w**: Weight of wet gelatin hydrogel (mg)
- **δ_a**: The acidity MPa¹/²
- **δ_b**: The basicity MPa¹/²
- **δ_d**: The dispersion forces MPa¹/²
- **δ_i**: The induction forces MPa¹/²
- **δ_o**: The orientation forces MPa¹/²
- **δ_t**: The total hilderbrand solubility parameter MPa¹/²
- **ρ**: Density (kg/m³)
\( \mu \) \hspace{1cm} \text{viscosity (kg/ms)}

\( \gamma \) \hspace{1cm} \text{Interfacial tension (N/m)}
Chapter 1

1 General Introduction and Literature Review

1.1 Ginseng and Ginsenosides

Ginseng is one of the perennial plants discovered more than 5,000 years ago in the mountain provinces of Manchuria. It is currently one of the best-selling medicinal plants in the world [1, 2]. There are different types of ginseng that have been discovered depending on their cultivation area; the most important being *Panax ginseng* (Korean or Asian), *Panax quinquefolius* (American), *Panax notoginseng* (Tienchi or Sanchi), *Panax Japonicus* (Japanese) and *Panax vietnamensis* (Vietnamese) [3]. North American ginseng grows in the northern areas of the United States and within both British Columbia and Ontario provinces of Canada. Native North Americans used the roots of this plant as a part of traditional medicine for relieving fever and stomach misery [4]. Successful cultivation of North American ginseng started from the 1800s. Nowadays ginseng is one of the most popular medicinal plants available as a herbal medicine; or nutritional supplement. Ontario produces around 2,000 tonnes of ginseng root annually, 90% of which is exported for commercial use to Asia. According to Agriculture and Agri-Food Canada, Canada exports about 3,000 tonnes of North American ginseng roots annually to China and other Asian markets, especially Hong Kong. The price of ginseng exports to all countries in total had 26.8% growth from 2007-2010 [5].

The extracts of this herbal plant have been studied and are known to have abundant beneficial health effects on the central nervous, cardiovascular, endocrine, and immune systems. Ginseng in general is composed of the active ingredients, saponins, called ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, fatty acids, sugars, amino acids, vitamins and minerals. The most active ingredients found in ginseng are ginsenosides, with more than 60 different kinds of ginsenosides being extracted and identified from different parts of North American ginseng plants [1]. The ginsenoside
composition varies depending on which part of the plant is used such as the roots, leaves, stems, flower buds and berries. The ginsenoside content in the leaves of North American ginseng has been reported to be between 1.9-4.2% of the dry weight, whereas it varies between 3-7% of the dry weight in the root [1]. The structure of different ginsenosides isolated from North American ginseng are shown in Figure 1.1.

![Figure 1.1](image_url)

**Figure 1.1.** Core structures of different types of triterpenoid saponins from American ginseng [6].

Differences in ginsenoside composition exist between the various types of ginseng. For instance, North American ginseng contains Rb₁, Re, Rd, Rc, Rg₁ and Rb₂ while it does not contain ginsenosides Rf and Rg₂. In comparison, Asian ginseng includes low concentrations of Rf and Rg₂. The other useful factor that can help to distinguish American from Asian ginseng is the ratio of Rg₁/Rb₁ and Rb₂/Rb₁. American ginseng exhibits both Rg₁/Rb₁ and Rb₂/Rb₁ ratios less than 0.4, whereas Asian ginseng shows higher ratios. Major ginsenosides identified in North American ginseng consist of

**Figure 1.1.** Core structures of different types of triterpenoid saponins from American ginseng [6].

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protopanaxadiol type (PPD) that has sugar moieties attached to the β-OH at C-3 and/or C-20 and protopanaxtriol type (PPT) that has sugar moieties attached to the α-OH at C-6 and/or β-OH at C-20. Four malonyl derivatives (also called “acidic” ginsenosides) have been isolated from North American ginseng. Minor ginsenosides isolated from North American ginseng include ocottillo-type and oleanane-type ginsenosides. Other extracted ginsenosides can be categorized as modified C-20 side chain ginsenosides, which can be divided into nine groups due to C-20 side chain differences. Malonyl-ginsenosides which have a malonic acid functionality at the R1 group (C-6 of the terminal glucosyl ring) attached to C-3 in the PPD structure (Figure 1.1) such as malonyl mRb₁, mRb₂, mRc and mRd are more polar and water soluble than original ginsenosides.

The malonyl-ginsenosides are unstable ginsenosides that are changed to neutral ginsenosides by hydrolysis, demalonylated by heat, or catalyzed by both acid and base. As other examples of thermal instability, ginsenosides Rg₃, Rh₂, and Rg₅ have been shown to have anti-cancer applications and are the thermal degradation products of neutral ginsenosides not naturally found in North American ginseng [6]. Polar ginsenosides often become less polar during the steaming or heating process due to structural changes of the sugar moieties. Moreover, it has been shown that ginsenosides can be degraded into novel structures using microbes, enzymes and intestinal bacteria within animals and humans [7]. For instance, ginsenoside Rb₁ is metabolized to compound K via biotransformation which has higher bioactivity as shown in Figure 1.2.
Ginseng is one of the best-selling herbal plants in the world with more than six million Americans consuming ginseng products to enhance resistance to physical, chemical and biological stress and improve general vitality [1]. Similar to Asian ginseng (Panax), American ginseng has been shown to provide various pharmacological activities such as antioxidant, anti-inflammatory and immunostimulatory activities. It is believed that most of the ginseng biological activities are related to the ginsenoside components, which are the main active components of ginseng root. Other ginseng ingredients such as polysaccharides, polypeptides, polyacetylenes, and alkaloids have been shown to participate in biological activity. The isolated diacetylenes, mainly panaxytriol, panaxynol, and panaxydol, demonstrate cytotoxic, antiplatelet and anti-inflammatory activities, respectively [10]. Also, quinquefolans A-C from American ginseng have demonstrated marked blood glucose-lowering effects after administration to both normal and alloxan-induced hyperglycemic mice [10]. Wang et al have shown a hypoglycemic
effect of ginseng polypeptides [11]. *In vitro* studies on polysaccharide extracts from American ginseng have shown stimulation of the proliferation of normal mouse spleen cells, of which the major responding subpopulation was identified as B lymphocytes. This American ginseng polysaccharide extract activated peritoneal exudates macrophages and increased interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and nitric oxide (NO) production. Moreover, it stimulated *in vivo* immunoglobulin G (IgG) production in treated mice [12]. A following *ex vivo* study using C57 BL/6 mice implied that the polysaccharide extract is capable of increasing Con-A-induced IL-2 and IFN-γ production in spleen cells in a dose-dependent manner. A study by Azike et al. also supported the previous results that North American polysaccharide extracts show an immunostimulatory effect both *in vitro* and *in vivo* [13]. Several studies have evaluated the anti-inflammatory activity of ginsenosides and their metabolites. For instance, ginsenosides Re and Rg1 are transformed to ginsenoside Rh1 or 20(S)-protopanaxatriol, which showed anti-inflammatory effects [14]. Park et al. examined the anti-inflammatory activity of ginsenoside Rb1 isolated from Panax ginseng and its metabolite compound K on LPS-induced RAW264.7 cells. Their results demonstrated that compound K and not ginsenoside Rb1 inhibited the production of NO in LPS-induced RAW 264.7 macrophage cells [15]. Park et al. also reviewed the *in vitro* and *in vivo* anti-inflammatory effects of ginsenosides and their metabolites. While ginsenosides Rb1 and Rb2 were shown to inhibit TNF-α production by LPS-stimulated RAW264.7 murine or U937 human macrophages in a dose-dependent manner, ginsenosides Re and Rg1 did not show any potency [16]. Similarly, ginsenosides Rb1, Rc or Re did not inhibit NO production by LPS-stimulated RAW264.7 cells [17]. Another study reported on the anti-inflammatory effects of ginsenosides on the inhibition of various inflammatory molecules such as PGE2 (produced by COX-2) by murine or human macrophages, isolated from Panax ginseng [17].

Based on the various pharmacological effects of North American ginsenosides [1], their biological activity can be summarized as shown in Figure 1.3.
As can be seen in Figure 1.3, North American ginsenosides have been shown to provide beneficial effects on aging, central nervous system (CNS) disorders, and neurodegenerative diseases [18]. Specifically Rb<sub>1</sub>, Rg<sub>1</sub>, Rg<sub>3</sub>, Rd, and Re effects on neurodegeneration have been examined in animals and in neuronal cell cultures [19-23]. Moreover, North American ginseng and ginsenosides are believed to increase cognitive performance and mood [24-26]. They are also believed to provide increased neuronal cell survival, extending neurite growth, and rescuing neurons from death either in vivo or in vitro. Their effect on neurodegenerative disease models of Parkinson’s and Alzheimer’s diseases have also been investigated [27].

The antioxidant property of North American ginseng has been shown to affect cardiovascular activity [28]. This herb has also demonstrated anti-ischemic, antiarrhythmic and anti-hypertensive effects [29]. The major antioxidant agent of North
American ginseng, ginsenoside Re, has been found to protect cardiomyocytes by scavenging \( \text{H}_2\text{O}_2 \) and hydroxyl radicals [30].

American ginseng and ginsenosides have also been shown to have cancer prevention activity by increasing the chemopreventive effect of 5-fluorouracil in human colon cells, the chromosomal aberration suppression induced by mitomycin C in mice, cancer related fatigue improvement, and radioprotective potential production in the lymphocytes of healthy individuals [1]. Due to the complexity of the anticancer mechanisms of ginseng, most studies have examined individual ginsenosides, but not North American ginseng extract or acetylated ginsenosides.

### 1.3 Extraction of bioactive compounds

Extraction of bioactive components from natural plants such as ginseng has attracted significant attention in recent years. Separation of bioactive compounds is possible utilizing a suitable extraction method. The available extraction methods are used to extract the bioactive compounds from natural plants, and can enhance the selectivity of analytical methods. Also, various extraction methods are common for the purpose of increasing sensitivity of a bioassay by enhancing the targeted component concentration. In addition, all extraction methods are desired to provide a reproducible technique that is not affected by variations in the plant matrix [20].

#### 1.3.1 Methods for extraction of ginsenosides from North American ginseng

The ginsenoside content in NA ginseng has been shown to be sensitive to the extraction technique and to the solvents used [31]. The conventional methods of solvent extraction are mainly based on the choice of solvent and the use of heat and/or mixing to increase the solubility of materials and the rate of mass transfer [32]. Traditional extraction methods such as Soxhlet are time consuming and require large amounts of organic solvents. By increasing the demand for reducing the extraction time and the organic solvent consumption, novel extraction methods such as pulsed-electric field extraction [11, 20, 33], enzyme-assisted extraction [20, 34, 35], ultrasound assisted extraction [32], microwave assisted extraction [36-38], supercritical fluid extraction [39-41], and
accelerated solvent extraction [32, 42, 43] have been utilized for the extraction of medicinal and biological components from herbal plants like ginseng [32, 44]. Below we will examine Soxhlet, ultrasound-assisted and supercritical fluid extraction which were utilized in this thesis.

1.3.1.1 **Soxhlet extraction method**

In Soxhlet extraction, as a standard and reference technique, the plant material is firstly placed in a cellulose thimble, and then placed in the Soxhlet distillation apparatus which is filled with condensed fresh solvent from a distillation flask after the solvent reaches the boiling point. After the solvent reaches the overflow level, a siphon starts to remove the solvent carrying the extracted material into the distillation flask. The solute is separated by means of distillation and remains in the flask and the solvent again passes back to the solid bed in the thimble. This procedure is repeated until the extraction is complete [32]. The main disadvantages of conventional Soxhlet extraction include: (1) the long extraction times; (2) a large amount of solvent is used; (3) agitation cannot be provided to intensify the process; (4) the large amount of solvent used needs an evaporation/concentration process; and (5) the possibility of thermal decomposition of materials as the extraction often happens at the boiling point of the solvent. However, this method is used for ginsenoside extraction usually as a reference method [2].

1.3.1.2 **Ultrasound-assisted extraction**

Ultrasound-assisted extraction is an inexpensive, simple and efficient alternative to conventional extraction techniques [32]. In ultrasound-assisted extraction, the extra external energy helps to increase mass transfer compared to conventional solvent extraction. The extraction mechanism by ultrasound consists of two physical phenomena. First, the diffusion across the cell wall and second the rinse of the cell contents after the walls are broken [20]. Natural plant characteristics such as the moisture content of the sample, milling degree, particle size and the solvent used in the extraction process are very important factors. Besides temperature, pressure, frequency and sonication time are important factors for the action of ultrasound. The main advantage of ultrasound in plant extraction is an increase of the extraction yield and faster kinetics. Ultrasound can also
decrease the operating temperature so it is a good technique for thermally unstable compounds. This method can also be used with a wide range of solvents for the extraction of natural compounds. However, the effects of ultrasound on extraction yield and kinetics may be related to the nature of the plant matrix. Wu et al. [45] evaluated the ultrasound-assisted extraction for separation of ginsenosides from both Panax ginseng and American ginseng and found that this extraction method is three times faster than traditional extraction methods such as Soxhlet. Moreover, many researchers have used the ultrasound-assisted extraction method as a comparison with other methods under different conditions [46, 47].

1.3.1.3 *Supercritical fluid extraction*

A supercritical fluid is a fluid which exists at a pressure higher than its critical pressure and a temperature greater than its critical temperature. A phase diagram of a supercritical fluid is illustrated in Figure 1.4. A liquid-like density of a supercritical fluid and its gas-like viscosity and higher diffusivity than liquid (as shown in Table 1.1) make supercritical fluids enabling solvents for extraction with sufficient solvation power and excellent mass transfer characteristics.

![Figure 1.4. Schematic of the phase diagram of a single substance [48].](image)
Table 1.1. Typical properties of gas, liquid, and supercritical fluids [48].

<table>
<thead>
<tr>
<th>Property</th>
<th>Units</th>
<th>Gas 1atm, 25°C</th>
<th>Liquid 1atm, 25°C</th>
<th>Supercritical fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density</td>
<td>$\rho$ (g cm$^{-3}$)</td>
<td>0.6-2×10$^{-3}$</td>
<td>0.6-1.6</td>
<td>0.2-0.5, 0.4-0.9</td>
</tr>
<tr>
<td>Diffusivity</td>
<td>$D_m$ (cm$^2$s$^{-1}$)</td>
<td>1-4×10$^{-1}$</td>
<td>0.2-2×10$^{-5}$</td>
<td>0.5-4×10$^{-3}$, 0.1-1×10$^{-3}$</td>
</tr>
<tr>
<td>Viscosity</td>
<td>$\mu$(g cm$^{-1}$s$^{-1}$)</td>
<td>1-3×10$^{-4}$</td>
<td>0.2-3×10$^{-2}$</td>
<td>1-3×10$^{-4}$, 3-9×10$^{-4}$</td>
</tr>
</tbody>
</table>

In Table 1.2, the critical properties of different materials used as supercritical solvents are shown. The most commonly used supercritical fluid is supercritical carbon dioxide, which is non-toxic, non-flammable, inexpensive, naturally abundant, chemically stable and has a convenient critical temperature (31.1°C) and pressure (73.8 bar) [49]. In addition, it is environmentally friendly (i.e. is not a volatile organic compound (VOC)), is a byproduct of fermentation, and has lower operation costs due to lower energy consumption.

Table 1.2. Materials useful as supercritical fluids [50].

<table>
<thead>
<tr>
<th>Materials</th>
<th>$T_c$ (K)</th>
<th>$P_c$ (bar)</th>
<th>$\rho_c$ (g cm$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene</td>
<td>282</td>
<td>50</td>
<td>0.22</td>
</tr>
<tr>
<td>Xenon</td>
<td>290</td>
<td>58</td>
<td>1.11</td>
</tr>
<tr>
<td>Fluoroform</td>
<td>299</td>
<td>49</td>
<td>0.53</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>304</td>
<td>74</td>
<td>0.47</td>
</tr>
<tr>
<td>Ethane</td>
<td>305</td>
<td>49</td>
<td>0.20</td>
</tr>
<tr>
<td>Nitrous oxide</td>
<td>310</td>
<td>72</td>
<td>0.45</td>
</tr>
<tr>
<td>Propane</td>
<td>370</td>
<td>43</td>
<td>0.22</td>
</tr>
<tr>
<td>Ammonia</td>
<td>406</td>
<td>114</td>
<td>0.23</td>
</tr>
<tr>
<td>Water</td>
<td>647</td>
<td>221</td>
<td>0.32</td>
</tr>
</tbody>
</table>
$T_c, P_c$ and $\rho_c$ are critical temperature, critical pressure and critical density, respectively.

Moreover, one of the most significant benefits of supercritical solvents is their solvation power, which can be tuned by changing the pressure and and/or temperature. Therefore, the solubility of the solute in the supercritical solvent can be decreased or increased by enhancing or reducing the pressure and/or temperature [49]. This property is advantageous in the selective separation and isolation of bioactive components from natural herbal plants. The main disadvantage of using pure supercritical CO$_2$ for separation and chromatography is the poor solubility of polar components in Sc-CO$_2$ [32]. To encounter this lack of solubility, modifiers such as methanol, ethanol and acetone are used to increase the solubility of materials in the supercritical CO$_2$.

The supercritical fluid extraction (SFE) technique is one of the most environmentally responsible and effective methods for the extraction of novel medicinal compounds from herbal plants. The application of a supercritical fluid for extraction applications was established with its discovery by Hannay and Hogarth (1879) [51]. However Zosel is also associated with the discovery of SFE by presenting a patent for decaffeination of coffee using SFE. Using this technique, selective extraction can be obtained since the solubility of a chemical in a supercritical fluid (SCF) can be manipulated by changing the pressure and/or temperature of the fluid. Moreover, the solute solubility in the SCF is enhanced with the density of the fluid that can be obtained at higher pressures. By reducing the SCF density during depressurization, the dissolved components can be recovered. Furthermore, the SCF diffusivity is one to two orders of magnitude higher than that of other liquids, thus increasing the mass transfer rate, and enhancing the extraction yield. Additionally, the low supercritical temperature of CO$_2$ makes it more useful for the extraction of thermally unstable compounds. Due to a minimum use of organic solvents in SFE, this method can be applied as an environmentally friendly extraction process compared to other conventional methods. By adding modifiers such as methanol to the SCF, its polarity can be increased for gaining more selective separation power. However, the economics and difficult operating conditions of the SFE method made some limitations in it's application. Many researchers have worked on the SFE method to extract plant materials, including lipids [52], and essential oils [53-55]. In addition,
extraction of biologically active components from plants has been carried out in previous studies [39-41]. In 2001, Wang et al. [2] evaluated a semi-continuous supercritical CO\textsubscript{2} process for ginsenoside extraction from Panax ginseng root in the presence of ethyl alcohol as the co-solvent. The authors implied that the crude oil extraction yield increased with pressure at constant temperature, and was only enhanced with temperature when the pressure was above 24.2 MPa. Wood et al. [31] demonstrated ginsenoside extraction from North American ginseng root by means of supercritical CO\textsubscript{2} extraction in the presence of methanol and DMSO as modifiers. The authors investigated the optimal conditions to obtain the maximum amount of target ginsenosides and found that the modifier had a significant effect on ginsenoside selectivity. Also, at constant pressure, temperature and modifier percentages, increasing flow rate was found to have no significant effect on the extraction yield. Furthermore, they found that in the presence of both methanol and DMSO as modifiers during supercritical CO\textsubscript{2} extraction, relatively large amounts of a novel mono-acetylated ginsenoside was detected providing potential for a higher bioactivity extract [31].

1.3.2 Comparison of different extraction methods for selected bioactive components from natural plant

In order to achieve the most effective and selective extract, several factors should be investigated including: the characteristics of the natural herbal plant and its bioactive components, the solvents used for extraction and the extraction method applied. It is important to know that a high yield of extract will not always guarantee a high yield of bioactive components in the extract. The extraction method should be carefully considered for separation of bioactive components which are sensitive to oxygen and heat. Moreover, the yield and quality of bioactive components should be taken into consideration when an extraction method is chosen [32].

1.4 Quantification and separation of bioactive components from natural herbal plants

There is an increasing demand for highly sensitive and selective analytical methods for the determination of ginseng extracts. The analytical methods can be used for either the
total saponin content determination or for analysis of the target compound, e.g. the analysis of a specific ginsenoside [19]. Also, some studies have evaluated the group-specific analysis, i.e. the analysis of a number (preferably all) ginsenosides. Others have used analytical methods for metabolite fingerprinting that exhibit a large number of primary and secondary metabolites from the extract, and contain (besides the ginsenosides) carbohydrates, lipids, amino acids, etc. [19].

1.4.1 Chromatographic techniques used in separation, qualitative and quantitative analysis

Various methods for the identification and quantification of ginsenosides in ginseng have been investigated. Ginsenoside fractionation has been obtained using both thin layer chromatography (TLC) and gas chromatography, although high performance liquid chromatography (HPLC) is the most widely used method. Due to HPLC's speed, sensitivity and compatibility with non-volatile polar materials; this technique is a precise method for saponin analysis. The chromatographic conditions of the HPLC method includes the use of, almost exclusively, a reversed-phase C18 column; a UV–Vis diode array detector, and a binary solvent system containing water (solvent A) and a polar organic solvent (solvent B). Reverse phase (RP) HPLC has become an important analytical tool for the separation and determination of ginsenosides with different detection systems, such as diode array detector (DAD), mass or tandem mass spectrometry. Evaporative light scattering (ELSD) and fluorescence were utilized with HPLC for ginsenosides detection [56]. Furthermore, new techniques such as near infrared spectroscopy (NIRS) and enzyme immunoassay (EIA) were recently used for the determination of ginsenosides [3]. Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry [22] are also valuable techniques used for interpretation of chemical structures of molecules, such as ginsenosides or other bioactive unknown compounds in ginseng extract [57-59]. Most of the literature available on ginsenoside extract identification has been analyzed by HPLC [56, 60, 61].
1.4.1.1 *Supercritical Fluid Chromatography (SFC)*

Supercritical fluid chromatography (SFC) is a relatively recent chromatographic technique used in the separation and identification of bioactive compounds from natural plants. SFC can be depicted as a chromatography system with similarities to both HPLC and GC, however the mobile phase is a supercritical fluid [49]. The first work on SFC is considered to be in 1962 by Kleper et al. The expansion of SFC during this period was slow compared to the rapid growth of HPLC which happened at about the same time [49]. The major increase in SFC use happened during the 1980s [49]. The number of publications on SFC represented in Figure 1.5 covers the academic literature from 1962-2012 [48].

![Figure 1.5. Number of publications on supercritical fluid chromatography [48].](image)

By using SFC, the amount of organic solvents commonly used in HPLC is reduced, thus this method can be considered a “green” technique. Moreover, in comparison to HPLC, separations can be faster by means of SFC because the diffusion of solutes in supercritical fluids is about ten times greater than that in liquids (and about three times less than gases). Compared with GC, capillary SFC can provide high resolution chromatography at much lower temperatures. This allows fast analysis of thermolabile
compounds [49]. Column technology and instrumentation research were very dynamic in the 1980s, leading to commercialization of SFC instruments. Normally, packed-columns are used for SFC with binary or ternary fluids as the mobile phase, composition programming, and a UV detector. Stationary phases used in packed-column SFC systems show higher surface areas to void volume ratios than capillaries and are much more retentive [48]. The SFC system is flexible due to its compatibility with multiple detectors such as UV, ELSD, and MS [48]. ELSD is a universal detector used in both HPLC and SFC usually for compounds with low UV absorption. In packed-column SFC, researchers have investigated organic modifiers in high percentage added to the CO$_2$ as mobile phase to increase the solvation power. Then, under regularly used chromatographic conditions such as 100-120 bar pressure and 40°C temperature, the mobile phase fluid is not in a supercritical state. However, SFC researchers use the phrase supercritical fluid chromatography despite the real state of the fluid employed [49]. In addition, research has demonstrated that preparative SFC can be utilized as a rapid isolation method in the separation and fractionation of unknown bioactive compounds for spectroscopic identification in pharmaceuticals and nutraceuticals [48, 49].

SFC has been reported in the literature as a useful tool in natural product applications and drug discovery. In drug analysis, the only study that used SFC to separate ginsenosides from ginseng extracts was reported by Li et al. [62]. They only used pure CO$_2$ as the mobile phase and pressure gradient from 100 to 350 bar and temperature of 300°C with a flame ionization detector to separate panaxadiol and panaxatriol saponins from ginseng. Although there is essentially no other studies on the SFC of ginseng itself, there is a large amount of literature regarding the use of SFC analysis for other natural products. Natural compounds such as resin acids, triterpenoid compounds (important in the cosmetics industry), glycolipids and phospholipids and carbohydrates, and ginko terpene trilactones were analyzed with SFC. Most of these research investigations used different columns, mobile phase modifiers and additives, and chromatographic conditions to obtain rapid high resolution isolation [63-68]. Buskov et al. [69] used SFC with methanol as a modifier for the determination and quantification of the different indol-3-ylmethyl, which has anticarcinogenic effects, including ascorbigens. They also investigated myrosinase catalysed degradation of 4-hydroxybenzylglucosinolate (sinalbin), which has anti-
nutritional and cancer preventive effects. Sinalbin occurs as an important glucosinolate in the seeds of *Sinapis alba* L., in different mustards and other food products [70]. In a separate study [71], they used SFC with CO$_2$-ethanol to isolate and identify furocoumarins, which are natural plant metabolites, mainly obtained from the Umbelliferae (celery, parsnip, parsley) and Rutaceae (citrus plants). The authors demonstrated that SFC identified the essential oil composition in a short time (around 10 min). Recently, Lesellier et al. [23] used SFC coupled with ELSD as a high resolution, fast analysis technique for separation of triperpenoids in natural plants that possess different biological activities such as anti-bacterial, anti-oxidant, anti-inflammatory, and anti-tumor. They tested various stationary phases, modifier percentage, from 10 to 30%; backpressure (from 120 to 180 bar) and temperature (from 15 to 25 °C) to improve the separation [23].

### 1.5 Delivery systems for herbal formulations

Over the past several years, significant progress has been directed for the improvement of novel delivery systems for bioactive compounds from plant extracts and natural health products such as nutraceuticals, probiotics, and traditional Chinese medicines [72]. These plant based formulations include tablets, capsules, teas, creams, oils, and liquids which consist of a mixture of generally low concentrations of biologically active compounds. These plant based extracts work more slowly and gently than pharmacologic drugs, and are less likely to cause serious side effects and toxicity. Recently, different formulations such as polymeric nanoparticles, nanocapsules, liposomes, phytosomes, nanoemulsions, microsphere, transferosomes, and ethosomes have been investigated for bioactive and plant extract delivery [73-75]. These novel systems have significant advantages over conventional delivery of plant actives and extracts although require basic development of pharmacological activity and sustained delivery.

#### 1.5.1 Microencapsulation techniques for bioactive extracts from herbal plants

One of the potential delivery carriers for bioactive compounds of plant extracts is polymer microspheres. There are various microencapsulation synthetic methods for
bioactive compounds. Choosing the best method depends on several factors such as polymer and drug characteristics, the site of action and therapy administration. The preparation process should be in a way that assures the chemical stability and biological activity of the encapsulated material. The encapsulation efficiency and the yield of the microspheres as well as the uniform size distribution of microspheres should be consistent with the targeted end-use. Furthermore, the encapsulation process should yield reproducible results and the release profile should be in agreement with the application requirements. Emulsion-solvent evaporation/extraction methods are widely used as an encapsulation method for bioactive compounds using either the so-called single emulsion or double emulsion methods [76-78]. Usually microspheres consist of either synthetic biodegradable polymers or natural polymers. Synthetic polymers used as a microsphere carrier usually contain poly-α-cyanoacrylate alkyl esters, polyvinyl alcohol, polylactic acid, and polylacticroglycolic acid, etc. Whereas the natural polymer as a microsphere carrier typically includes either proteins (albumin, gelatin and vegetable protein) or polysaccharides (cellulose, starch and its derivatives, alginate, chitin and chitosan, etc.).

So far, a series of plant active ingredients as shown in Table 1.3 has been made into microspheres. It should be mentioned that delivery of bioactive extracts may need other administration routes rather than oral such as parenteral formulations in order to prevent degradation in the digestive tract and first pass metabolism. Therefore, the injectable and/or transdermal biodegradable and biocompatible PLGA microparticles can potentially be used for bioactive and natural herbal products delivery.
Table 1.3. Microspheres encapsulated herbal formulations [73].

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Active ingredients</th>
<th>Applications</th>
<th>Biological activity</th>
<th>Administration route</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin–alginate–chitosan microcapsules</td>
<td>Rutin</td>
<td>Targeting into cardiovascular region</td>
<td>Cardiovascular diseases</td>
<td>In vitro</td>
</tr>
<tr>
<td>Zedoary oil microsphere</td>
<td>Zedoary oil</td>
<td>Sustained release and higher bioavailability</td>
<td>Hepatoprotective</td>
<td>Oral</td>
</tr>
<tr>
<td>CPT loaded microspheres</td>
<td>Camptothecin</td>
<td>Prolonged-release of camptothecin</td>
<td>Anticancer</td>
<td>Intraperitoneally and intravenously</td>
</tr>
<tr>
<td>Quercetin microspheres</td>
<td>Quercetin</td>
<td>Significantly decreases the dose size</td>
<td>Anticancer</td>
<td>In vitro</td>
</tr>
<tr>
<td>Cynara scolymus microspheres</td>
<td>Cynara scolymus extract</td>
<td>Controlled release of neutraceuticals</td>
<td>Nutritional supplement</td>
<td>Oral</td>
</tr>
</tbody>
</table>

1.5.1.1 PLGA as biodegradable delivery carrier

Polyester PLGA is a FDA-approved biodegradable polymer that is a copolymer of poly lactic acid (PLA) and poly glycolic acid (PGA). Due to its long clinical experience, high biocompatibility, appropriate degradation characteristics and possibilities for sustained drug delivery, PLGA is one of the most well-known biodegradable polymers that have been studied as delivery carriers for drugs, proteins, and peptides. Based on its physicochemical properties, PLGA can be formed into any shape and size, and can be used to encapsulate a variety of materials. Moreover, the overall physical properties of the PLGA-bioactive compound system can be adjusted by controlling parameters such as polymer molecular weight, ratio of lactide to glycolide and bioactive compound concentration in order to obtain a desired release behavior. As can be seen in Figure 1.6, biodegradation of PLGA occurs by hydrolysis of its ester linkages into oligomers and, finally monomers, lactic and glycolic acids. Lactic acid can be metabolized and subsequently eliminated from the body as carbon dioxide and water. Glycolic acid can also be excreted unaffected by the kidneys or can be metabolized and ultimately eliminated as carbon dioxide and water. Moreover, lactic acid is more hydrophobic than glycolic acid due to presence of methyl groups. As a result, when the ratio of lactide to glycolide is higher, PLGA shows more hydrophobicity and the degradation process lowers. Therefore, the release and degradation rates of encapsulated bioactive compounds
will be effected by changes in PLGA properties during the polymer biodegradation process [79].

![Figure 1.6](image)

**Figure 1.6.** Hydrolysis of poly lactic-co-glycolic acid [79].

Conventional microencapsulation methods generate microspheres with broad size distributions influences the kinetics of encapsulant release and limits their clinical application [80]. Recently, microfluidic devices have attracted attention due to the adjustability to create microspheres with controlled morphology [81]. Microfluidic devices utilize low-priced and convenient systems for the control of fluid flow [82]. A large number of microfluidic devices are engineered by poly(dimethyl) siloxane (PDMS), a fairly inexpensive and simply moldable elastomeric polymer. Other materials with greater solvent resistance such as glass and silicon have been used due to swelling and deformation of PDMS in the presence of strong organic solvents.

In general, the microsphere generation process relies on different parameters, including the flow rates of dispersed and continuous phases, the interfacial tension between the fluids and the wetting effect of the channel wall, of which the last two are difficult to adjust experimentally [83]. In order to avoid adherence to the channel walls, the hydrophilic inner wall of a microfluidic device are treated with silanization and siliconization to provide hydrophobic channels for water/oil (W/O) droplets. The surface wettability can also be changed by adding surfactants such as Span 80 and Tween 20 [84]. Moreover, surfactants are employed to avoid unwanted coalescence between microspheres by decreasing the surface tension between the continuous and disperse
phases. Two significant types of microfluidic devices used for monodisperse particles generation are T-junctions [85] and flow-focusing [82] which are dependent on the channel geometry for controlling monodisperse particles which can be shown in Figure 1.7.

![Diagram of T-junction and flow-focusing](image)

**Figure 1.7.** Droplet formation with different mechanisms: (a) Flow focusing; (b) T-junction [86].

In the T-junction device, the inlet channel including the dispersed phase vertically crosses the main channel which contains the continuous phase (Figure 1.7b). The droplet sizes can be "tuned" by adjusting the fluid flow rates, the channel widths, or by altering the relative viscosity between the two phases. In the flow-focusing geometry, the dispersed and continuous phases are carried to a narrow section in the device (Figure 1.7a). The sizes of the droplets are decreased by increasing the flow rates of the continuous phase. Different factors such as geometry of the device, the viscosity of the disperse and continuous phases, use of surfactants, and hydrophilicity or hydrophobicity of the channel surface have an effect on the size ranges of formed droplets [87].

Monodisperse microspheres made from biodegradable polymers have been generated by microfluidic devices widely used in drug delivery systems [80]. Xu et al. [82] used a flow-focusing microfluidic device for encapsulation of a model amphiphilic drug (bupivacaine) with a size of 11 µm and 41 µm. The size of the microspheres have been shown to be control with the disperse and continuous phases flow rate. Also, the kinetics of drug release show that the release profile from monodisperse microspheres is slower
than that from the conventional methods followed by a lower initial burst. Moreover, He et al. [80] modified a microfluidic chip for encapsulation of paclitaxel within poly(L-lactic acid) microspheres. They modified a PDMS surface by using poly(vinyl alcohol)/glycerol (PVA/Gly) solution immersion in order to produce monodisperse droplet within a range of different sizes depending on the relative flow rates. However, there is no study available regarding to the fabrication of monodisperse microspheres containing bioactive plant extract.

1.5.2 Release behavior and modeling approach

The rationale for using controlled release systems is to preserve active component concentration in the blood or in target tissues at a desired value for as long as possible. The concentration of the active component in the blood circulation varies over successive doses of most conventional formulations due to fast release right after administration (i.e. burst release effect). Therefore, the concentration of drug or herbal extract in blood rises quickly to a high value (peak) followed by a rapid decrease to a very low value (trough) due to component elimination (Figure 1.8). In fact, the therapeutic window is the range of concentration that causes an optimal therapeutic effect with concentrations above this range leading to side effects while concentrations below this range may result in decreased efficacy. The object of many controlled release technologies is to lessen the time during which the concentration of active component is above or below the therapeutic window, while lengthening the duration of the active component’s existence within the desired concentration range.
Figure 1.8. Release profile for conventional and controlled release within effective concentration region [88].

The release mechanism of the active pharmaceutical ingredient (API) has been used with different definitions. True release mechanisms refer to the way in which a drug is released while rate-controlling release mechanisms refer to the processes that control the drug release rate. There are four true release mechanisms including diffusion through water filled pores, diffusion through the polymer, osmotic pumping and erosion (i.e. no drug transport) as shown in Figure 1.9.

Figure 1.9. True release mechanisms: A) diffusion through water filled pores, B) diffusion through the polymer, C) osmotic pumping and D) erosion [89].
The complex picture of the different parameters that have an affect on the drug release from PLGA is shown in Figure 1.10. The rate-controlling release mechanisms including water absorption or swelling, dissolution, hydrolysis, erosion, drug-polymer interaction, etc. seem to be more informative in order to modify how a drug can release. However, due to the complexity of the delivery system it is not generally clear which of the processes is governing and which are rate-governing for API release.

**Figure 1.10.** The complex picture of various parameters that effect drug release from PLGA matrices [89].

Mathematical modeling is a very useful tool to predict controlled release in order to understand the mechanism of release. A diversity of mathematical models have been investigated for drug release. These can be categorized into two main groups: empirical/semi-empirical models and mechanistic mathematical models [89]. Empirical/semi-empirical models are simply mathematical explanations, and are not
based on any real chemical, physical or biological phenomenon. Several methods can be employed as empirical/semi-empirical models such as the Peppas, Higuchi, and Weibull models. These methods are useful, for instance, in representing diverse phases of the drug release, which are useful in product development [72, 80, 90]. However, mechanistic mathematical models are derived from real phenomena, such as diffusion, degradation and erosion, and are useful tools for a detailed understanding of the release process.

1.6 Scope of the research

Since ginsenosides can be selectively extracted from North American ginseng and actively targeted to various disease areas; different extractions, characterizations and bioassays should be used to test this hypothesis.

The following were identified as specific objectives of this project:

I. Ultrasound-assisted extraction of North American ginseng with DMSO and methanol as solvents.

II. Isolation and identification of 6”-O-acetylginsenoside Rb1 extracted from North American ginseng.

III. Study the Immunosuppressive and Anti-angiogenic effects of 6”-O-acetylginsenoside Rb1 extracted from North American ginseng.

IV. Analyze of North American ginseng extract using supercritical fluid chromatography.

V. Encapsulation of acetyl ginsenoside Rb1 within monodisperse PLGA microspheres using microfluidic technique.

VI. PLGA microspheres crosslinked gelatin hydrogels systems for acetyl ginsenoside Rb1 delivery.
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Chapter 2

2 Ultrasound Assisted Extraction of North American Ginseng and Isolation of 6”-O-acetylginsenoside Rb₁, Immunosuppressive and Anti-angiogenic Effects

Abstract

Extraction of medicinally-active components from natural health products has become an emerging source for drug discovery. Of particular interest for this work was the finding and testing of new ginsenosides from North American ginseng (Panax quinquefolius). In the present study, the yield and selectivity of ginsenoside extraction from North American ginseng root was determined by an ultrasonication method with methanol and DMSO as solvents and their aqueous mixtures. Quantitative analysis of individual ginsenosides from the extracts were measured by HPLC. These results showed that ultrasonication significantly enhanced the extraction efficiency, with the best efficiency found using 80% solvent (methanol, and DMSO) and 20% water. A large amount of 6”-O-acetylginsenoside Rb₁ was found using ultrasonic extraction of North American ginseng with DMSO aqueous solution. This new ginsenoside was well identified with MS, FTIR, and 1D (¹H and ¹³C) and 2D (gCOSY, gHSQC, and gHMBC) NMR. Subsequent bioassay experiments confirmed that acetyl ginsenoside Rb₁ demonstrated additional immunosuppressive activity towards inhibiting the production of nitric oxide (NO) and tumor necrosis factor (TNF)-α in lipopolysaccharide (LPS)-induced macrophage cells in a dose-dependent manner using murine macrophages. In addition, using a human umbilical vein cell line, a strong anti-angiogenic effect was found, similar to ginsenoside Rb₁. Due to this unique combination of pharmacological properties, this new ginsenoside is encouraging to be further explored for future development of novel drugs.
2.1 Introduction

North American ginseng (*Panax quinquefolius*) is a medicinal plant used in traditional herbal medicines grown for its roots, similar to Asian ginseng (*Panax ginseng C.A. Meyer*), which also has a long history as a medicinal herb [1]. Native North Americans used the roots both as a health food [2] and as a traditional medicine for relieving fever, stomach ailments, etc [3, 4]. Ginseng extracts have demonstrated significant anti-stress, anti-angiogenic, immunosuppressive, and anti-oxidant activity [5-10]. The components isolated from North American ginseng and characterized include ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids [11, 12]. The biological and pharmacological effects of ginseng are mainly attributed to the ginseng saponins, especially the class referred to as ginsenosides, with the structure of the six common ginsenosides displayed in Figure 2.1. In order to make use of these ginsenosides efficiently, it is necessary to separate them from ginseng root, identify them, and study their characteristic biological activities. Extraction is the first and most critical step in the separation of active ingredients from medicinal plants like ginseng. Not only are the ginseng extracts dependent on the genetic, growing and harvesting factors [13], they are also affected by the method of extraction and the utilized solvent [2, 14]. Different conventional extraction methods, such as Soxhlet extraction, reflux extraction, microwave-assisted extraction, and supercritical fluid extraction have been employed for extraction of ginsenosides from ginseng [3, 15, 16]. However, ginsenosides are thermally sensitive biologically active components they can be degraded to other ginsenosides at high temperatures [15, 17]. Degradation can be minimized by using the ultrasound assisted extraction technique. Ultrasonic extraction has been recognized for its simplicity and unique ability in the recovery and purification of active pharmaceutical ingredients from herbal plants providing higher efficiency, lower extraction times and lower solvent consumption [17-19]. Of additional benefit, the mechanical effects of acoustic cavitation from the inputted ultrasonic energy improves the diffusion of solvent into the plant tissue while also enhancing product release through the ruptured plant cell wall [14, 20, 21]. Wu et al. demonstrated that Asian and American ginseng extraction using ultrasonication with alcohols was approximately 3-times faster than conventional Soxhlet extraction [20]. Also, Liu et al. showed that higher content of malonyl ginsenosides, which are
thermally unstable and can degrade into corresponding neutral ginsenosides, were obtained using ultrasound assisted extraction with alcohols and water in comparison with other conventional techniques [16].

Different ginsenosides isolated from various types of ginseng have different pharmacological effects. By using an in vitro survival assay, ginsenosides Rb\textsubscript{1} and Rg\textsubscript{1} were found to protect spinal neurons from excitotoxicity induced by glutamate and kainic acid and oxidative stress induced by hydrogen peroxide, making them efficient neuroprotective agents for spinal cord neurons, while compound Re did not exhibit such activity [22]. It has been reported that isolated ginsenosides Rc and Rg\textsubscript{1} showed an immunomodulatory effect on cellular immune response by stimulating T cell proliferation as well as NK cell activity [23]. Ginsenosides Rd, Re and Rg\textsubscript{1} affected the immune system by enhancing specific-antibody responses [23]. Recently, it has been shown that ginsenoside Rg\textsubscript{1} isolated from Korean ginseng partially supported immunomodulatory function and improved bone marrow suppression [24]. As the major ginsenoside component in North American ginseng, ginsenoside Rb\textsubscript{1} demonstrated inhibition of proinflammatory cytokine responses for both lipopolysaccharide (LPS)-induced interleukin (IL)-6 and tumor necrosis factor (TNF)-α production in mice, but did not display the same patterns of inhibition as cell culture data [25, 26]. Ginsenoside Rb\textsubscript{1} also exhibited anti-angiogenic activity in-vivo [9], inhibiting the growth of new blood vessels and thus being used to treat cancer and age-related macular degeneration, etc. As an intestinal bacterial metabolite of ginsenoside Rb\textsubscript{1}, compound K (20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol, CK) has demonstrated immunosuppressive activity [10]. This activity could be utilized to prevent the body from rejecting an organ transplant, treating graft-versus-host disease after a bone marrow transplant, or for the treatment of auto-immune diseases. The other ginsenoside possessing this activity is ginsenoside Rh\textsubscript{2}, a biotransformation product of ginsenosides Rg\textsubscript{1} and Rd [27]. Qi et al. reported that acetylation of protopanaxadiol ginsenosides could increase their anti-cancer activity [11]. Acetyl ginsenosides are expected to have higher bioactivity as the acetyl ginsenosides are more lipophilic and consequently may have a higher cellular uptake [28]. Gebhardt et al. acetylated ginsenoside Rb\textsubscript{1} by means of lipase B of Candida antarctica, obtaining a mixture of 6″,6‴″-di-O-acetylginensoside Rb\textsubscript{1} (33%), 6‴″-O-
acetylginsenoside Rb₁ (60%) and 6”-O-acetylginsenoside Rb₁ (7%) [28]. Wood et al. demonstrated that a significant amount of mono-acetate ginsenoside Rb₁ could be obtained using supercritical CO₂ extraction with DMSO as co-solvent. However, they did not distinguish the type of mono-acetate compound [3]. Among the known ginsenosides, acetyl ginsenosides are normally reported as very minor components of the total ginsenoside content [29].

![Structure of common Panax quiquefolius ginsenosides](image)

<table>
<thead>
<tr>
<th>Ginsenoside</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb₁</td>
<td>-glc²-glc</td>
<td>-glc⁸-glc</td>
<td>-H</td>
</tr>
<tr>
<td>Rb₂</td>
<td>-glc²-glc</td>
<td>-glc⁹-ara (p)</td>
<td>-H</td>
</tr>
<tr>
<td>Rc</td>
<td>-glc²-glc</td>
<td>-glc⁹-ara (f)</td>
<td>-H</td>
</tr>
<tr>
<td>Rd</td>
<td>-glc²-glc</td>
<td>-glc</td>
<td>-H</td>
</tr>
<tr>
<td>Re</td>
<td>-H</td>
<td>-glc</td>
<td>-O-glc²-rha</td>
</tr>
<tr>
<td>Rg₁</td>
<td>-H</td>
<td>-glc</td>
<td>-O-glc</td>
</tr>
</tbody>
</table>

Glc=glucose, ara (p)=alpha-L arabinopyranose, ara (f)=alpha-L arabinofuranose, rha= rhamnose

**Figure 2.1.** Structure of common *Panax quiquefolius* ginsenosides [3].
In the present study, ultrasonication extraction of ginseng was investigated using both pure solvents (methanol, DMSO) and their aqueous mixtures. The isolated high concentration of ginsenoside from North American ginseng extract is characterized for the first time to identify its molecular structure as 6”-O-acetylginsenoside Rb₁, using mass spectroscopy, Fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR). Also, the immunosuppressive and anti-angiogenic effects of the extracts and the isolated new ginsenoside were evaluated in-vitro using the murine macrophage and a vascular endothelial cell line, respectively, to determine the biological activity for its potential immunosuppressive and anti-angiogenic applications.

2.2 Materials and methods

2.2.1 Materials

Roots of *Panax quinquefolius* came from Delhi, Ontario and collected by Dr. Dan Brown who is the leader for the Plant Biotechnology/Agriculture Platform Technology Group. A reference plant was deposited in Western University, Department of Biology Herbarium, entry number 52100. The dried root samples were ground in liquid nitrogen. Solvents (ethanol, methanol, acetonitrile, DMSO) of HPLC grade (>99.9%) were obtained from Sigma-Aldrich Canada. Purified water was produced from a Milli-Q water purification system (18.2 MΩ·cm resistivity, Barnstead EasyPureII, Thermo Scientific, USA). Ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂ and Rd were purchased from Indofine Chemical Company (Somerville, New Jersey, USA) and used as standards for HPLC analysis. Stock solutions were prepared in methanol at a concentration of 1000 µg/mL. Working standard solutions were prepared in methanol in the range 25-150 µg/mL for ginsenosides and then stored at -20°C. RAW 264.7 (ATCC TIB 67) murine macrophage cell lines and human umbilical vein cell lines (EA.hy926) were provided by Dr. Jeff Dixon (Department of Physiology and Pharmacology, Western University, Canada). BD OptEIA ELISA kits tumour necrosis factor-α were provided by BD Biosciences (Bedford, MA, USA). LPS (Lipopolysaccharides) from *Escherichia coli* and Griess reagent were purchased from Sigma-Aldrich (USA). Trypsin-EDTA was supplied by
Cellgro (Mediatech, Inc, Manassas, VA). Standard matrigel was provided by BD Bioscience (Bedford, MA, USA). Cell culture medium and reagents were purchased from Gibco laboratories (USA).

2.2.2 General experimental procedures

Ginsenoside concentrations were determined by a Shimadzu HPLC (Shimadzu Corporation) with an LC-20AB gradient pump, an SIL-20AC autosampler, and an SPD-20A UV-Vis detector based on calibration with the ginsenoside standards. The column was an end-capped Luna 3u PFP (2) (Phenomenex Inc, USA) 100A of 150 × 4.60 mm with 5µm packing, kept at room temperature. The mobile phase, modified from [30], was a binary gradient of acetonitrile (A) and HPLC grade filtered water (B) at a constant composition of 21% A from 0–20 min followed by a linear gradient to 42% from 20–40 min. The column was flushed and equilibrated after each analysis. The flow rate was 1 mL/min, and the ginsenosides peaks were detected by a UV detector at 203 nm. For the analysis, 10 µL of the sample solution was injected from the HPLC vial onto the column. Unknown ginsenoside was collected manually using a Shimadzu HPLC (Shimadzu Corporation) with the same system as the analytical HPLC analysis using a semi-preparative Atlantis T3 Waters column (250 × 10 mm with 5µm packing) kept at room temperature with purity of >99% (Appendix A). A full loop injection was employed (100 µL) with the flow rate set at 4 mL/min and the unknown ginsenoside peak was detected by a UV detector at 203 nm. Data were collected using a Shimadzu class VP 7.4 SP3 HPLC system software. All samples were filtered through 13 mm, 0.2 µm PTFE filters (Canadian Life Science, Peterborough, ON) attached to disposable 3 mL syringes prior to injection. In the case of semi-preparative analysis, the mobile phase was a binary gradient of acetonitrile (A) and HPLC grade filtered water (B) at a constant composition of 35% (A) for 2 min followed by a linear gradient to 50% from 2–30 min. The column was flushed and equilibrated for 35 min after each analysis. Fractions obtained were evaporated under vacuum to isolate the unknown extract. Semi-preparative HPLC assays were performed in duplicate. Molecular weight identification of ginsenoside Rb₁ and unknown ginsenoside were obtained by mass spectrometry on a QTof Micro mass spectrometer (Waters) equipped with a Z-spray source and run in negative ion mode.
Mass survey data range between 200-1800 amu was recorded with a 3.2 kV capillary voltage and a 50 V cone voltage. Melting points were measured with a DSC Q200 (TA Instrument, New Castle, Delaware, USA). The scanning rate was 10 °C /min, with a scanning temperature range of 25–250 °C. FTIR-ATR (Nicolet 6700, Thermo Scientific) was used to investigate the chemical structure of the unknown ginsenoside in the range of 500-4000 cm\(^{-1}\). Moreover, NMR spectra were measured using either a Varian INOVA 600 or a Varian INOVA 400 spectrometer at 25°C. Pyridine-d5 was used as the solvent and chemical shifts were referenced to tetramethyl silane (TMS; 0.0 ppm).

### 2.2.3 Extraction and isolation

Ginseng samples (100 mg) were placed in 20 mL sample vials and mixed with extracting solvent (DMSO and methanol and their aqueous solution), with the mixture sonicated for 30 min in a sonicating bath (VWR scientific products model 50D). This bath has a frequency of 35 kHz with the extraction temperature set initially at 25°C, with a maximum rise to no higher than 33°C. After extraction, the samples were centrifuged at 4500 rpm for 10 min. The supernatant was subsequently collected with the residue washed with solvent and the procedure repeated twice, resulting in three collected samples which were then combined and evaporated to dryness under vacuum at 40°C. For each sample, three to four replicates were performed for statistical validation. DMSO being a high boiling point solvent (\(T_b=189°C\) versus 65°C for methanol) required vacuum drying in the presence of a lower boiling co-solvent, ethanol [31]. The ginseng extract was then characterized using analytical HPLC with an unknown component obtained which showed a different retention volume in the HPLC chromatogram from those of the known ginsenosides including \(\text{Rg}_1\), \(\text{Re}\), \(\text{Rb}_1\), \(\text{Rc}\), \(\text{Rb}_2\) and \(\text{Rd}\) [3]. The unknown ginsenoside was collected using a semi-preparative HPLC column.

Moreover, the conventional extraction method of boiling and refluxing the solvent in a Soxhlet extractor for 10 h was used for comparison with the ultrasonic extraction method. 1g ginseng powder was placed in a cellulose thimble and 30 mL methanol as a solvent was allowed to come to its boiling point.
2.2.4 Immunosuppressive effect of North American ginseng extracts

The mouse macrophage cell line RAW 264.7 was cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 25 mM HEPES, 2 mM Glutamine, 100 IU penicillin/mL and streptomycin 100 µg/mL. The cells were kept at 37°C in a humidified incubator with 5% CO₂ and seeded in 96-well tissue culture plates at a density of $1.5 \times 10^5$ cells per well. The inhibitory effect of ginseng extracts inflammatory mediators profile in vitro was evaluated as previously described [32]. The LPS-stimulatory response was measured by incubating macrophages with 1 µg/mL lipopolysaccharide (LPS) for 24. To examine the inhibitory effects of ginseng extracts, 5-150 µg/mL of 80% DMSO extract and 80% methanol extract as well as ginsenoside Rb₁ and acetyl ginsenoside Rb₁ were added to the macrophages with 2 hr prior to the addition of LPS. In this study, dexamethasone (DEX) was used as a positive control for the suppression of LPS-induced stimulation of macrophages. Dexamethasone which is used as an anti-inflammatory agent, has been shown to inhibit the production of NO and TNF-α in cells stimulated with LPS [10, 33-36]. The 24 hr cytokine production induced by LPS was determined by measuring NO and TNF-α levels in the culture medium. TNF-α concentrations in supernatants from cultured cells were analyzed with ELISA. Samples were assessed with mouse cytokine-specific BD OptEIA ELISA kits (BD Biosciences, USA) according to the manufacturer's protocol. Sample nitrite concentrations in culture medium were determined by means of Griess reagent (0.5% sulfanilic acid, 0.002% N-1-naphthyl-ethylenediamine dihydrochloride, 14% glacial acetic acid). Culture supernatant of each sample and the Griess reagent (50 µL of each) were added to a 96 well plate. The measurements of absorbance were done at 550 nm wavelength using a Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Finland) with SkanIt software (version 2.4.2, Thermo Fisher Scientific, Finland). Nitrite concentrations were calculated from a sodium nitrite standards calibration curve.
2.2.5 Anti-angiogenesis effect of North American ginseng extracts

Human umbilical vein cell lines (EA.hy926) were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with ECGS (20 mg/mL), 10% fetal bovine serum (FBS), 1% antibiotic, and 1 mL (5X) per 50 mL of medium Hat media supplement. The cells were grown at 37°C in humidified air with 5% CO₂ incubator. When the cell cultures were 80%–90% confluent, cells were harvested following trypsin treatment and the released cells were resuspended to provide cell densities required for tube formation assays. To examine the anti-angiogenic activity of individual ginsenosides in vitro, human umbilical vein cell lines (EA.hy926) were seeded in 96 well tissue culture plates coated with matrigel at a density of $4.5 \times 10^4$ cells per well and incubated with medium and various concentrations of ginsenoside Rb₁ and acetyl ginsenoside Rb₁ (0.5-50 ppm) at 37°C for 20 hr. The images of tube formation were captured by an inverted microscope (Nikon TMS, Japan) using a 40x objective. Images from a total of five microscopic fields per well were analyzed by Motic Image Plus 2.0 software (Motic Instruments Inc., Richmond, Canada). The anti-angiogenic activities were estimated by counting the branch points of the formed tubes and the average numbers of branch points were calculated. Each experiment was repeated in triplicate.

2.2.6 Statistical analysis

All experiments were performed at least three times with statistical analysis accomplished using GraphPad prism 4.0a Software (GraphPad Software Inc., USA). Data was tested based on mean ± standard deviation (SD) of three sets of experiments. Data sets with multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Tukey’s and Dunnett’s post-hoc test. p < 0.05 was considered to be statistically significant.
2.3 Results and Discussion

2.3.1 Identification of ginsenosides in North American ginseng extract

North American ginsenosides extracted with the ultrasonic extraction method were analyzed by HPLC as shown in Figure 2.2, with excellent resolution of ginsenosides obtained (for Rb₁, Rb₂, Rc, Rd, Re and Rg₁). By comparing the retention times of the ginsenosides in the extracts with known standards, peaks were identified as provided in Table 2.1. As shown in Table 2.1, by using the ultrasonic extraction method, the highest yield of ginsenosides was obtained using DMSO as solvent, giving a total ginsenoside yield of almost 10 (% w/w). The results are comparable to literature where sonication extraction of powdered American and Asian ginseng roots and extracts in methanol showed a similar range of ginsenoside content (4.8 – 10.93 (%w/w)) [21]. Also, the ginsenoside content (7.29 % w/w) obtained using the 10 h methanol Soxhlet extraction is consistent with previous studies on ginsenosides extraction from ginseng roots [3]. A substantial peak was detected in the ginsenoside region using this ambient temperature ultrasonic approach that did not correspond to the ginsenoside standards (see Figure 2.2a&b at 29 min), particularly when using DMSO as the extraction solvent. This peak concentration was calculated as an average of all calibration curves studied as provided in Table 2.1.
Figure 2.2. HPLC analysis of North American ginsenoside extract: a) DMSO ultrasonic extraction; b) Methanol ultrasonic extraction.
Table 2.1. Ginsenosides content from extraction of North American ginseng.

<table>
<thead>
<tr>
<th>Method</th>
<th>Solvent</th>
<th>Rb₁</th>
<th>Rb₂</th>
<th>Rc</th>
<th>Rd</th>
<th>Re</th>
<th>Rg₁</th>
<th>Acetyl Rb₁</th>
<th>Ginsenoside content</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 h Soxhlet extraction</td>
<td>Methanol</td>
<td>3.78</td>
<td>0.14</td>
<td>0.73</td>
<td>1.06</td>
<td>1.33</td>
<td>0.25</td>
<td>0</td>
<td>7.29 (1.27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.83)</td>
<td>(11.18)</td>
<td>(4.11)</td>
<td>(2.79)</td>
<td>(3.36)</td>
<td>(10.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultrasonic extraction</td>
<td>DMSO</td>
<td>3.58</td>
<td>0.12</td>
<td>0.92</td>
<td>1.11</td>
<td>2.32</td>
<td>0.28</td>
<td>2.22</td>
<td>10.55 (2.07)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.09)</td>
<td>(32.99)</td>
<td>(7.27)</td>
<td>(3.47)</td>
<td>(33.33)</td>
<td>(27.15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>4.43</td>
<td>0.15</td>
<td>0.85</td>
<td>1.05</td>
<td>1.43</td>
<td>0.26</td>
<td>0.17</td>
<td>8.34 (0.61)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9.04)</td>
<td>(13.02)</td>
<td>(7.67)</td>
<td>(2.60)</td>
<td>(5.97)</td>
<td>(16.01)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values in (%, w/w). All runs are average of at least three repeats. Values shown in parentheses are percent relative standard deviations.

For determination of the chemical identity of this peak, MS was performed in negative ion mode. The MS spectrum at 29 min showed a peak at 1149.9 m/z which was subjected to CID fragmentation, with the obtained MS/MS spectrum shown in Figure 2.3. This MS/MS spectrum illustrates the [M−H]− ion at 1149.9 m/z and its major fragment ions. The m/z of the peak and its fragmentation pattern correspond to the previously observed peak from LC-MS/MS of ginseng extracted using supercritical CO₂ + DMSO [3]. In the MS spectrum of Figure 2.3, the facial loss of an acetyl group is evidenced by the major peak at 1107.97 m/z, with the sequential loss of an acetyl-hexose and hexose group also observed (to generate peaks at 945.84, 783.76, 621.63 and 459.4 amu respectively). The acetyl ginsenoside Rc and acetyl ginsenoside Rd were also determined in the LC-MS/MS of ultrasonic extracts although the quantity of these ginsenosides was below the detection limit of the UV detector used for HPLC analysis (Figure 2.4).
Figure 2.3. Mass spectrum of peak at m/z 1149.95 for [M−H]− ions of acetyl ginsenoside Rb₁ eluted at 29 min from ultrasonic extraction with DMSO.
Figure 2.4. Mass spectrum of peak at a) m/z 1119.93 for [M−H]− ions of acetyl ginsenoside Rc eluted at 25 min and b) m/z 987.86 for [M−H]− ions of acetyl ginsenoside Rd eluted at 26 min from ultrasonic extraction with DMSO.

It can be observed that DMSO was found to be the best solvent for both Re and the unstable acetyl ginsenoside Rb1, with acetyl ginsenoside Rb1 extraction efficiency more than 10 times higher compared to methanol. As well, more efficient extraction of acetyl ginsenoside Rb1 was found with DMSO ultrasonication 2.22 (%, w/w), compared to around 1.00 (%, w/w) using supercritical fluid extraction [3]. Acetyl ginsenosides are typically thought to make up a very small amount of conventional ginseng extracts; they are believed to be thermally unstable and may convert to neutral ginsenosides under normal extraction conditions e.g. hot water extraction which would be used in conventional tea preparation [28]. Our results showed that the Soxhlet extraction method with methanol yielded no significant quantity of acetyl ginsenoside Rb1 which is in good agreement with literature [3]. The ability of ultrasound assisted extraction of ginseng employed at ambient temperatures in this study (25-33°C) with DMSO as a solvent yielded high amounts of acetyl Rb1 in less than 2 hr. This is a major advantage over Gebhardt et al's method of enzymatic synthesis which requires pure Rb1 to be converted into mono- and di- acetyl ginsenosides Rb1 in a 4 hr reaction [28].
2.3.2 Identification of acetyl ginsenoside Rb\textsubscript{1} in North American ginseng extract

The root of North American ginseng was extracted using DMSO or methanol. These extracts were subjected to HPLC analysis to yield six common ginsenosides (Figure 2.1) as well as one unknown ginsenoside. In order to chemically identify this unknown component, mass spectroscopy, FTIR, and NMR were used for characterization of the isolated sample. Figure 2.5 compares the mass spectrum of the unknown fraction with that of the commercial ginsenoside, Rb\textsubscript{1}. While the ginsenoside Rb\textsubscript{1} showed a significant peak at \textit{m/z} 1107.72, the unknown fraction demonstrated a major peak at \textit{m/z} 1149.66. The mass difference of \textit{m/z} 41.94 may be attributed an acetyl group attached to the ginsenoside Rb\textsubscript{1}. As previously reported \cite{37, 38} in the negative ion mass spectra, the ionization of ginsenosides resulted in strong deprotonated molecular ions [M−H]\textsuperscript{−} accompanied by their corresponding adduct ions [M+Cl]\textsuperscript{−}, which might occur from contamination of the ion source. Hence, the other two major peaks at \textit{m/z} 1143.73 for ginsenoside Rb\textsubscript{1} and \textit{m/z} 1185.61 for the unknown sample might be attributed to the Figure adduct ions [M+Cl]\textsuperscript{−} with a higher \textit{m/z} of 36 than the [M−H]\textsuperscript{−} ions.

![Mass spectra](https://via.placeholder.com/150)

**Figure 2.5.** Mass spectra of: a) ginsenoside Rb\textsubscript{1} and b) the isolated unknown fraction obtained by the semi preparative HPLC of North American ginseng extract analysed by TOF-MS-ES.
The unknown component was then examined with FTIR. Figure 2.6 compares the FTIR spectra of ginsenoside Rb₁, DMSO extract, and the isolated unknown fraction. According to the literature [39-41] major peaks around 3300 cm⁻¹ are due to O-H vibrations. The peaks around 2900 cm⁻¹ are assigned to C-H stretching and the peaks at 1650 cm⁻¹ are attributed to the C=C alkene stretching. The peaks at ~1010 cm⁻¹ are attributed to the vibrations of C-C-O or C-C-OH in ginsenosides. The unknown fraction (Figure 2.6c) showed all the peaks possessed by the commercial ginsenoside Rb₁ (Figure 2.6a), with additional peaks at 1730 and 1248 cm⁻¹, which are attributable to the C=O and C-O stretching vibrations of an acetic ester group, respectively. These two additional peaks were also observed in the spectrum of the DMSO extract (Figure 2.6b) which contained the unknown component, but their height was much lower than that of the isolated pure unknown component due to its low concentration in the extract. Along with the mass spectroscopic result, the FTIR result confirmed the identity of the unknown component to be acetylginsenoside Rb₁.
As a widely-recognized tool in the determination of molecular identity and structure for natural products, NMR was employed in the characterization of the isolated acetylginsenoside Rb\(_1\). According to the 1D (\(^1\)H and \(^{13}\)C) and 2D (gCOSY, gHSQC, and gHMBC) NMR measurements, the isolated acetylginsenoside Rb\(_1\) was found to be \(\delta''\)-O-acetylginsenoside Rb\(_1\), with its molecular structure provided in Figure 2.7. In order to obtain complete structural information about the acetylginsenoside Rb\(_1\), \(^1\)H and \(^{13}\)C NMR spectra of the acetylginsenoside Rb\(_1\) were measured and compared with those of a commercial ginsenoside Rb\(_1\) sample. A comparison of the \(^1\)H NMR spectra of the extracted acetylginsenoside Rb\(_1\) and the commercial ginsenoside Rb\(_1\) is shown in Figure 2.8.
Figure 2.7. Proposed molecular structure of the extracted 6’’-O-acetylginsenoside Rb₁.
Figure 2.8. $^1$H NMR spectra of commercial ginsenoside Rb$_1$ (bottom, blue) and the extracted acetylginsenoside Rb$_1$ (top, red) in pyridine-$d_5$.

A few $^1$H peaks of the acetylginsenoside Rb$_1$ are different from those of the commercial ginsenoside Rb$_1$, including the C1” proton shifting from 5.38 to 5.33 ppm, the C3” proton shifting from 4.35 to 4.29 ppm, the C4” proton shifting from 4.36 to 4.15 ppm, the C5” proton shifting from 3.95 to 4.03 ppm, and the C6” protons shifting from 4.36 to 4.82, and 4.50 to 4.96 ppm. All these differences occur in the 2’-O-glucose unit, indicating that the acetyl group is attached to the C6” position. Moreover, a significant singlet at 2.05 ppm (8”) in the spectrum of the acetylginsenoside Rb$_1$ confirmed the presence of an acetyl group in the sample, which was absent in the spectrum of ginsenoside Rb$_1$. Figure 2.9 compares the $^{13}$C NMR spectra of the extracted acetylginsenoside Rb$_1$ and the commercial ginsenoside Rb$_1$. 
Figure 2.9. $^{13}$C NMR spectra of commercial ginsenoside Rb$_1$ (bottom, blue) and the extracted acetylginsenoside Rb$_1$ (top, red) in pyridine-$d_5$.

In addition to some minor differences, the major differences were found to be the C2’, C4”, and C6” carbon peaks shifting from 83.9 to 84.7 ppm, from 72.0 to 71.4 ppm, and from 63.1 to 65.1 ppm, respectively. With a close look at the spectra, the C5” carbon peak shifted from 78.7 to 75.7 ppm. Along with the additional acetyl peaks at 171.4 (7”) and 21.3 ppm (8”) in the spectrum of the extracted acetylginsenoside Rb$_1$, these differences in the $^{13}$C peaks also provided evidence of the attachment of an acetyl group to the C6”.

In addition to the 1D NMR measurements, the acetylginsenoside Rb$_1$ was also examined with 2D (gCOSY, gHSQC, and gHMBC) NMR. The gHSQC and gHMBC spectra of the extracted acetylginsenoside Rb$_1$ are displayed in Figure 2.10. In the gHSQC spectrum, the correlation peaks of C1”, C2”, C3”, C4”, C5”, C6”, and 8” are located at H5.33-C106.6, H4.13-C77.1, H4.29-C78.9, H4.15-C71.4, H4.03-C75.7, H4.82&4.96-C65.1, and H2.05-C21.3, respectively. In the gHMBC spectrum of the extracted acetylginsenoside Rb$_1$, the correlation peaks of H1”-C2’, H2”-C1”, and H8”-C7” can be clearly identified at H5.33-C84.7, H4.13-C106.6, and H2.05-C171.4, respectively. All these results confirmed the
molecular structure of 6''-O-acetylginsenoside Rb₁, with the \(^1\text{H}\) and \(^{13}\text{C}\) peaks of the commercial ginsenoside Rb₁ and the extracted 6''-O-acetylginsenoside Rb₁ being summarized in Table 2.2.

**Figure 2.10.** gHSQC (top) and gHMBC (bottom) NMR spectra of the extracted acetylginsenoside Rb₁ in pyridine-\(d_5\).
Table 2.2. Assignment of $^1$H and $^{13}$C NMR peaks of ginsenoside Rb$_1$ and 6"-O-acetylginsenoside Rb$_1$ (in pyridine-$d_5$).

<table>
<thead>
<tr>
<th>Position</th>
<th>Commercial Ginsenoside Rb$_1$</th>
<th>Extracted 6&quot;-O-Acetylginsenoside Rb$_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^1$H Chemical Shift (ppm)</td>
<td>$^{13}$C Chemical Shift (ppm)</td>
</tr>
<tr>
<td>1</td>
<td>0.74, 1.57</td>
<td>39.6</td>
</tr>
<tr>
<td>2</td>
<td>1.85, 2.21</td>
<td>27.2</td>
</tr>
<tr>
<td>3</td>
<td>3.28 (dd, J=11.52, 4.1 Hz)</td>
<td>89.3</td>
</tr>
<tr>
<td>4</td>
<td>0.67</td>
<td>56.8</td>
</tr>
<tr>
<td>5</td>
<td>1.54</td>
<td>18.8</td>
</tr>
<tr>
<td>6</td>
<td>1.21, 1.47</td>
<td>35.5</td>
</tr>
<tr>
<td>7</td>
<td>1.37</td>
<td>50.6</td>
</tr>
<tr>
<td>8</td>
<td>40.4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.97</td>
<td>31.2</td>
</tr>
<tr>
<td>10</td>
<td>4.20</td>
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<td>14</td>
<td>1.02, 1.57</td>
<td>31.1</td>
</tr>
<tr>
<td>15</td>
<td>1.37, 1.85</td>
<td>27.0</td>
</tr>
<tr>
<td>16</td>
<td>2.60</td>
<td>52.0</td>
</tr>
<tr>
<td>17</td>
<td>0.98</td>
<td>16.4</td>
</tr>
<tr>
<td>18</td>
<td>0.83</td>
<td>16.6</td>
</tr>
<tr>
<td>19</td>
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</tr>
<tr>
<td>20</td>
<td>83.8</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>1.67</td>
<td>22.8</td>
</tr>
<tr>
<td>22</td>
<td>1.85, 2.41</td>
<td>36.6</td>
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<tr>
<td>23</td>
<td>2.41, 2.60</td>
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<td>5.33</td>
<td>126.3</td>
</tr>
<tr>
<td>25</td>
<td>131.4</td>
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</tr>
<tr>
<td>26</td>
<td>1.62</td>
<td>26.2</td>
</tr>
<tr>
<td>27</td>
<td>1.67</td>
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<tr>
<td>29</td>
<td>1.12</td>
<td>17.0</td>
</tr>
<tr>
<td>30</td>
<td>0.98</td>
<td>17.8</td>
</tr>
<tr>
<td>3-O-glc-1’</td>
<td>4.93 (d, J=7.42 Hz)</td>
<td>105.5</td>
</tr>
<tr>
<td>2’</td>
<td>4.26</td>
<td>83.9</td>
</tr>
<tr>
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<td>5’</td>
<td>3.95</td>
<td>78.3</td>
</tr>
<tr>
<td>6’</td>
<td>4.36, 4.58</td>
<td>63.3</td>
</tr>
<tr>
<td>2’-O-glc-1”</td>
<td>5.38 (d, J=7.42 Hz)</td>
<td>106.5</td>
</tr>
<tr>
<td>2’’</td>
<td>4.14</td>
<td>77.5</td>
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<td>78.7</td>
</tr>
<tr>
<td>6’’</td>
<td>4.36, 4.50</td>
<td>63.1</td>
</tr>
<tr>
<td>7’’</td>
<td>171.4</td>
<td></td>
</tr>
</tbody>
</table>
Previously, Gebhardt et al. [28] synthesized 6”-O-acetyl ginsenoside Rb₁ by means of an enzyme-catalyzed reaction of ginsenoside Rb₁. However, 6”-O-acetyl ginsenoside Rb₁ was the minor product in comparison to the major products of 6”’,6”’-di-O-acetyl ginsenoside Rb₁ and 6”,6”’-di-O-acyl ginsenoside Rb₁, indicating difficulties in obtaining a large amount of 6”-O-acetyl ginsenoside Rb₁ using this strategy. We obtained pure 6”-O-acetyl ginsenoside Rb₁ using ultrasonic extraction of North American ginseng root without the need of separation of the mono- and di- acetyl ginsenoside Rb₁. This method should be considered as an efficient and green approach, and thus preferred for obtaining 6”-O-acetyl ginsenoside Rb₁.

### 2.3.3 Effect of solvents on the yields of ginsenosides

It is known that different solvents will yield varying amounts and compositions of extract. The effect of extracting solvents can be seen in Table 2.1. The results show that DMSO is a good solvent for extraction of ginsenosides Rg₁, Re, Rc, Rd, and specially acetyl Rb₁ whereas methanol is a good choice for extraction of Rb₁ and Rb₂. According to the literature [32], to study the biological activity of ginseng extracts, usually 70-75% \( \nu_{\text{solvent}}/\nu_{\text{water}} \) has been used for ginseng extraction. Therefore, for studying the effect of adding water to the extraction solvent, a series of different solvent/water ratios (60-100% \( \nu_{\text{solvent}}/\nu_{\text{water}} \)) were examined on the extraction of ginsenosides from North American ginseng. Figure 2.11 compares the different solvent:water ratios for ginsenoside
extraction with DMSO and methanol solvents. The results show that using aqueous solvent mixtures of both methanol and DMSO solvents in the ultrasonic extraction of ginseng has a small effect on the yield of ginsenosides R$_{b2}$, R$_c$, R$_d$, R$_{g1}$, and acetyl R$_{b1}$. However, changing the solvent/water ratios (60-100% $v_{\text{solvent}}/v_{\text{water}}$) has a significant effect on the ginsenoside R$_{b1}$ yield which is the major and bioactive component of American ginseng. The highest extraction efficiency was obtained for most of the ginsenosides when using 80% solvent: 20% water. Therefore, in this study, 80% DMSO and 80% methanol extracts were employed in examining the biological activity of North American ginseng.
Figure 2.11. Comparison of different solvent: water ratios for ginsenoside extraction. a) DMSO:water ratios, b) Methanol:water ratios. All runs are average of at least three repeats.

The higher amount of acetyl ginsenoside Rb$_1$ in ginseng extracts obtained with ultrasonication using DMSO at ambient temperatures (25-33°C) compared to methanol is potentially due to the higher solubility parameter of DMSO. The individual components of the solubility parameters of the solvents used in this study are shown in Table 2.3, which provides us with information on why the acetyl ginsenoside Rb$_1$ could be selectively obtained when using DMSO as solvent. As shown in Table 2.3, compared to methanol, DMSO has a higher dispersion, orientation, and induction values which would enhance the solubility and stability of the acetyl ginsenoside Rb$_1$. The Lewis acid–base interactions of DMSO's sulfonyl group with the carbonyl groups of acetyl ginsenoside could also enhance the stability of the acetyl ginsenoside. Moreover, DMSO has a zero acidity value which enables it to act as a proton acceptor for accepting hydrogen bonding that can lead to high solubility of acetyl compounds in DMSO. It has been reported that the solute-DMSO hydrogen bonding interaction is related to the proximity of the DMSO
molecule to the solute [42, 43]. For instance, as can be seen in Figure 2.12, the hydrogen bonding may happen when the oxygen atom of a DMSO molecule position is close to the hydrogen atom of the methyl group of acetyl ginsenoside Rb$_1$. Also, secondary hydrogen bonding may occur between the hydrogen atom of the methyl group of DMSO and the oxygen atom of the carbonyl group of the acetyl ginsenoside Rb$_1$. Poopari et al. studied different solvation models such as vibrational absorption (VA) in combination with density functional theory (DFT) calculations for testing the ester compound, methyl mandalate, solubility. They simulated this compound's solubility in organic solvents such as methanol, DMSO, and chloroform and showed that the solubility of methyl mandalate is related to the solvent–solute hydrogen-bonding interactions as well as the compounds structure. Therefore, future investigation is required to calculate the intermolecular hydrogen bond lengths between the acetyl ginsenoside Rb$_1$ and the solvents which is a complex phenomenon.

Table 2.3. The solubility parameters of solvents used in this study [44].

<table>
<thead>
<tr>
<th></th>
<th>$\delta_d$</th>
<th>$\delta_o$</th>
<th>$\delta_i$</th>
<th>$\delta_a$</th>
<th>$\delta_b$</th>
<th>$\delta_t$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPa$^{1/2}$</td>
<td>MPa$^{1/2}$</td>
<td>MPa$^{1/2}$</td>
<td>MPa$^{1/2}$</td>
<td>MPa$^{1/2}$</td>
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<tr>
<td>DMSO</td>
<td>17.2</td>
<td>12.5</td>
<td>4.3</td>
<td>0</td>
<td>10.6</td>
<td>24.5</td>
</tr>
<tr>
<td>Methanol</td>
<td>12.7</td>
<td>10</td>
<td>1</td>
<td>17</td>
<td>17</td>
<td>29.7</td>
</tr>
</tbody>
</table>

$\delta_d$ the dispersion forces, $\delta_o$ the orientation forces, $\delta_i$ the induction forces, $\delta_a$ the acidity and $\delta_b$ the basicity, $\delta_t$ the total hilderbrand solubility parameter.
2.3.4 Effect of North American ginseng extracts on LPS-stimulated production of NO and TNF-α in macrophages in vitro

Although the effect of Panax ginseng and the isolated ginsenosides such as ginsenosides Rc, Re and Rg₁ on the immune system for controlling inflammatory diseases and microbial infections has been studied extensively [23, 24, 45], there has not been many reports on the immunomodulation effects of North American ginsenosides. Therefore, it was decided to investigate whether or not acetyl ginsenoside Rb₁ isolated from North American ginseng would possess immunomodulation effects. Although the 80% DMSO extract and the 80% methanol extract examined in this study did not show immunostimulatory activity (Figure 2.13), they demonstrated varying degrees of suppression of LPS stimulation of macrophages (Figure 2.14).

**Figure 2.12.** Possible hydrogen bonding between acetyl ginsenoside Rb₁ and DMSO. Sulfur atom is yellow, oxygens are red, carbons are gray and hydrogens are white.
Figure 2.13. Immuno-stimulatory effects of 80% DMSO ginseng extracts (first row), 80% methanol ginseng extracts (second row) on 24 hours macrophage production of NO (a,b), TNF-α (c,d). Murine macrophages (RAW 264.7 cells) were treated with or without ginseng extracts and LPS (1 µg/ ml) for 24 hours and the culture supernatants were analysed for NO and TNF-α by Griess reaction assay and ELISA respectively. Three independent experiments were performed and the data were shown as mean ± SD.

Figure 2.14a&b display a significant inhibition of the production of NO and TNF-α in LPS-induced RAW 264.7 macrophages by the 80% DMSO ginseng extracts. This inhibition occurred in a dose-dependent manner within the dose range of 5-150 µg/mL extract, with a higher dose resulting in better inhibition. The 80% methanol ginseng extracts showed similar inhibitory effects, in spite of a relatively lower effect than that
using the DMSO ginseng extract (Figure 2.14c&d). With 5 µM of NO production being observed in the presence of 150 µg/mL DMSO extract, the more significant inhibition effect of this extract on NO production compared to the methanol extract could be due to the higher concentration of 6’-O-acetylginsenoside Rb₁ in the DMSO ginseng extract. In order to investigate this hypothesis, the immunosuppressive activity of both the isolated 6’-O-acetylginsenoside Rb₁ and commercial Rb₁ were examined by bioassay analysis using macrophages in-vitro. The 6’-O-acetylginsenoside Rb₁ was isolated by prep HPLC. While ginsenoside Rb₁ did not show any inhibitory effect on LPS-stimulation of macrophages (Figure 2.14e&f), which validated previous findings [10], 6’-O-acetylginsenoside Rb₁ significantly inhibited the production of NO and TNF-α in LPS-induced RAW 264.7 macrophage cells in a dose-dependent manner within the dose range of 5-150 µg/mL (Figure 2.14g&h). Therefore, 6’-O-acetylginsenoside Rb₁ isolated from North American ginseng significantly enhanced the immunosuppressive potency. It should be noted that transformed ginsenosides and ginsenoside metabolites have shown more potency toward cytotoxic activity than neutral ginsenosides [46, 47]. For instance, when 20(S)-ginsenoside Rg₃ was metabolized to 20(S)-ginsenoside Rh₂ or 20(S)-protopanaxdiol by human intestinal microflora, the cytotoxicity of ginsenosides against tumor cell lines was enhanced [46]. Also, Ke-Ke et al. [48] isolated and identified four new ginsenosides from Panax ginseng and investigated their cytotoxicity against HL-60 cells in vitro which showed medium and minor cytotoxicity. Therefore, the cytotoxicity of 6’-O-acetylginsenoside Rb₁ should be further evaluated.
NO Production (µM)  

80% DMSO ginseng extract

80% methanol ginseng extract

ginsenoside Rb₁

TNF-α Production (pg/mL)
Figure 2.14. Inhibitory effect of 80% DMSO ginseng extracts (first row), 80% methanol ginseng extracts (second row), ginsenoside Rb$_1$ (third row), and 6”-O-acetylginsenoside Rb$_1$ (fourth row) on LPS-stimulated 24hr macrophage production of NO (a, c, e, g) and TNF-α (b, d, f, h). Murine macrophages (RAW 264.7 cells) were pre-treated with the ginseng extracts and dexamethasone (5 µM) for two hours after which LPS (1 µg/mL) was added, and the culture supernatants were analyzed for NO and TNF-α by Griess reaction assay and ELISA, respectively after 24 h. Dexamethasone (DEX) at 5 µM was used as a positive control. Three independent experiments were performed and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. #Values p<0.05 Significantly different from LPS-induced group.

2.3.5 Anti-angiogenesis effect of North American ginseng extracts

Ginsenosides are known to have paradoxical effects on angiogenesis. Ginsenosides Rb$_1$ and Rg$_1$ are known to possess anti and pro-angiogenic effects, respectively; however the structure-activity relationship is poorly understood [9]. To understand the anti-angiogenic effect of the isolated 6”-O-acetylginsenoside Rb$_1$, this material was tested and compared with that of the commercial ginsenoside Rb$_1$. As shown in Figure 2.15, under the influence of matrigel, a tube like network is observed in the control experiment. However, the magnitude of tubulization was reduced in the presence of either ginsenoside Rb$_1$ or 6”-O-acetylginsenoside Rb$_1$. The reduction of tube formation increased with increasing treatment concentration dosage from 0.5 to 50 µg/mL. By comparing the anti-angiogenic effect of the two ginsenosides, it was found that both the 6”-O-acetylginsenoside Rb$_1$ and the commercial ginsenoside Rb$_1$ showed similar anti-
angiogenic activity and exhibited similar potency towards inhibiting tube-like structure formation of endothelial cells. This finding is in agreement with a previous report by Qi et al. which showed that acetylation of protopanaxadiol ginsenosides could increase their anti-cancer activity [49]. Acetyl ginsenosides are expected to have higher bioactivity as the acetyl ginsenosides are more lipophilic and consequently may have a higher cellular uptake [50].

Figure 2.15. Anti-angiogenesis activity of ginsenoside Rb₁ and mono-o-acetylated ginsenoside Rb₁. bottom panel, data represent the mean ± SD of the number of branch points relative to the control, which was set to 100%, counted in 10 microscopic fields of three independent experiments. #Values p<0.05 compared to control were determined to be significant.

2.4 Conclusions

In conclusion, ultrasonication extraction with DMSO and methanol as solvents at temperatures 25-33°C were investigated on North American ginseng powdered root. It
was successfully shown that DMSO and methanol were both effective solvents for ultrasonic extraction of ginsenosides, with DMSO in particular showing higher extraction and better selectivity. Aqueous solvents were found to increase the extraction efficiency in comparison with the pure solvents, with the best extraction efficiency obtained using 80/20 $v_{\text{solvent}}/v_{\text{water}}$. Large quantities of $6''$-$O$-acetylginsenoside Rb$_1$ were found using ultrasonic extraction with DMSO which was fractionated and isolated using a preparative HPLC column and subsequently identified by MS, FTIR, and NMR. This study was the first to examine DMSO extracts, and $6''$-$O$-acetylginsenoside Rb$_1$ exhibiting significant potency towards inhibiting the NO and TNF-$\alpha$ production in LPS-induced macrophage cells in a dose dependent manner. $6''$-$O$-acetylginsenoside Rb$_1$ was shown for the first time to have an immunosuppressive effect, attributed to existence of the acetyl group, compared with ginsenoside Rb$_1$, which did not show any immunosuppressive activity. Both ginsenoside Rb$_1$ and $6''$-$O$-acetylginsenoside Rb$_1$ were shown to have similar anti-angiogenic properties, suggesting that the presence of a acetylated moiety did not modify this biological effect.
2.5 References


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Chapter 3

3 Supercritical Fluid Chromatography of North American Ginseng Extract

Abstract

Supercritical fluid chromatography (SFC) using supercritical carbon dioxide (scCO$_2$) is considered as a “green” separation method, particularly suitable for the isolation of thermally unstable bioactive components. However, co-solvent and additives are often required in the mobile phase due to the poor solubility of polar components in scCO$_2$. In the present study, the effect of temperature and pressure on the separation of ginsenosides was studied with methanol being added to the CO$_2$ mobile phase. Acidic, basic, and ionic additives were introduced to the mobile phase, respectively, to study their effect on the separation of ginsenosides. The best separation conditions were achieved by adding trifluoroacetic acid in methanol (0.05% v/v) at 50°C and 150 bar. A high-concentration component in the extracts from the supercritical fluid extraction of North American ginseng was isolated by SFC and identified as sucrose using NMR, HPLC, and ESI-MS. These results show that SFC is a promising technique for the separation, isolation, and identification of ginseng extracts.
3.1 Introduction

North American ginseng (*Panax quinquefolius*) is a medicinal plant used in traditional herbal medicines mainly grown for its roots, similar to Asian ginseng (*Panax ginseng C.A. Meyer*), which also has a long history as a medicinal herb [1]. Native North Americans used the roots of ginseng both as a health food [2] and as a traditional medicine for relieving fever, stomach ailments, etc [3, 4]. The components isolated and characterized in North American ginseng include ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids [5, 6]. The most active ingredient of North American ginseng are known as ginsenosides which are polar triterpenoid saponins (Figure 3.1) and have been shown to provide valuable health effects including anti-inflammatory, anti-stress, and anti-cancer properties [7, 8]. However, due to the thermal instability of some ginsenosides, the yield and quality of these extracts in North American ginseng are dependent on the extraction method [4].

Conventional extraction methods for ginsenoside isolation from ginseng include Soxhlet, ultrasound assisted extraction, and microwave assisted extraction [9-11]. Some of the conventional extraction methods require long extraction times and a larger amount of solvent, and may result in the thermal decomposition of the target components. Moreover, a subsequent filtration and/or concentration step is often required to remove the solid residue [9]. Supercritical fluid extraction (SFE) using CO$_2$ and polar modifier has shown significant advantages in the extraction of medicinal plants [4, 12-15]. Previously we reported the isolation of ginsenosides and essential oils from North American ginseng using SFE [4]. Generally, ginsenosides are separated and analyzed by high performance liquid chromatography (HPLC) using water or organic solvents such as methanol and acetonitrile as mobile phases [4]. The SFE technique requires less separation time, and smaller amount of solvents, being considered as a “green” methodology [9]. In addition, better selectivity in the extraction of bioactive components has been obtained using the SFE approach [4]. The concentration step could be eliminated in SFE by simply lowering the density of supercritical fluids with reduced pressure. Moreover, the low critical temperature of CO$_2$ makes this method very suitable for the extraction of thermally unstable compounds [9].
Figure 3.1. Chemical structures of six common North American ginsenosides [4].

However, as medicinal and natural plants always consist of a variety of complex phytochemical ingredients, SFE cannot be used for the isolation of individual components from the extraction mixture. Different ginsenosides have been reported to have various biological and medicinal activities such as the diverse effects of Rb₁ and Rg₁ on the central nervous system and the unique anti-cancer effect of Rg₃ [6]. Hence,
pharmacological studies require pure individual ginsenoside components. Therefore, the preparative chromatography systems based on supercritical fluids provide an interesting approach to separate and isolate the most precious components of medicinal plant extracts [16].

Possessing the advantages of SFE, supercritical fluid chromatography (SFC) is an attractive separation method with better selectivity and shorter analysis time due to the low viscosity, high diffusivity, and high solvating power of supercritical fluids compared to the traditional gas chromatography and high performance liquid chromatography (HPLC) [17, 18]. Under regularly used chromatographic conditions such as 100-120 bar pressure and 40°C temperature, the mobile phase fluid is not in the supercritical state, although SFC researchers use the terminology of SFC despite the state of the fluid employed [19]. Moreover, in a preparative SFC system, the desired material fractions are isolated in the dry form, removing most of the solvent residue by simply venting CO₂. Therefore, SFC is a promising approach for separation and isolation of both pharmaceutical and nutraceutical active ingredients. Analytical and preparative SFC has been utilized to isolate valuable components such as lipids, vitamins and other bioactive compounds from extracts [16-18, 20-22]. The only application of SFC reported in the separation of ginsenosides was from Asian ginseng extracts by Li et al. [23]. In that study, pure CO₂ was used as the mobile phase with pressure gradient from 100 to 350 bar and temperature at 300°C to separate panaxadiol and panaxatriol which are the acid-hydrolyzed products of ginseng. As pure CO₂ has a low solvation power for polar components, the individual ginsenosides could not be selectively separated. For separation and isolation of polar components such as ginsenosides, organic polar modifiers are often required to add to the CO₂ mobile phase to increase the solvating power of the mobile phase [24].

In the present study, we examine the SFC of both individual ginsenosides and mixtures of ginsenosides at various temperatures and pressures while examining several typically used additives. We previously reported that DMSO was a useful co-solvent in SFE of North American ginseng, extracting unusual lipophillic ginsenosides (mono-acetylated Rb₁s) which may have higher biological activity [4]. We hypothesized that better
separation of ginsenosides could be achieved by adding polar organic solvents such as methanol. Hence, we then examined the isolation and identification of individual ginsenosides in the SFE extracts of North American ginseng by SFC. The effect of a small amount of polar additives such as acid, base, and aqueous ionic solution on the separation of ginsenosides are examined in this work. An unknown high-concentration component in the SFE extracts of North American ginseng was also found, characterized and identified.

3.2 Materials and methods

3.2.1 Materials

Ground, dried, root of North American ginseng was obtained from Agriculture and Agri-Food Canada. The volume weighted mean diameter of the powder was found as 550 µm, as determined by dynamic light scattering (Hydro 2000MU Malvern Instruments Ltd.). Solvents (ethanol, methanol, and DMSO) of HPLC grade (>99.9%), ammonium acetate (99%, ACS grade), trifluoroacetic acid (TFA) (99%), and diisopropylamine (DIPA) (99%) were obtained from Sigma-Aldrich Canada. The carbon dioxide SFC grade was purchased from Praxair, Inc., USA. Purified water was produced from a Milli-Q water purification system (18.2 MΩ·cm resistivity, Barnstead EasyPureII, Thermo Scientific, USA). Ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂ and Rd were provided by Dr. Ed Lui’s lab (Department of Physiology and Pharmacology, Western University, Canada) and used as standards for SFC analysis. Stock solutions were prepared in methanol at a concentration of 1000 µg.ml⁻¹ and then stored at -20°C.

3.2.2 Ginseng supercritical extracts preparation

An Isco SFE unit was employed for North American ginseng extract preparation as previously described [4]. Briefly, 1 g of ginseng root powder was mixed with 19 g of sand and then placed in a 10-mL extraction vial. 3.2 (g DMSO/g ginseng) was spread directly onto the solids, prior to placing them in the extraction vessel. The experimental conditions were at a temperature of 110°C, and pressure of 34.5 MPa which were shown
to form a single phase with DMSO [4]. After 60-min extraction, a dynamic CO₂ and DMSO as modifier was charged to the system to recover the extracted product into ethanol as the trapping liquid. Extracted materials were evaporated under vacuum at 40°C and the total mass was weighted and kept at -20°C prior to supercritical fluid chromatography. All SFE experiments were done by triplicate.

### 3.2.3 Supercritical fluid chromatography (SFC) of ginseng extracts

The SFC unit used for all analytical and semi-preparative separations is a Thar SFC instrument (Pittsburgh, PA) including a fluid delivery module with high pressure pumps for CO₂ and modifier delivery, a column oven for temperature control, autosampler, automated back pressure regulator (ABPR) for column pressure controlling, and an UV-Vis detector as well as an evaporate light scattering detector (ELSD) (Polymer Laboratories Inc, MA, USA) with drift tube temperature at 80°C (Figure 3.2).

![Figure 3.2. Schematic of the supercritical fluid chromatography (SFC) system. (A) CO₂ cylinder; (B) filter; (C) cooler; (D) CO₂ pump; (E) modifier reservoir; (F) modifier pump; (G) mixer; (H) sampler; (I) column and oven; (J) UV detector; (K) ELSD detector; (L) automated back pressure regulator (ABPR); (M) vent; (N) fraction collector.](image-url)
In the present study, ELSD was considered as an ideal detection system due to low UV wavelength of ginsenosides which is around 203 nm. The SFC column used was a cyanopropyl packed column (250 × 4.6 mm, dₚ 5 μm, 60 Å) (Princeton Chromatography Inc., NJ, USA) which is a polar stationary phase. The SFC system includes six fraction collector vessels for sample fractionation. SFC conditions examined were pressures from 100-200 bar, and temperatures from 32-50°C. Methanol was used as the modifier due to its high solubility in CO₂ and high polarity and ability to disturb solute–plant matrix bonding [4]. The mobile phase (CO₂:methanol ratio) flow rate was 1.5 mL/min. For the analysis, a partial loop (20 μL) of sample solution was injected to the SFC system and the mobile phase gradient composition was as 15% modifier in CO₂ for 1 min after injection followed by an increase in mobile phase concentration to 31% at rate of 2% per min, and was kept at 31% until the end of run. Additives were added into methanol as modifier (v/v) before mixing with CO₂. To help achieve stable chromatographic conditions, the mobile phase went through the column for 30 min before sample injection. Also, prior to each change of mobile phase additive, the column was washed with pure methanol for at least one hour at 1 mL/min for preventing previous analyte column contamination. Then, for column equilibration, the mobile phase with the next additive was introduced to the system 30 min prior to sample injection. In order to collect sample fractions, a full loop (100 μL) of sample solution at 10 mg/mL was injected to the SFC system. Fractions collected in the SFC vessels were dried under vacuum at room temperature prior to further characterization.

3.2.4 Analysis of supercritical fluid chromatography unknown fraction

Several techniques were employed to identify the unknown isolated fraction including nuclear magnetic resonance (NMR), HPLC-RID, and electrospray ionization mass spectrometry (ESI-MS). 1D (¹H and ¹³C) and 2D (gCOSY, gHSQC, gHMBC) NMR spectra were measured using either a Varian INOVA 600 or a Varian INOVA 400 spectrometer at 25°C. Methanol-d₄ (CD₃OD) was used as the solvent and chemical shifts were referenced to tetramethyl silane (TMS; 0.0 ppm). HPLC-RID system was used to identify the unknown semi-preparative SFC fraction. HPLC analysis was performed
using HPLC-RID system (Agilent Technologies Inc., USA) with a Hi-Plex H column (Zorbax, Agilent Technologies Inc., USA) 50 x 7.7 mm with 0.005 M sulfuric acid in water solution as mobile phase, at a temperature of 65°C isocratically and a flow rate of 0.6 mL/min. Molecular weight identification of the unknown semi-preparative SFC peak was obtained by ESI-MS analysis on PE-Sciex API 365 triple quadrupole mass spectrometer with a mass range (m/z) of up to 3000.

3.3 Results and Discussion

3.3.1 Supercritical fluid chromatography of North American ginsenosides

Our preliminary results showed that pure CO₂ was a poor mobile phase for separation of the polar components of North American ginseng extract, particularly for the ginsenoside components of interest. This is attributed to the low solubility of the polar ginsenosides in pure CO₂; therefore methanol was added as a modifier in the SFC mobile phase. Isocratic methanol concentration (e.g. 5%, 15% and 31%) was first tested for the separation of a ginsenoside standards mixture (Appendix B). The results showed that 5% methanol in scCO₂ caused co-elution and poor separation of all ginsenosides. The use of 15% methanol in scCO₂ resulted in better separation of ginsenosides, with Rg₁ and Re being eluted as a mixture. Using 31% methanol in scCO₂ led to a better separation of the ginsenoside Rd, but gave poorer separation of the mixture of ginsenosides Rg₁ and Re. Also, ginsenosides Rb₁, Rb₂ and Rc co-eluted with large tailing in the case of using isocratic methanol concentration (e.g. 15% and 31%). A higher concentration of modifier did not improve the peak shapes. However, a gradient composition of methanol in scCO₂ from 15-31% resulted in better peak shapes and symmetry of ginsenosides with no tailing and gave relatively good separation of ginsenoside Rd and partial separation of ginsenosides Rg₁ and Re in the mixture. Therefore, for the purpose of better fractionation of ginsenosides, a gradient composition of methanol in CO₂ was employed with the mobile phase consisting of 15% methanol in CO₂ for 1 min after injection, then increasing the methanol concentration to 31% at a rate of 2% per minute and kept at 31%
until the end of run. In order to select an optimal condition for the SFC experiments, the effect of temperature and pressure on the retention time and peak shape of the ginsenosides standard was investigated first. The effect of temperature on the retention time was examined at three different temperatures, 32, 40 and 50°C under a constant pressure of 150 bar with a flow rate of 1.5 mL/min. In order to identify the valuable ginsenoside components, each of the six common ginsenosides standards (Figure 3.1) was injected in the SFC-ELSD system, with the retention times listed in Table 3.1. A mixture of the six ginsenosides standards was injected to examine the effect of temperature and pressure on the separation efficiency. As shown in Figure 3.3 and Table 3.1, the retention time of all the ginsenosides increased with increasing temperature in the range of 32-50°C. Also, it can be seen that at T=32°C, ginsenosides Rg1 and Re co-eluted within 7.8-8 min whereas ginsenosides Rd, Rc, Rb2, and Rb1 eluted within 9.3-9.8 min. By increasing temperature from 32°C to 40°C, better separation was obtained. At T=40°C, ginenoside Rd separated as an individual peak at 11.5 min, although ginsenosides Rb2, Rc, and Rb1 still eluted as one peak within the range of 11.8-12.3 min. At T=50°C, ginsenoside Rg1 and Re could be partially isolated while better isolation of ginsenoside Rd was achieved. It should be noted that the elution order of these ginsenosides did not change with increasing temperature from 32 to 50 °C except Rc at T=32 °C. Also from the separation point of view, our results show that at P=150 bar and T=50°C ginsenoside Rd was well separated from the panaxidol ginsenosides (Rb1, Rb2, Rc) and the panaxitrol ginsenosides (Rg1, Re). However, when using lower separation temperatures (i.e. T=40°C and T=32°C), only partial separation was achieved.
Table 3.1. Retention time of individual ginsenoside standard at different temperatures under 150 bar, with 15-31% methanol in CO₂ as mobile phase at a flow rate of 1.5 mL/min.

<table>
<thead>
<tr>
<th>Ginsenoside</th>
<th>Retention Time (min)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>T=32°C</td>
</tr>
<tr>
<td>Rg₁</td>
<td>7.8</td>
</tr>
<tr>
<td>Re</td>
<td>8.0</td>
</tr>
<tr>
<td>Rd</td>
<td>9.3</td>
</tr>
<tr>
<td>Rb₂</td>
<td>9.6</td>
</tr>
<tr>
<td>Rc</td>
<td>9.4</td>
</tr>
<tr>
<td>Rb₁</td>
<td>9.8</td>
</tr>
</tbody>
</table>

Figure 3.3. Effect of temperature on the retention time of ginsenoside standards mixture at 150 bar, with 15-31% methanol in CO₂ as mobile phase at a flow rate of 1.5 mL/min. a) T=32 °C, b) T=40 °C, and c) T=50 °C.
In explanation, under a constant pressure, the density of the mobile phase decreased with increasing temperature, leading to a lower solvating power of the mobile phase. The lower solubility of the ginsenosides in the mobile phase resulted in longer retention times. This explanation was echoed during the study of the effect of pressure on the retention time.

As shown in Figure 3.4 and Table 3.2, the retention time of all the ginsenosides decreased with increasing pressure from 100 to 200 bar at a constant temperature of 50°C. The increased pressure at constant temperature resulted in increased density and solving power of the mobile phase, leading to a shorter retention time. By investigating the pressure effect, at first the best separation seemed to be obtained at P=100 bar, however the peak shapes and the baseline were noisy which may be due to lower solubility of ginsenosides in the mobile phase under these conditions. At P=200 bar, ginsenosides Rg₁ and Re eluted as one peak whereas ginsenoside Rd was partly isolated from Rc, Rb₂, and Rb₁. By comparing the chromatographic profiles of the mixture of ginsenoside standards under these conditions, the best condition for the separation of ginsenosides was found to be T=50°C and P=150 bar. It should be noted that the elution order of ginsenosides Rb₂ and Re was altered at P=100 bar and P=200 bar. Usually, the elution order in SFC is determined by the number of hydroxyl groups in the analyte. When a polar stationary phase is used, the retention time increases with increasing number of hydroxyl groups in the analyte [25]. However, for ginsenoside Re with the same number of hydroxyl groups as Rd, the hydroxyl group on carbon 3 is very close to the two methyl groups on carbon 4. Hence, the interactions between this hydroxyl group and the stationary phase could be reduced by steric hindrance. Therefore, ginsenoside Re eluted earlier than ginsenoside Rd [25].
Table 3.2. Retention time of individual ginsenoside standard under different pressures at 50°C, with 15-31% methanol in CO₂ as mobile phase at a flow rate of 1.5 mL/min.

<table>
<thead>
<tr>
<th>Ginsenoside</th>
<th>Retention Time (min)</th>
<th>P=100 bar</th>
<th>P=150 bar</th>
<th>P=200 bar</th>
</tr>
</thead>
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<tr>
<td>Rg₁</td>
<td>12.8</td>
<td>12.4</td>
<td>11.2</td>
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</tr>
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<td>Re</td>
<td>13.4</td>
<td>12.6</td>
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<tr>
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<td>Rb₁</td>
<td>15.8</td>
<td>14.2</td>
<td>12.6</td>
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Figure 3.4. Effect of pressure on the retention time of ginsenosides standard mixture at 50°C, with 15-31% methanol in CO₂ as mobile phase at a flow rate of 1.5 mL/min. a) P=100 bar, b) P=150 bar, and c) P=200 bar.
3.3.2 Effect of additives on elution of North American ginsenosides

As the utilized binary mobile phase (CO₂/methanol) was not ideal in the separation of the standard ginsenoside mixture, we hypothesized that better separation could be achieved by adding additives to the mobile phase. Three different compounds including trifluoroacetic acid (TFA), diisopropylamine (DIPA), and aqueous ammonium acetate solution were added to the CO₂ mobile phase, respectively. These compounds represent three different types of additives, i.e., acidic, basic, and ionic additives, respectively. Figure 3.5 displays the chromatograms of the mixture of the ginsenosides standards by adding a small amount of TFA to the mobile phase (gradient 15-31% methanol in CO₂) under the selected chromatographic conditions of temperature 50°C, pressure 150 bar, at a flow rate of 1.5 mL/min. According to the retention time of the individual ginsenoside standards, it was found that the elution of ginsenosides Rg₁, Re, and Rd was improved with 0.05% v/v TFA being added to the mobile phase. The co-elution issue of ginsenoside Rg₁ with Re was resolved, although ginsenoside Re and Rd eluted partially together. Hence, the separation efficiency of ginsenosides Rg₁, Re, and Rd was improved. By increasing the percentage of TFA in the mobile phase from 0.05% v/v to 0.1% v/v, ginsenoside Re and Rd co-eluted within 11-12 min and no difference was observed for other ginsenosides elution. Therefore, 0.05% v/v TFA in the mobile phase seems to be the optimum acidic additive amount that is useful for isolation of ginsenosides. In addition, the retention times of the ginsenosides decreased slightly with increasing TFA concentration in methanol from 0.05% v/v to 0.1% v/v. This decrease in retention time may be attributed to the improved solvating power of the mobile phase due to the addition of the acidic additive [26]. TFA can potentially enhance separation of the ginsenosides by either protonation of their hydroxyl groups or protonating the unmodified Si-OH groups on the separation column, leading to enhanced solvent/solute interactions [27].
Figure 3.5. SFC-ELSD chromatogram of the mixture of ginsenosides standard at 50°C, 150 bar, 15-31% methanol in CO$_2$ as mobile phase at a flow rate of 1.5 mL/min with the addition of a) 0.0% of TFA; b) 0.05% TFA; c) 0.1% TFA.

To investigate the effect of basic additive, 0.1% v/v and 0.5% v/v of DIPA was added to methanol and then introduced to the mobile phase. Under the chromatographic condition of temperature 50°C, pressure 150 bar, at a flow rate of 1.5 mL/min, the mixture of ginsenoside standard was injected to the SFC-ELSD system. The chromatograms with addition of DIPA are displayed in Figure 3.6. Based on the retention times of the ginsenosides standard under the same conditions, ginsenosides Rg$_1$ and Re were better separated by adding 0.1% v/v of DIPA in methanol. However by increasing the concentration of DIPA from 0.1% v/v to 0.5% v/v in the mobile phase, further improvement was not observed for isolation of ginsenosides. Similarly, the introduction of 0.1% v/v of DIPA into methanol resulted in decreased retention times of the ginsenosides (Figure 3.6). This suggests that the interactions of DIPA with the utilized cyano SFC column increased, decreasing ginsenoside-column interactions. Further
increasing the amount of DIPA from 0.1%v/v and 0.5%v/v in methanol did not affect the retention times of the ginsenosides but led to poorer peak shape and separation of the ginsenosides, possibly due to partial phase separation.

Figure 3.6. SFC-ELSD chromatogram of the mixture of ginsenosides standard at 50°C, 150 bar, 15-31% methanol in CO₂ at a flow rate of 1.5 mL/min with the addition of: a) 0.0% DIPA; b) 0.1% DIPA; c) 0.5% DIPA.

The effect of ionic additive on the SFC was evaluated by adding 10 mM of AA aqueous solution to the mobile phase. As shown in Figure 3.7, adding the ionic additive into the mobile phase gave no improvement on the separation of ginsenosides with ginsenoside Rg₁ and Re being co-eluted with ginsenoside Rd. Increasing the amount of water to 5% v/v with 10 mM AA in methanol could not improve the ginsenoside elution and the baseline became noisy with poor signal/noise ratio. In contrast to the addition of TFA or DIPA, there was no significant change in the retention times of ginsenosides by adding
AA aqueous solution. Along with the poorer peak shape and separation with increased amount of AA aqueous solution, the introduction of ionic additive is believed to cause phase separation of the neutral ginsenosides in the mobile phase.

Figure 3.7. SFC-ELSD chromatogram of the mixture of ginsenosides standard at 50°C, 150 bar, 15-31% methanol in CO₂ at a flow rate of 1.5 mL/min with addition of: a) no ionic additive; b) 3% v/v solution of 10 mM AA; c) 5% v/v solution of 10 mM AA.

By comparing the addition of the three types of additives, under the same chromatographic conditions with the cyanopropyl stationary phase, the best peak shape, symmetry, efficiency, and resolution for mixture of ginsenoside standards was obtained by using the acidic additive TFA. In general, due to the presence of various site isomers on different carbon atoms, not all of North American ginsenosides could be isolated by SFC-ELSD under the studied conditions. These results are in good agreement with reports in the literature under the same conditions and in the presence of additives where
hydrogen bond donor/acceptor analytes retention time were additive dependent [18, 24, 28]. Therefore, in all cases the retention time of ginsenosides slightly decreased by increasing the additive concentration in the mobile phase.

### 3.3.3 Supercritical fluid chromatography of North American ginseng extracts

The extracts of North American ginseng was obtained with the SFE method using supercritical CO$_2$ as described above. By employing a modifier DMSO, a total quantity of extracted material of 350 mg/g with a relative standard deviation of 3.21% based on three replicates was achieved. The extracts were subsequently fractionated by means of SFC-ELSD. The optimum chromatography conditions of 50°C, 150 bar, 15-31% methanol in CO$_2$ as mobile phase at a flow rate of 1.5 mL/min with the addition of 0.05% TFA performed for separation and isolation of ginsenosides from SFE North American ginseng extracts. According to the retention times of the ginsenoside standards under the same conditions, the peaks of the ginsenosides in the extracts were identified between 10 and 13 min (Figure 3.8). As can be observed in Figure 3.8a, ginsenoside Rg$_1$ separated within 10-11 min followed by ginsenoside Re, in addition ginsenoside Re and Rd isolated partially within 11.5-12.3 min. However, ginsenosides Rb$_2$, Rc, and Rb$_1$ in the SFE North American ginseng extract co-eluted within 12.4-13 min. Our results are consistent with the previous study [4] which showed that the main ginsenosides compound of SFE North American ginseng extract is ginsenoside Rb$_1$, and in a lower degree, ginsenosides Rd and Re. Ginsenosides Rc, Rg$_1$, and Rb$_2$ are present in North American ginseng extract in lower amounts, respectively. Figure 3.8b shows the SFC chromatograph of SFE North American ginseng extracts in optimum selected condition. Interestingly, an extensive peak was detected at 8 min that did not correspond to any of the ginsenoside standards (Figure 3.8b). As the literature contained no information on this observation, we decided to isolate and identify the unknown component.
Figure 3.8. SFC-ELSD chromatograph of a) SFE North American ginsenoside separation (enlargement of ginsenosides region); b) the SFE North American ginseng extracts at 50°C, 150 bar, 15-31% methanol in CO₂ as mobile phase at a flow rate of 1.5 mL/min with the addition of 0.05% TFA.

3.3.4 Identification of the unknown component

During the SFC separation of the SFE extracts of North American ginseng, an extensive peak was detected between 7-9 min, which was not attributed to any ginsenosides and has not been reported in any literature. Due to its high concentration in the SFE extracts, it was deemed necessary to identify this component. NMR, HPLC-RID, and ESI-MS were employed in the characterization of this component, which was fractionated by the semi-
preparative SFC system at pressure 150 bar, temperature 50 °C, flow rate of 1.5 mL/min, mobile phase of CO₂/methanol with a gradient composition of 15-31% at 2% per min. NMR is a powerful tool in identifying the chemical structure of an organic chemical. As shown in Figure 3.9, the \(^1\)H peaks appeared between 3.42 and 5.39 ppm in the \(^1\)H NMR spectrum while 12 distinct \(^{13}\)C peaks were present between 62.4 and 105.5 ppm in the \(^{13}\)C NMR spectrum (Figure 3.10).

**Figure 3.9.** \(^1\)H NMR spectrum of the unknown fraction (in CD₃OD).

In order to understand the relationship among these \(^1\)H and \(^{13}\)C peaks, 2D NMR spectra of gCOSY, gHSQC, and gHMBC of the unknown fraction were also measured (Figure 3.11). By analyzing these 1D and 2D NMR spectra, the chemical structure of the unknown fraction was proposed in Figure 3.12, with the peaks being assigned and listed in Table 3.3. Interestingly, this structure is identical to the commonly known disaccharide, sucrose.
Figure 3.10. $^{13}$C NMR spectrum of the unknown fraction (in CD$_3$OD).
Figure 3.11. 2D NMR spectra of gCOSY (top), gHSQC (middle), and gHMBC (bottom) of the unknown fraction (in CD$_3$OD).
Figure 3.12. The proposed structure of the unknown fraction based on the 1D and 2D NMR spectra.

Table 3.3. Assignment of the peaks in the $^1$H and $^{13}$C NMR spectra of sucrose (in CD$_3$OD).

<table>
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<th>No</th>
<th>$^1$H Chemical Shift (ppm)</th>
<th>$^{13}$C Chemical Shift (ppm)</th>
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<tr>
<td>1</td>
<td>5.39d (J=3.66Hz)</td>
<td>93.8</td>
</tr>
<tr>
<td>2</td>
<td>3.42dd (J1=9.52Hz, J2=3.66Hz)</td>
<td>73.4</td>
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<tr>
<td>3</td>
<td>3.70</td>
<td>74.8</td>
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<tr>
<td>4</td>
<td>3.36dd (J1=10.14Hz, J2=8.97Hz)</td>
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<td>5</td>
<td>3.81-3.84</td>
<td>74.6</td>
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<tr>
<td>6</td>
<td>3.72-3.73,3.79-3.81</td>
<td>62.4</td>
</tr>
<tr>
<td>7</td>
<td>3.62q (J=12.45Hz)</td>
<td>64.2</td>
</tr>
<tr>
<td>8</td>
<td>n.a.</td>
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</tr>
<tr>
<td>9</td>
<td>4.09</td>
<td>79.5</td>
</tr>
<tr>
<td>10</td>
<td>4.02t (J=8.06Hz)</td>
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</tr>
<tr>
<td>11</td>
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<td>84.0</td>
</tr>
<tr>
<td>12</td>
<td>3.75-3.77</td>
<td>63.5</td>
</tr>
</tbody>
</table>
The HPLC-RID chromatogram of the unknown fraction was collected and compared with that of a reference sample (commercial sucrose) in Figure 3.13. It should be pointed out that the presence of sulfuric acid in the mobile phase and the temperature of 65°C caused the commercial sucrose to be hydrolyzed to monosaccharides glucose and fructose [29]. Hence, two distinct peaks appeared in the chromatogram of the reference sample, at 11.273 and 12.028 min, respectively (Figure 3.13a), with the former peak assigned to glucose and the latter one attributed to fructose. The chromatogram of the unknown fraction also showed two peaks at 11.284 and 12.039 min, respectively (Figure 3.13b), which are essentially at the same retention times as that of the reference sample.

![HPLC-RID chromatogram](image)

**Figure 3.13.** HPLC-RID chromatogram of a) sucrose standard; b) unknown fraction obtained by the preparative SFC of North American ginseng extract.

In addition, the unknown fraction was also characterized with ESI-MS, with the mass spectrum being displayed in Figure 3.14. Due to the application of NaI as an external standard, Na⁺ was adducted to the sample molecule, showing a dominant peak at
365.1 Da, which can be assigned to [sucrose+Na]+. Another quasimolecular ion, [sucrose$_2$+Na]$^+$ showed a peak at 707.2 Da (684+23). These results are in good agreement with the spectra of sucrose reported in the literature [30, 31].

![Mass spectra of unknown fraction obtained by the preparative SFC of North American ginseng extract analysed by ESI-MS.](image)

**Figure 3.14.** Mass spectra of unknown fraction obtained by the preparative SFC of North American ginseng extract analysed by ESI-MS.

Although the most biologically active ingredients of ginseng extracts are ginsenosides, ginseng extracts have been reported to consist of different components such as amino acids, fatty acids, carbohydrates, alkaloids, polysaccharides, sesquiterpenes, polyacetylenes, peptidoglycans, minor elements, vitamins, and phenolic compounds [6]. In a recent report analyzing North American Ginseng by HPLC, Lui et al. [32] found glucose as the major monosaccharide at 77-86 % (w/w), galactose at 6.8-7.5 % (w/w), arabinose at 4.5-5.9 % (w/w), and galacturonic acid at 8.7-9.5 % (w/w). This analysis did not report finding sucrose although significant quantities of higher molecular weight bioactive carbohydrates were reported. Supercritical CO$_2$ in combination with polar co-solvents would increase the solubility of disaccharide. Montanez et al. found that different polar co-solvents such as isopropanol and ethanol and water mixtures were
capable of enhancing solubility, selectivity, and yield for the fractionation of binary carbohydrate mixtures [33]. Hence in this work SFC was successfully employed in the separation of the SFE extracts of North American ginseng using supercritical CO$_2$, co-solvent, and appropriate additives, leading to fractionation of several ginsenosides and the disaccharide sucrose.

3.4 Conclusions

Supercritical fluid chromatography coupled with an evaporative light scattering detector was employed in the separation of the SFE extracts of North American ginseng with 15-31% of methanol in supercritical CO$_2$ as the mobile phase. The effect of temperature and pressure on the separation of mixture of ginsenoside standard was tested with the best condition being found at T=50°C and P=150 bar. The effect of three types of additives including the acidic TFA, basic DIPA, and ionic AA aqueous solution on the separation efficiency was examined, with the best selectivity being found in the case of the addition of 0.05% v/v TFA in methanol. By adding the additives, the retention time changed with the varying amount of additives, indicating the varying solubility of the extract fractions. A high-concentration fraction in the SFE extracts of North American ginseng was isolated with the SFC system and characterized with NMR, HPLC-RID, and ESI-MS, with its identification being confirmed to be sucrose.
3.5 References


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Chapter 4

4 Encapsulation of acetyl ginsenoside Rb₁ within PLGA Microspheres using Microfluidic Technique

Abstract

Ginsenosides are the active pharmaceutical ingredients (APIs) from ginseng, whose herbal extracts have been recognized to have unique pharmaceutical effects. In the present study, 6"-O-acetylinsenoside Rb₁ (ac-Rb₁) isolated from North American ginseng, which we have previously shown to have potent immunosuppressive activity, was encapsulated within biodegradable poly (DL-lactic-co-glycolic acid) (PLGA) microspheres. Both a conventional double emulsion and a microfluidic technique were examined for microsphere formation. The ac-Rb₁ encapsulation within PLGA microspheres was characterized by various physico-chemical techniques including FTIR, DSC and XRD. The experimental data showed that the PLGA microspheres produced from the microfluidic technique were uniform with tunable mean diameters from 7 to 59 µm and standard deviations less than 10%. The size was independent of the capillary number and could be tuned by altering the continuous and disperse phase flow rate ratios. High encapsulation efficiencies were obtained of 75.2%-96.7%. Those obtained from a conventional double emulsion method gave sizes in the range of 39 µm to 48 µm and standard deviations less than 20%, with lower encapsulation efficiencies of 77.5%-78.8%. Release profiles of uniform microspheres produced by microfluidics were investigated and quantified by UV-Vis spectrophotometry, showing controlled release of ac-Rb₁ while retaining potency toward immunosuppressive activity using macrophages in vitro. This study shows that ac-Rb₁ encapsulated PLGA microspheres obtained using the microfluidic approach can be utilized for the development of next-generation biomedical agents that are immunosuppressive in drug-release devices.
4.1 Introduction

Of the well known traditional Chinese medicines with biological activity, ginsenosides, which are the main active components of plant ginseng, have been widely used as a medicinal herbal plant extract [1]. Ginsenosides have been shown to have various beneficial health effects including anti-inflammatory, anti-stress and anti-cancer properties [2-4]. For instance, ginsenosides Rb₁ and Rg₁ have been shown as efficient neuroprotective agents for spinal cord neurons, while ginsenoside Re did not exhibit such activity [5]. We showed that ac-Rb₁ ginsenoside (Figure 4.1), which was isolated and characterized from North American ginseng, has potent immunosuppressive effects [6] and anti-angiogenic effects (Chapter 2). It has also been reported that transformed ginsenosides and ginsenoside metabolites show more potency toward cytotoxic activity than neutral ginsenosides [7, 8]. However, the oral bioavailability of ginsenosides from the gastrointestinal (GI) tract is known to be low, i.e. 5% or less. This is due to biotransformation of ginsenosides in the human GI tract by enzymes and intestinal bacteria to other ginsenoside-like compounds such as compound K, which is also biologically active [9]. Due to the cytotoxicity and poor bioavailability of ginsenosides, there is a growing need for more advanced delivery vehicles for ginseng extracts.

Over the past several years, significant progress has been directed towards developing novel delivery systems for bioactive compounds from plant extracts and natural health products including nutraceuticals, probiotics, and traditional Chinese medicines [10]. Currently, traditional approaches using tablets, capsules, teas, creams, oils, and liquids are used for the mixtures of generally low concentrations of biologically active compounds. Although some initial work has started investigating various formulations for bioactive and plant extracts such as polymeric nanoparticles, nanocapsules, liposomes, and microspheres [11-13], very little has been done on investigating ginsenosides. Of the microspheres that may be suitable for ginsenosides, PLGA-based microspheres are of particular interest due to their long clinical experience, FDA compliance, high biocompatibility, appropriate degradation characteristics and possibilities for sustained drug delivery. PLGA is one of the most well-known biodegradable polymers and delivery carriers for drugs, proteins, peptides and steroids.
due to its ability to encapsulate a variety of materials [14, 15], although it has not been examined with ginsenosides.

To prepare microspheres, various techniques are possible such as emulsion solvent evaporation/extraction methods, phase separation, spray drying and supercritical fluids [12, 16]. Conventional microencapsulation methods generate microspheres with broad size distributions which influences the kinetics of encapsulant release and limits their clinical application [17]. Recently, microfluidic devices have attracted attention due to their easy adjustability to create microspheres with controlled morphologies [18]. Microfluidic devices utilize low-priced and convenient systems for the control of fluid flow [19]. Monodisperse microspheres made from biodegradable polymers have been generated by microfluidic devices widely used in drug delivery systems [17]. Monodisperse microspheres significantly affect the drug release kinetics, reproducibility, and bioavailability of the encapsulant [20]. Several organic (PLA and PLGA) and inorganic (chitosan, poly(N-isopropylacrylamide) pNIPAAM, hydrogelator, silk protein, pectin, hydrazide and aldehyde-functionalized carbohydrates, dextranhydroxyethyl methacrylate (dex-HEMA) and silica) materials have been used for microsphere fabrication via microfluidic approach. However, there is no study available regarding making monodisperse microspheres containing bioactive plant extract with a microfluidic device.

In this study, we investigate both a microfluidic and a conventional double emulsion technique for ac-Rb1 loaded PLGA microspheres. Their encapsulation, release and bioactivity are measured.
Figure 4.1. a) Acetyl ginsenoside Rb₁ (ac-Rb₁) structure, b) PLGA structure.

4.2 Materials and Methods

4.2.1 Materials

6”-O-acetylginsenoside Rb₁ (ac-Rb₁) was isolated and fractionated from North American ginseng root as previously described [6]. Poly (dl-lactide-co-glycolide) (PLGA) (75:25, MW 66-107 kDa), poly (vinyl alcohol) (PVA) (87-89% hydrolyzed, MW 13-23 kDa), 0.1 mol/L phosphate buffered solution (PBS, pH 7.4), dichloromethane (DCM) of HPLC grade (>99.9%), methanol and ethanol were purchased from Sigma-Aldrich, Canada and used as received. Poly(dimethylsiloxane) (PDMS; Sylgard 184) was obtained from Dow Corning (Midland, USA) used for preparation of microfluidic microchip. Purified water was produced from a Milli-Q water purification system (18.2 MΩ-cm resistivity, Barnstead EasyPureII, Thermo Scientific, USA). RAW 264.7 (ATCC TIB 67) murine macrophage cell lines were provided by Dr. Jeff Dixon (Department of Physiology and Pharmacology, Western University, Canada). BD OptEIA ELISA kits tumour necrosis factor-α were provided by BD Biosciences (Bedford, MA, USA). LPS (Lipopolysaccharides) from Escherichia coli (0111:B4) purity>99%, Dexamethasone purity>98% and Griess reagent were purchased from Sigma-Aldrich (USA). Cell culture medium and reagents were purchased from Gibco laboratories (USA).
4.2.2 Preparation of ac-Rb\textsubscript{1} loaded PLGA microsphere

A microfluidic method was used to prepare the ac-Rb\textsubscript{1} encapsulated PLGA microspheres. The schematic of the microfluidic apparatus is shown in Figure 4.2. A microfluidic chip with channel dimensions of 100 µm in height and 200 µm in width was fabricated by soft lithography techniques [21] in collaboration with Dr. Jun Yang, Mechanical and Material Engineering Department, Western University, Canada. The microfluidic chips were molded by a mixture of PDMS elastomer base/curing agent in 10:1 ratio from SU-8 mold and bonded to a flat PDMS substrate under plasma treatment for 35 s in a PX-250 plasma chamber (March Instruments, Concord, MA) and then immediately placed in contact to bond the surfaces irreversibly. The devices were then bonded using a homemade UV-ozone generator. We dissolved 10 mg of ac-Rb\textsubscript{1} in 5 mL of solution (methanol: de-ionized water (2:3 v/v)) and 100 mg of PLGA was dissolved in 10 mL of DCM. The ac-Rb\textsubscript{1} solution was emulsified in PLGA organic solution (oil-phase) to form the disperse phase. The micro channels were first filled with a continuous phase of 2% PVA aqueous solution before introducing the organic (disperse) phase [17, 19]. Digitally controlled syringe pumps (KD scientific pump, Inc. Legato 2200, 1100) delivered liquid phase into the microfluidic device at constant flow rates using Teflon tubing (1.6 mm OD, 0.6 mm ID). The flow rate of the continuous phase was in the range between 0.05-0.2 mL/min while that of disperse phase was kept at a constant value of 0.01 mL/min as summarized in Table 5.1. At the end of the device outlet, the Teflon tubing was submerged into a 10-mL beaker containing 1% of PVA aqueous solution at 40°C to collect the droplets. Next, the solvent was removed from the droplets by evaporation. The formed microspheres were then centrifuged at 4500 rpm for 5 min followed by washing with de-ionized water three times to remove excess PVA and freeze-drying then stored at −20°C.
Figure 4.2. Schematic drawing of microfluidic system: (1) dispersed phase inlet (Acetyl ginsenoside Rb₁ solution dissolved in PLGA in DCM solution), (2) continuous phase inlet (2% PVA aqueous solution), (3) outlet.

As a comparison to the microfluidic technique, the widely applied water-in-oil-in-water (w/o/w) double emulsion method [22] was utilized for the formation of ac-Rb₁-loaded PLGA microspheres (Figure 4.3). Briefly, 10 mg of ac-Rb₁ was dissolved in 5 mL of solution (methanol: de-ionized water (2:3 v/v)) and 100 mg of PLGA was dissolved in 10 mL of DCM. The ac-Rb₁ solution was emulsified in the PLGA solution using a magnetic stirring bar at three different speeds of 800, 1000 and 1200 rpm summarized in Table 4.1. The entire mixture (w/o) was added to a 2% of PVA aqueous solution and emulsified again to generate the double emulsion (w/o/w), which was subsequently poured into 40 mL of de-ionized water and stirred at 400 rpm for 3 hr. After removing DMC by evaporation, the formed microspheres were centrifuged at 4500 rpm for 5 min, followed by washing with de-ionized water three times to remove PVA and freeze-drying to remove water. The ac-Rb₁ loaded PLGA microspheres were stored at −20°C.
4.2.3 Determination of ac-Rb\(_1\) content in the PLGA microspheres

The ac-Rb\(_1\) concentration encapsulated inside the PLGA microspheres was measured using a UV spectrophotometer (Shimadzu UV-Vis 3600) in triplicate at 203 nm. First, a standard calibration curve of ac-Rb\(_1\) was established using the UV spectrophotometer by measuring the absorbance as a function of concentration. To measure the ac-Rb\(_1\) content after PLGA encapsulation, 5 mg of ac-Rb\(_1\)-loaded microspheres were dissolved in 1 mL of DCM, which was subsequently evaporated with the residue then dissolved in 1 mL of methanol. The ac-Rb\(_1\) content was analyzed by the UV spectrometer based on the calibration curve established from ac-Rb\(_1\) standard solution mixtures. The ac-Rb\(_1\) encapsulation efficiency (EE\%) was calculated via the following equation

\[
EE\% = \frac{\text{Drug Loading (DL\%)}}{\text{Theoretical Loading (TL\%)}} \times 100, \tag{4.1}
\]

where

\[
\text{Drug Loading (DL\%)} = \frac{\text{weight of ac - Rb1 in PLGA measured by UV spectrometer}}{\text{weight of PLGA microspheres}} \times 100, \tag{4.2a}
\]

\[
\text{Theoretical Loading (TL\%)} = \frac{\text{weight of ac - Rb1 added to system}}{\text{weight of ac - Rb1 + weight of PLGA}} \times 100. \tag{4.2b}
\]
4.2.4 Ac-Rb$_1$ delivery carriers characterization

The surface morphology of the PLGA microspheres systems was measured using a scanning electron microscope (Hitachi S-4500). Samples were sputtered with gold coating, and placed on a copper stub. Also, an Axioimager Z1 motorized upright fluorescence microscope was used to acquire images of the polydisperse and monodisperse PLGA microspheres. Melting points were measured by a DSC Q200 (TA Instrument, New Castle, Delaware, USA). The scanning rate was 10 °C/min, with a scanning temperature range of 25–250 °C. XRD data were obtained for ac-Rb$_1$, placebo microspheres, and ac-Rb$_1$-loaded microspheres to evaluate the crystallinity after encapsulation (Inel CPS Powder Diffractometer). Scanning was done up to a 2Θ angle of 43° using a powder diffractometer instrument which was equipped with a Cu-X-ray radiation Tube, an Inel XRG3000 generator and an Inel CPS 120 detector. ATR-FTIR (Nicolet 6700, Thermo Scientific) was used to investigate the cross-linked core-shell composites in the range of 500-4000 cm$^{-1}$.

4.2.5 Kinetic release studies of ac-Rb$_1$

The ac-Rb$_1$ concentration encapsulated within the PLGA microspheres was measured using a UV spectrophotometer (Shimadzu UV-Vis 3600) in triplicate at 203 nm as described in section 4.2.3. For the release studies, 5 mg of ac-Rb$_1$-loaded PLGA microspheres were suspended in 10 mL PBS (pH=7.4) at 37°C with shaking at 50-100 rpm. At predetermined time intervals, the solution was centrifuged at 2000 rpm for 5 min, with 2 mL of supernatant then collected and replaced with 2 mL of fresh PBS. The ac-Rb$_1$ content in the supernatant was determined at a wavelength of 203 nm using a UV spectrophotometer (Shimadzu UV-Vis 3600). All experiments for each time interval were repeated in triplicate. The cumulative release of ac-Rb$_1$ profiles were acquired with the volume loss correction.

4.2.6 Bioactivity of released ac-Rb$_1$

The mouse macrophage cell line RAW 264.7 was cultured in Dulbeccos Modified Eagle's Medium supplemented with 10% FBS, 25 mM HEPES, 2 mM Glutamine, 100 IU penicillin/ml, and streptomycin 100 µg/mL. The pH of the medium was around 7.4. The
cells were kept at 37°C in a humidified incubator with 5% CO₂, and seeded in 96-well tissue culture plates at a density of 1.5×10⁵ cells per well. Our previous study showed that ac-Rb₁ has potency toward immunosuppressive activity on murine macrophages [6]. In this study, the inhibitory effect of released ac-Rb₁ from the microspheres in vitro was evaluated. To examine the inhibitory effect of ac-Rb₁ released from PLGA microspheres after 400 h, 5-150 µg/mL of ac-Rb₁ before encapsulation and after being released from PLGA microspheres were added to the macrophages 2h prior to the addition of LPS. In this study, dexamethasone (DEX) was used as a positive control for the suppression of LPS-induced stimulation of macrophages. So, cultured macrophages were incubated with DEX (5 µM) for 2 hr prior to the addition of LPS. The 24-hour cytokine production induced by LPS was determined by measuring NO and TNF-α levels in the culture medium. TNF-α concentrations in supernatants from cultured cells were analyzed with ELISA. Samples were assessed with mouse cytokine-specific BD OptEIA ELISA kits (BD Biosciences, USA) according to the manufacturer’s protocol. Sample nitrite concentrations in culture medium were determined by means of Griess reagent (0.5% sulfanilic acid, 0.002% N-1-naphtyl-ethylenediamine dihydrochloride, 14% glacial acetic acid). Culture supernatant of each sample and the Griess reagent (50 µL each) were added to a 96-well plate. The measurements of absorbance were conducted at a wavelength of 550 nm using a Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Finland) with SkanIt software (version 2.4.2, Thermo Fisher Scientific, Finland). Nitrite concentrations were calculated from a sodium nitrite standards calibration curve.

4.2.7 Statistics

All experiments were performed at least three times with statistical analysis accomplished using GraphPad prism 4.0a Software (GraphPad Software Inc., USA). Data were reported based on mean ± standard deviation (SD) of three sets of experiments. Data sets with multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Tukey’s and Dunnett’s post-hoc test. p < 0.05 was considered to be statistically significant.
4.3 Results and Discussion

4.3.1 Characterization of the microspheres

Ac-Rb$_1$-loaded PLGA microspheres were prepared comparing both a conventional double emulsion method and by microfluidics. The experimental parameters and characteristics of the examined formulations of microspheres are listed in Table 4.1.

Table 4.1. Formulation parameters and characteristics of ac-Rb$_1$-loaded PLGA microspheres with microfluidic and conventional double emulsion methods.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>disperse phase flow (mL/min)</th>
<th>continuous phase flow (mL/min)</th>
<th>average size (µm)</th>
<th>Std. Dev. (µm)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF-1</td>
<td>0.01</td>
<td>0.05</td>
<td>*n.a</td>
<td>*n.a</td>
<td>*n.a</td>
</tr>
<tr>
<td>MF-2</td>
<td>0.01</td>
<td>0.08</td>
<td>59</td>
<td>6</td>
<td>96.7</td>
</tr>
<tr>
<td>MF-3</td>
<td>0.01</td>
<td>0.1</td>
<td>57</td>
<td>5</td>
<td>96.5</td>
</tr>
<tr>
<td>MF-4</td>
<td>0.01</td>
<td>0.15</td>
<td>40</td>
<td>9</td>
<td>93.3</td>
</tr>
<tr>
<td>MF-5</td>
<td>0.01</td>
<td>0.2</td>
<td>7</td>
<td>2</td>
<td>75.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>First emulsion</th>
<th>Second emulsion</th>
<th>average size (µm)</th>
<th>Std. Dev. (µm)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE-6</td>
<td>800</td>
<td>400</td>
<td>48</td>
<td>19</td>
</tr>
<tr>
<td>DE-7</td>
<td>1000</td>
<td>400</td>
<td>41</td>
<td>24</td>
</tr>
<tr>
<td>DE-8</td>
<td>1200</td>
<td>400</td>
<td>39</td>
<td>15</td>
</tr>
</tbody>
</table>

* under this experimental condition, no microsphere was formed.
Figure 4.4a-c shows the size and morphology of typical samples produced from the double emulsion method by SEM and ZIESS light microscopy. The samples have a spherical morphology with a broad size distribution of 10-70 µm. The size distribution of the microspheres was analyzed by using Image J software and are displayed in Figure 4.5, revealing an average size of 39-48 µm with a standard deviation of 15-24 µm. The size of the microspheres decreased with increasing stirring speed, agreeing well with the results reported in the literature [23].

Figure 4.4. PLGA microspheres from double emulsion method a, b) Scanning Electron Microscopy results and c) Zeiss Light Microscopy results.

![Figure 4.4a](image1.png)

![Figure 4.4b](image2.png)

![Figure 4.4c](image3.png)
Figure 4.5. Image J number of size distribution for ac-Rb1-loaded PLGA microsphere: a) DE-6, b) DE-7 and c) DE-8.

Figure 4.6a-c shows the size and morphology of microspheres produced from the microfluidic method by SEM and ZIESS light microscopy. The samples have a similar spherical morphology to the microspheres prepared using the double emulsion method, however the microfluidic approach gave much more uniform microspheres (50-65 µm) compared to the double emulsion method (10-70 µm). Also, Figure 4.7 shows the size distributions of the microspheres which were obtained using Image J software, showing a uniform distribution with standard deviations between 2 µm and 9 µm.

Figure 4.6. PLGA microspheres from microfluidic method where the disperse phase flow included ac-Rb1 solution emulsified in PLGA organic solvent solution, and continuous phase flow contained 2% PVA aqueous solution. The continuous phase flow rate was 0.1 mL/min while the disperse phase flow rate was 0.01 mL/min. a, b) Scanning Electron Microscopy results and c) Zeiss Light Microscopy results.
In the microfluidic approach, the flow rate ratio of the continuous phase to the disperse phase had a significant effect on the formation of microspheres. It should be pointed out that increasing the disperse phase flow rate from 0.001 mL/min to 0.01 mL/min led to blocking of the inlet microchannel, preventing the formation of microspheres. Also, when the disperse phase flow rate was higher than 0.01 mL/min and higher than the continuous phase flow rate, microsphere particles were not formed. The relationship between the average microsphere size and the continuous phase flow rate is given in Figure 4.8. As the flow rate of the continuous phase increased from 0.08 mL/min to 0.20 mL/min, while that of the disperse phase was kept at 0.01 mL/min, the average size of the formed microspheres decreased from 59 µm to 7 µm. However, by lowering the flow rate of the continuous phase to 0.05 mL/min, no microspheres were formed. Our experimental results show that the microsphere size obtained with the microfluidic method are tunable.
by changing the flow rate, although only a narrow experimental window enabled microsphere formation.

![Graph showing microsphere size vs. continuous phase flow rate](image)

**Figure 4.8.** Effect of continuous phase flow rate on the microsphere size at the disperse phase flow rate of 0.01 mL/min.

In order to provide a better understanding of these results, the Reynolds number (Re) and the capillary number (Ca) need to be considered. Flow in microfluidic systems usually have a low value of Reynolds number which indicates that the flow is laminar. The Reynolds number defined as: 

\[ Re = \frac{\rho ud}{\mu} \]

where \( \rho \) is the density of the continuous phase, \( u \) is the velocity of the continuous phase, \( d \) is the characteristic length scale (e.g. the hydraulic diameter of the channel cross-section), and \( \mu \) is the viscosity of the continuous phase. The dimensionless capillary number has a significant role in determining the droplet break off. The capillary number is defined as: 

\[ Ca = \frac{\mu u}{\gamma} \]

where \( \mu \) is the viscosity of the continuous phase, \( u \) is the velocity of the continuous phase, and \( \gamma \) is the interfacial tension between the oil and water phases. In the microfluidic system, the formation of microspheres is governed by the competition between the viscous forces and the interfacial forces. At high values of \( Ca \) (greater than 0.01), the viscous forces play a significant role in the process of the microsphere break-up in the junction. As the droplet...
enters the main channel, it is exposed to a viscous force from the continuous phase to the point that it breaks off near the junction without blocking the entire cross-section, which is called the shearing regime [24]. At low values of $Ca$ (typically less than 0.01), interfacial forces dominate over viscous forces. As a result, the viscous force on the interface of the droplet which enters the main channel are not adequate to deform it. Consequently, the droplet blocks almost the whole cross-section of the main channel. If the flow rate of the disperse phase is higher than the flow rate of the continuous phase, the droplet is elongated and the thin film was created from the extending neck. Ultimately, break-up occurs after the disconnection of the dispersed phase from the junction due to the pressure drop across the droplet. However, when the disperse phase flow rate is lower than the continuous phase flow rate, the droplet does not find enough time to elongate and break-up happens directly after the droplet blocks the entire cross-section of the main channel. In this study, we chose the disperse flow rate lower than the continuous phase flow rate so the disconnection occurs at the junction which is believed to be a more stable process in the droplet formation [25]. Table 4.2 shows the values for $Ca$ and $Re$ numbers used in this work which were calculated based on the utilized microfluidic device geometry and the continuous phase properties. The low values of Reynolds number confirm that the flow is laminar. The results show that $Ca$ is lower than 0.01 which according to the literature [24] implies that the size of microspheres is independent of $Ca$ and mainly determined by the ratios of the disperse and the continuous phases flow rates.
Table 4.2. Values of Capillary (Ca) and Reynolds (Re) at different continuous phase flow rate ($Q_c$).

<table>
<thead>
<tr>
<th>Qd (mL/min)</th>
<th>Qc (mL/min)</th>
<th>Ca</th>
<th>Re</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.05</td>
<td>1.7×10⁻³</td>
<td>6.18</td>
</tr>
<tr>
<td>0.01</td>
<td>0.08</td>
<td>2.7×10⁻³</td>
<td>9.89</td>
</tr>
<tr>
<td>0.01</td>
<td>0.1</td>
<td>3.4×10⁻³</td>
<td>12.37</td>
</tr>
<tr>
<td>0.01</td>
<td>0.15</td>
<td>5.1×10⁻³</td>
<td>18.55</td>
</tr>
<tr>
<td>0.01</td>
<td>0.2</td>
<td>6.8×10⁻³</td>
<td>24.74</td>
</tr>
</tbody>
</table>

There are several numerical methods using computational fluid dynamics (CFD) that have been developed to model microsphere formation and the mechanism of droplet formation [24, 26, 27]. Accurate simulation however remains challenging as it deals with microsphere formation of a disperse phase within a continuous phase which is an unsteady process of moving fluid-fluid interfaces and is therefore a complex flow problem [25]. Figure 4.9 represents the preliminary results showing the above explained phenomena in this studied condition when the disperse flow rate is lower than the continuous phase flow rate. For this case, the droplet does not have time to elongate and therefore break-up happens immediately after the droplet blocks the entire cross-section of the main channel as is shown in Figure 4.9. However, future investigation is needed to simulate the process and mechanism of microsphere formation which is a complex phenomenon.
Figure 4.9. Schematic of T-junction microchannel. Where the disperse phase flow included ac-Rb<sub>1</sub> solution emulsified in PLGA organic solvent solution (red) and continuous phase flow contained 2% PVA aqueous solution (blue). The continuous phase flow rate was 0.1 mL/min while the disperse phase flow rate was 0.01 mL/min. The droplet does not have time to elongate and break-up happens immediately after the droplet blocks the entire cross-section of the main channel.

4.3.2 Encapsulation of ac-Rb<sub>1</sub> within PLGA microspheres

In order to measure the ac-Rb<sub>1</sub> concentration encapsulated within the PLGA microspheres required to determine encapsulation efficiency and subsequent drug release, UV spectrometry was utilized as shown in Figure 4.10. A standard calibration curve of ac-Rb<sub>1</sub> in the range of 100 µg/mL to 300 µg/mL was plotted by measuring the absorbance as a function of concentration. The ac-Rb<sub>1</sub> has a maximum absorbance at 203 nm. The relation between the concentration and the intensity of absorbance can be described as shown in Figure 4.10.
Figure 4.10. UV-Vis absorption spectra and the calibration curve of standard ac-Rb₁.

Figure 4.11 shows the encapsulation efficiency calculated for microspheres obtained from both the double emulsion and microfluidic methods. According to Equation (4.1) and Figure 4.11, the ac-Rb₁ encapsulation efficiency of 96.7%, 96.5%, 93.3% and 75.2% was obtained using four formulations of MF-2, MF-3, MF-4 and MF-5, respectively. For the microfluidic method, the ac-Rb₁ encapsulation efficiency increased with increasing average size of the microspheres. However, microspheres obtained from the conventional double emulsion method gave a lower encapsulation efficiency of 77.5% - 78.8% with the narrowest size distribution obtained using DE-8 at 10 - 70 µm.
Figure 4.11. Encapsulation efficiency of the microspheres obtained from a) double emulsion method and b) microfluidic method.

These results (Table 4.1 and Figure 4.7) reveal that compared to the double emulsion method, more uniform microspheres with higher encapsulation efficiency were obtained using the microfluidic method. These results are in agreement with those reported in the literature where the poly(L-lactic acid) polymeric microspheres obtained from microfluidics were significantly better in terms of encapsulation efficiency than the microspheres fabricated by other methods such as conventional double emulsion method, spray drying and supercritical anti-solvent methods [17]. Encapsulation efficiency is an important factor in drug delivery, especially for valuable and expensive bioactive compounds. Formulation MF-3 generated the most uniform microspheres with the narrowest size distribution (Figure 4.7b) with an average size of 57 µm and EE% of 96.5. It was selected for further development of the drug delivery systems as examined in more detail below.

To confirm the presence of ac-Rb₁ in the PLGA microspheres, the FTIR spectra of ac-Rb₁, pure PLGA, and ac-Rb₁ loaded microspheres are compared in Figure 4.12. In the FTIR spectrum of the ac-Rb₁ (Figure 4.12a), the peaks at 1726 and 1021 cm⁻¹ are attributed to the C=O and C-O stretching vibrations of an acetic ester group, respectively. Also, the peaks at 3304 and 1643 cm⁻¹ are assigned to the OH of the sugar moieties in the ac-Rb₁ [6]. In the FTIR spectrum of pure PLGA (Figure 4.12b), the peak at 1740 cm⁻¹ is assigned to the absorbance of C=O in PLGA [28-30]. The spectrum of ac-Rb₁ loaded PLGA microspheres (Figure 4.12c) showed all the peaks appearing in the spectrum of
pure PLGA and additional hydroxyl peaks from ac-Rb$_1$ at 3303 and 1643 cm$^{-1}$, indicating successful encapsulation of ac-Rb$_1$ in the PLGA matrix. It should be noted that in the encapsulation process of ac-Rb$_1$ in PLGA microsphere, the products were freeze-dried at the end to eliminate any residual water which may have similar peaks in the region of 3304 and 1643 cm$^{-1}$.

Figure 4.12. FTIR results for a) ac-Rb$_1$; b) pure PLGA; c) ac-Rb$_1$ encapsulated in PLGA microsphere obtained from microfluidic method.

In order to investigate the physical state of the encapsulated ac-Rb$_1$ in the microspheres, DSC and XRD were employed [31]. DSC thermograms of ac-Rb$_1$, ac-Rb$_1$-loaded PLGA micropheres, and placebo PLGA microspheres are shown in Figure 4.13A. Ac-Rb$_1$ shows a sharp endothermic peak (Figure 4.13A-a) at 54 °C attributed to the melting point of ac-Rb$_1$. A small peak at 99°C is due to the loss of moisture. In Figure 4.13A-c, the placebo microspheres show two endothermic peaks at 51°C and 100°C which are due to the PLGA glass transition temperature ($T_g$) [32, 33] and the loss of moisture, respectively. In Figure 4.13A-b, the ac-Rb$_1$-loaded microspheres demonstrate all the endothermic peaks available in the thermograms of placebo microspheres, however there is no peak
corresponding to ac-Rb$_1$, which is consistent with molecular-level dispersion of ac-Rb$_1$ in the polymer microspheres. The amorphous state of ac-Rb$_1$ in the microspheres was also confirmed by XRD (Figure 4.13B), as the crystalline peak at 2θ=4°, which shows the semi-crystalline nature of ac-Rb$_1$, disappeared after being encapsulated within the microspheres.

**Figure 4.13.** A) DSC thermograms of a) ac-Rb$_1$, b) ac-Rb$_1$-loaded PLGA microspheres obtained from microfluidic method, and c) placebo PLGA microspheres obtained from microfluidic method; B) XRD patterns of: a) ac-Rb$_1$-loaded PLGA microspheres obtained from microfluidic method, b) placebo PLGA microspheres obtained from microfluidic method, and c) ac-Rb$_1$.

### 4.3.3 In Vitro ac-Rb$_1$ release and release kinetics

Release kinetics of the ac-Rb$_1$ from both the monodisperse and polydisperse microspheres are compared in Figure 4.14. Each point on this figure represents the average of three experimental data points. The ac-Rb$_1$ release profile shows faster release kinetics in the first 100 h followed by a slow steady release from 100 h to 400 h. The observed burst effect may be due to ac-Rb$_1$ molecules that are at or near the PLGA microsphere surface. Our FTIR results showed that ac-Rb$_1$ was observable, which would indicate some surface enrichment, although the XRD indicated molecular integration, thus either complete micromixing or some surface dispersion cannot be ruled out. As can be seen in Figure 4.14B, the initial burst release of ac-Rb$_1$ from the monodisperse
microspheres in the first 100 h is considerably smaller than that from the polydisperse microspheres [19]. From Figure 4.14A, the total cumulative ac-Rb₁ release is 46% from the monodisperse microspheres and 81% from the polydisperse microspheres. The release profile of ac-Rb₁ from the monodisperse microspheres prepared by the microfluidic method is slower than that from the polydisperse samples. Although the large burst effect can release a therapeutic agent ac-Rb₁ relatively fast, it might also damage tissues around the treatment site [22]. Therefore the smaller burst effect may be advantageous.

**Figure 4.14.** A) Release profiles of ac-Rb₁ from ○ PLGA microspheres produced by double emulsion method (DE-8); ● PLGA microspheres produced by microfluidic method (MF-3); B) enlargement of release profile to magnify the burst effect. Releasing medium was PBS (pH=7.4) at 37°C with shaking at 50-100 rpm.
4.3.4 Bioactivity of released ac-Rb₁

Our results previously showed that ac-Rb₁ ginsenoside has potent immunosuppressive effects [6]. For the current study, the immunosuppressive activity of released ac-Rb₁ from both types of microspheres obtained via the microfluidic method and the double emulsion method was examined by bioassay analysis using macrophages in vitro. The effect of ac-Rb₁ treatment on LPS-stimulated NO and TNF-α production in macrophages can be seen in Figure 4.15. This figure shows that ac-Rb₁ released from the PLGA microspheres from both the microfluidic and double emulsion methods significantly inhibits the production of NO and TNF-α, in LPS-induced RAW 264.7 macrophages. This inhibition occurs in a dose-dependent manner within the dose range of 5-150 µg/mL released ac-Rb₁ extract. The immunosuppressive activity of ac-Rb₁ did not change significantly before encapsulation within the carrier and after release from the delivery carrier. Also, the microsphere preparation method does not have significant effect on the immunosuppressive activity of ac-Rb₁. Figure 4.15 also shows that the influence of ac-Rb₁ is inflammatory mediator-specific i.e. the magnitude of inhibition by ac-Rb₁ before encapsulation and after release from the delivery carriers was much greater with respect to NO production. The released ac-Rb₁ inhibited the LPS-induced NO production more significantly with the lowest level of NO production around 5 µM and 7 µM in presence of 150 µg/mL released ac-Rb₁ from PLGA microspheres produced by microfluidic method and double emulsion method, respectively.
Figure 4.15. Inhibitory effect of ac-Rb₁ released from microspheres obtained via (first row) microfluidic method and (second row) double emulsion method. Inhibitory effect of ac-Rb₁ on LPS-stimulated 24 hr macrophage production of a, c) NO and b, d) TNF-α. Murine macrophages (RAW 264.7 cells) were pre-treated with the ac-Rb₁ ginsenoside and dexamethasone (DEX) for two hours after which LPS (1 µg/mL) was added, and the culture supernatants were analyzed for NO and TNF-α by Griess reaction assay and ELISA, respectively. Dexamethasone (DEX) at 5 µM was used as a positive control. Three independent experiments were performed and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. #Values p<0.05 Significantly different from LPS-induced group.

4.4 Conclusions

Ac-Rb₁ was encapsulated in PLGA microspheres using different variations of the microfluidic method and conventional double emulsion method, with the first method generating microspheres of more uniform size. The microspheres were characterized by SEM, FTIR, DSC, and XRD to examine the morphology and the physical state of ac-Rb₁ in the microspheres. The monodisperse microspheres produced by a microfluidic method (50-65 µm) were found to exhibit a slower release rate and a smaller burst effect than the polydisperse microspheres prepared using a double emulsion method (10-70 µm). Moreover, the released ac-Rb₁ from delivery systems showed immunosuppressive effect on LPS-induced macrophages, revealing that the microsphere preparation method does not change the bioactivity of ac-Rb₁ in vitro. The bioactive plant extract ac-Rb₁
encapsulated within PLGA microsphere could provide many potential applications in pharmaceutical industries.
4.5 References


33 Rouse JJ, Mohamed F, van der Walle CF. Physical ageing and thermal analysis of PLGA microspheres encapsulating protein or DNA. International journal of pharmaceutics. 2007;339:112-120.
Chapter 5

5 PLGA Microsphere/Gelatin Hydrogel Combination Systems for the Delivery of acetyl ginsenoside Rb₁

Abstract

Recently we showed that 6’’-O-acetylginsenoside Rb₁ (ac-Rb₁) could be isolated from North American ginseng, and that is showed potent immnosuppressive activity using in vitro studies (Chapter 2). However, application of ginsenosides using conventional herbal release strategies has been limited due to their low oral bioavailability and cytotoxicity. In the present study, a drug delivery system for release of ac-Rb₁ was developed and investigated for the first time by forming core-shell structures of biodegradable poly (DL-lactic-co-glycolic acid) (PLGA) microspheres as the core with a gelatin hydrogel shell. The core-shell systems were characterized by various physico-chemical testing (SEM, FTIR, DSC, TGA). Release profiles were studied and quantified by UV-Vis spectrophotometry showing that the release of ac-Rb₁ from the gelatin shell slowed any burst effect and allowed tuning of the release profile by controlling the cross-linking density. The core-shell structures releasing ac-Rb₁ showed potency toward immnosuppressive activity using macrophages in vitro. The release data results were further analyzed using empirical equations which followed a Fickian diffusion release mechanism that could be controlled by tuning these hybrid structures. This approach shows utility for the development of next-generation drug-release devices using ginsenosides.
5.1 Introduction

Recent interest has turned to plants as a source of either new drugs or of compounds from which more efficacious or less toxic pharmaceutics can be developed based on their long-standing use in traditional medicine [1]. These compounds are believed to work more slowly and gently than pharmacologic drugs, and are less likely to cause serious side effects and toxicity [2-4]. Of the well known traditional Chinese medicines with biological activity, ginseng has been widely used as a medicinal herbal plant [5]. Similar to Asian ginseng, North American ginseng is also bioactive in which the main active components are ginsenosides, which have been shown to have various beneficial health effects including anti-inflammatory, anti-stress, and anti-cancer properties [6-8].

Recently, interest has shifted to study the effects of individual ginsenosides [6, 9-11] due to their unique therapeutic properties which may play a role in next-generation pharmaceutics [12, 13]. For instance, ginsenoside Rb₁ which is the major ginsenoside in North American ginseng, has exhibited inhibition of proinflammatory cytokine responses for both lipopolysaccharide (LPS)-induced interleukin (IL)-6 and tumor necrosis factor (TNF)-α production in mice [14, 15]. This ginsenoside has also shown anti-angiogenic activity in-vivo, inhibiting the growth of new blood vessels and thus is being used to treat cancer [10]. Moreover, ginsenosides Rb₁ and Rg₁ have been examined as efficient neuroprotective agents for spinal cord neurons, while ginsenoside Re did not exhibit such activity [11]. We have been investigating 6”-O-acetylgsenoside Rb₁ (ac-Rb₁), which was isolated from North American ginseng root, giving potent immunosuppressive properties [16]. It has also been reported that transformed ginsenosides and ginsenoside metabolites have shown more potency toward cytotoxic activity than neutral ginsenosides [17, 18]. However, the oral bioavailability of ginsenosides from the gastrointestinal (GI) tract is known to be low, i.e. 5% or less. This is because the ginsenosides are extensively biotransformed in the human GI tract by enzymes and intestinal bacteria to other ginsenoside-like compounds such as compound K, which is also biologically active [13]. To date, no work has been done in the delivery of individual ginsenosides with different biological activities.
Different formulations such as polymeric nanoparticles, nanocapsules, liposomes, and microspheres have been investigated for bioactive and plant extract delivery [19-21]. For ginseng extracts, tablets and capsules are available on the market [22]. Recently Xiao et al. [23] developed an injectable biocompatible hydrogel system that can release dexamethasone which has similar immunosuppressive activity as ginsenoside ac-Rb₁ (which in Chapter 2 is used as a control for studying the inhibitory effect of ac-Rb₁). This hydrogel injected into joint tissue could release drug in response to mechanical forces. Non-oral delivery systems are of interest for ginsenosides to be delivered via other administration routes such as intranasal, injection or transdermal patches to take advantage of these immunosuppressive and anti-angionenic properties [12, 24-26]. In designing these new delivery systems for ginsenosides, similar to conventional drug delivery systems, a high encapsulation efficiency, controlled release behavior, superior bioavailability, low toxicity, and low side effects are required [19, 20, 27]. Potential delivery carriers for bioactive compounds of plant extracts are polymer microspheres such as poly-α-cyanoacrylate alkyl esters, polyvinyl alcohol, natural polymers (albumin, gelatin and vegetable protein) or polysaccharides (cellulose, starch and its derivatives, alginate, chitin and chitosan) [20]. PLGA-based microspheres are of particular interest due to PLGA’s excellent biocompatibility, complete biodegradability, FDA compliance, and easy administration [28]. However, the effectiveness of PLGA microspheres may be limited by continued removal by macrophages [29, 30]. Gelatin, a natural polymer derived from collagen with a variety of pharmaceutical and medical applications, has been extensively used as a hydrogel carrier for site-specific drug delivery via different administration routes such as intranasal, injection or transdermal [31]. However, release from gelatin hydrogels can have high initial burst rates at the beginning before slowing to a low, constant level. While the burst effect can provide a therapeutic function in the treatment of acute disease, it can also lead to tissue damage around the injection or dermal site. Therefore, a microsphere/hydrogel combination system could be a better choice to achieve localized delivery which would take advantage of both individual carriers, providing a tunable delivery approach, as previously shown by Liu et al. [28].

Previously in Chapter 4 [32], we examined the encapsulation of ac-Rb₁ within PLGA microspheres prepared by both a microfluidic and a double emulsion method. In this
study, the microspheres containing ac-Rb₁ were embedded within a gelatin hydrogel to form core-shell structures with tunable shells. The release behavior of ac-Rb₁ from the gelatin encapsulated hybrids was investigated in simulated body fluid at 37°C by UV-Vis spectrometry. Their immunosuppressive activity using macrophages in vitro was also confirmed. The kinetics of ac-Rb₁ release from these systems was compared using empirical equations (Hixon-Crowell, Korsmeyer-Peppas, Weibull, and first-order models) to determine the best mechanism of experimentally obtained in vitro release data.

5.2 Materials and methods

5.2.1 Materials
6”-O-acetylginsenoside Rb₁ (ac-Rb₁) was isolated and fractionated from North American ginseng root as previously described [16]. Gelatin from porcine skin, glutaraldehyde 25% in H₂O, 0.1 mol/L phosphate buffered solution (PBS, pH 7.4), dichloromethane (DCM) of HPLC grade (>99.9%), methanol, ethanol, acetic acid, and ammonium hydroxide were purchased from Sigma-Aldrich, Canada and used as received. Purified water was produced from a Milli-Q water purification system (18.2 MΩ·cm resistivity, Barnstead EasyPureII, Thermo Scientific, USA). RAW 264.7 (ATCC TIB 67) murine macrophage cell lines were provided by Dr. Jeff Dixon (Department of Physiology and Pharmacology, Western University, Canada). BD OptEIA ELISA kits tumour necrosis factor-α were provided by BD Biosciences (Bedford, MA, USA). LPS (Lipopolysaccharides) from Escherichia coli (0111:B4) purity>99%, Dexamethasone purity>98% and Griess reagent were purchased from Sigma-Aldrich (USA). Cell culture medium and reagents were purchased from Gibco laboratories (USA).

5.2.2 Preparation of gelatin hydrogel shell
PLGA microsphere cores containing ac-Rb₁ were prepared as previously described [32]. The monodisperse microspheres prepared from the microfluidic approach were used for further delivery and release studies due to their narrow size distribution and more
uniform morphology. The schematic illustration of the process for preparing ac-Rb$_1$ loaded PLGA microsphere encapsulated into gelatin hydrogel is shown in Figure 5.1. For core encapsulation, 5% (w/w) of gelatin aqueous solution was prepared by dissolving gelatin powder in distilled water at 55-60 °C for 2 hr. Then 10 mg of ac-Rb$_1$-loaded PLGA microspheres were dispersed in 500 µL of distilled water, and added to 4 mL of the above gelatin solution with stirring. Glutaraldehyde 25%, was examined as a crosslinking agent at various concentrations: 0, 10, 50, and 100 µL by adding dropwise under stirring at room temperature to form stable gels. The product was washed with double distilled water and then centrifuged at 4500 rpm for 10 min followed by washing with de-ionized water 3X to separate the solid gels, which were subsequently poured into Petri dishes, freeze-dried, then stored in a desiccator.

![Figure 5.1](image)

**Figure 5.1.** Schematic illustration of the process for preparing ac-Rb$_1$ loaded PLGA microsphere encapsulated into gelatin hydrogel.

### 5.2.3 Characterization of core-shell structures

The surface morphology of the gelatin coated core-shell systems were determined using scanning electron microscope (Hitachi S-4500). Samples were sputtered with gold coating, then placed on a copper stub. Melting points were measured by a DSC Q200 (TA Instrument, New Castle, Delaware, USA). The scanning rate was 10 °C/min, with a scanning temperature range of 25–250 °C. Thermal degradation measurements were carried out using a TGA (TA Instrument, New Castle, Delaware, USA). Samples were heated from 35 °C to 700 °C at a constant heating rate of 10 °C /min under nitrogen
atmosphere. ATR-FTIR (Nicolet 6700, Thermo Scientific) was used to investigate the cross-linked core-shell composites in the range of 500-4000 cm\(^{-1}\).

5.2.4 Swelling behavior of gelatin hydrogel

To evaluate the swelling behavior of the gelatin hydrogels, the dry hydrogel was weighed, then immersed in different buffer solutions with various pH's. Then, the wet hydrogel solution was poured onto a Petri dish for 30 seconds to eliminate the bulk solution. The wet samples were then weighed to measure the swelling ratio of the hydrogel. The effect of crosslinker amount on the swelling behavior of the gelatin hydrogel was evaluated to determine the releasing kinetics mechanism. The swelling ratio was calculated according to the following equation

\[
\text{Swelling \%} = \left(\frac{W_w - W_d}{W_d}\right) \times 100, \tag{5.1}
\]

where \(W_w\) and \(W_d\) represent the weights of wet and dry gelatin hydrogels, respectively. Each experiment was carried out in triplicate.

5.2.5 Kinetic release studies of ac-Rb\(_1\)

The ac-Rb\(_1\) concentration encapsulated within the PLGA microspheres was measured using a UV spectrophotometer (Shimadzu UV-Vis 3600) in triplicate at 203 nm. First, a standard calibration curve of ac-Rb\(_1\) was established using the UV spectrophotometer by measuring the absorbance as a function of concentration. To measure the ac-Rb\(_1\) content after PLGA encapsulation, 5 mg of ac-Rb\(_1\)-loaded microspheres was dissolved in 1 mL of DCM, which was subsequently evaporated with the residue then dissolved in 1 mL of methanol. The ac-Rb\(_1\) content was analyzed based on the calibration curve established from ac-Rb\(_1\) standard solution mixtures. To measure the release of ac-Rb\(_1\) from the core-shell system, the crosslinked gelatin core-shells containing ac-Rb\(_1\)-loaded PLGA cores were placed in a 6-well tissue culture plates (Corning), and incubated with PBS (pH=7.4) at 37\(^{\circ}\)C. At predetermined time intervals, the supernatant was removed from each well and replaced with fresh PBS. The ac-Rb\(_1\) content in the supernatant was determined at a wavelength of 203 nm using a UV spectrophotometer (Shimadzu UV-Vis 3600). All
experiments for each time interval were repeated in triplicate. The cumulative release of ac-Rb$_1$ profiles were acquired with the volume loss correction.

5.2.6  Bioactivity of released ac-Rb$_1$

The mouse macrophage cell line RAW 264.7 was cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 25 mM HEPES, 2 mM Glutamine, 100 IU penicillin/ml, and streptomycin 100 µg/mL. The pH of the medium was around 7.4. The cells were kept at 37°C in a humidified incubator with 5% CO$_2$, and seeded in 96-well tissue culture plates at a density of 1.5×10$^5$ cells per well. Our previous study showed that ac-Rb$_1$ has potency toward immunosuppressive activity on murine macrophages [16].

Also, based the results in Chapter 4, the preparation method of the PLGA microspheres does not have significant effect on the inhibitory effect of ac-Rb$_1$. In this study, the inhibitory effect of released ac-Rb$_1$ from the core-shell system in vitro was evaluated. To examine the inhibitory effect of released ac-Rb$_1$, 5-150 µg/mL of ac-Rb$_1$ and released ac-Rb$_1$ from PLGA microsphere/ gelatin hydrogel crosslinked 50 µL were added to the macrophages 2h prior to the addition of LPS. In this study, dexamethasone (DEX) was used as a positive control for the suppression of LPS-induced stimulation of macrophages. So, cultured macrophages were incubated with DEX (5 µM) for 2 hr prior to the addition of LPS. The 24-hour cytokine production induced by LPS was determined by measuring NO and TNF-α levels in the culture medium. TNF-α concentrations in supernatants from cultured cells were analyzed with ELISA. Samples were assessed with mouse cytokine-specific BD OptEIA ELISA kits (BD Biosciences, USA) according to the manufacturer's protocol. Sample nitrite concentrations in culture medium were determined by means of Griess reagent (0.5% sulfanilic acid, 0.002% N-1-naphtyl-ethylenediamine dihydrochloride, 14% glacial acetic acid). Culture supernatant of each sample and the Griess reagent (50 µL each) were added to a 96-well plate. The measurements of absorbance were conducted at a wavelength of 550 nm using a Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Finland) with SkanIt software (version 2.4.2, Thermo Fisher Scientific, Finland). Nitrite concentrations were calculated from a sodium nitrite standards calibration curve.
5.2.7 Statistics

All experiments were performed at least three times with statistical analysis accomplished using GraphPad prism 4.0a Software (GraphPad Software Inc., USA). Data were reported based on mean ± standard deviation (SD) of three sets of experiments. Data sets with multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Tukey’s and Dunnett’s post-hoc test. p < 0.05 was considered to be statistically significant.

5.2.8 Model fitting of *in vitro* release of ac-Rb₁

In order to investigate the kinetics of ac-Rb₁ release from the PLGA microspheres as well as ac-Rb₁ release from the PLGA microspheres/gelatin hydrogel combination system, four commonly used empirical models were used to fit the *in vitro* release data, i.e. the Hixson-Crowell, Korsemeyer-Peppas, Weibull and first-order models [4, 33-35]. The curve fitting toolbox of Matlab R2010a software was used to determine both the regression coefficients, $r^2$, and the best fit model parameters to understand the release mechanism for the studied systems.

5.3 Results and Discussion

5.3.1 Synthesis and characterization of the delivery systems

PLGA microspheres loaded with ac-Rb₁ were used in the preparation of core/hybrid composite delivery systems by adding gelatin and glutaraldehyde crosslinker. The morphology of the prepared delivery system containing the microspheres and the gelatin shell was examined by SEM and is shown in Figure 5.2. The morphology of the ac-Rb₁-loaded PLGA microspheres did not change after being entrapped in the gelatin. In addition, it can be seen from the SEM images that the microspheres were uniformly distributed within the gelatin hydrogel structure.
Figure 5.2. Scanning Electron Microscopy results of a) PLGA microspheres obtained from microfluidic method and b) gelatin hydrogels cross-linked by 50 µL glutaraldehyde embadded PLGA microspheres obtained from microfluidic method.

To examine the chemical interactions between the gelatin hydrogel and glutaraldehyde (crosslinker), FTIR, DSC, and TGA were used. The FTIR spectra of non-crosslinked gelatin hydrogel and crosslinked gelatin hydrogel are displayed in Figure 5.3. The gelatin spectrum gives absorption bands at peaks around 3300, 2900, 1630 and 1520 cm\(^{-1}\) which are assigned to -OH, amine (N-H), amide I (C=O) and amide II (NH\(_2\)) functionalities, respectively. Our results show that in addition to the previously mentioned peaks, the crosslinked gelatin hydrogel gives a peak at around 1400 cm\(^{-1}\) assigned to the aldimine, which is not seen in the non-crosslinked gelatin hydrogel. This aldimine peak has been previously assigned at 1450 cm\(^{-1}\) whereas the two main amide peaks have been reported at 1650 and 1540 cm\(^{-1}\) [36-38].
**Figure 5.3.** FTIR results for a) non-crosslinked and b) crosslinked gelatin hydrogel. The crosslinked hydrogel was prepared by using 100 uL of glutaraldehyde.

In order to better understand the gelatin crosslinking reaction, DSC was carried out. As can be seen in Figure 5.4A, two glass-transition temperatures (T_g’s) for gelatin are observed. The minor T_g is at 97°C which is related to the T_g of α-amino acid blocks in the peptide chain [36]. This T_g detection is complicated by moisture loss of the hydrophilic gelatin, which occurs in the same temperature range. The second T_g is at 135°C which is related to the blocks of imino acids, i.e. proline, hydroxyproline, and glycine, which mainly control the overall physical behavior of gelatin [36]. The two T_g’s are shifted from 97°C to 106°C and from 135°C to 155°C, respectively, as a result of crosslinking, which confirms the FTIR results.

TGA analysis was used to further examine the crosslinking reaction between gelatin hydrogel and glutaraldehyde crosslinker (Figure 5.4B). The first weight loss of around 5.5% and 5% happened in the temperature range of 35-135°C due to the loss of moisture
for non-crosslinked and crosslinked gelatin hydrogels, respectively. Additional weight loss occurred from 135-450°C due to breaking the polymer linkages and decomposition of the polymer backbone. Weight losses of around 65% and 59% were observed at 400°C for the non-crosslinked and crosslinked gelatin hydrogels, respectively. The addition of glutaraldehyde to the gelatin network increased the thermal stability of the gelatin hydrogel slightly, which is in agreement with the literature on the thermal degradation behavior of crosslinked proteins [39, 40].

**Figure 5.4.** A) DSC thermograms of non-crosslinked (dash blue line) and crosslinked gelatin hydrogel (solid red line). B) TGA traces of non-crosslinked (dash blue line), and crosslinked gelatin hydrogel (solid red line). The crosslinked hydrogel was prepared by using 100 µL of glutaraldehyde.

For potential application of these materials in oral formulations, for instance, ginsenoside Rg$_3$ has been shown effective in colon cancer [41] and our previous results using ac-Rb$_1$ showed anti-angiogenic and immunoactivity activities [16], swelling behaviour is of interest. The swelling performance of the gelatin hydrogel was studied with respect to the amount of crosslinker and also at different pH values. Figure 5.5A shows the swelling behavior of gelatin hydrogel in PBS (pH 7.4) as a function of time (t) prepared with different amounts of crosslinker. The results show that swelling significantly increased with time up to 300 min, which then reaches a maximum. Also, the gelatin hydrogel
swelling decreased upon increasing the degree of crosslinking attributed to a decrease in porosity and polymer chain elasticity of the hydrogels by adding crosslinker. Highly crosslinked hydrogels have a hardened matrix and show additional resistance to the penetration of water due to their network structure. These results are in good agreement with the literature showing that swelling of gelatin sponge decreased with an increasing degree of crosslinking [28]. Moreover, the swelling behavior of gelatin hydrogels containing ac-Rb₁-PLGA microspheres was investigated at different pH values including 1.2, 4.5, and 7.5 (Figure 5.5B). The results show that all systems gave similar swelling behavior; however, they revealed different water affinity at different pH values, attributed to the pendant acidic groups on the polymeric chains [42]. At the lowest pH investigated, i.e. 1.2, the acidic pendant group of gelatin was un-ionized (-COOH) so the water affinity was low. When the pH was increased to 7.4, the hydrogel swelled gradually with the increased pH due to ionization of acidic groups, i.e. forming -COO⁻ ions.
5.3.2  *In Vitro* ac-Rb₁ release and release kinetics

The release profile of ac-Rb₁ from the core-shell structures are examined and compared in Figure 5.6. The release behavior was monitored for 400 hr at pH = 7.4. The non-crosslinked gelatin hydrogels containing ac-Rb₁ swelled and dissolved in the release medium, with around 80% of ac-Rb₁ released within 1 h (Figure 5.6A-a). The release profile of ac-Rb₁ from the non-crosslinked gelatin hydrogels remained constant and at the same level for the entire release time. The release profile from the cross-linked gelatin hydrogels containing ac-Rb₁-PLGA microspheres was slow (Figure 5.6A-c). These results are in accordance with the reported results on bovine serum albumin (BSA), a model protein drug, where the investigators utilized PLGA microspheres prepared by a double emulsion method, encapsulated within alginate hydrogels. Their results showed that almost 10% of BSA was released from the combined system after 10 h (initial burst) whereas 25% of BSA was released after 2 weeks [29, 30]. Our results show a smaller burst effect (less than 10% within 10 h) attributed to the utilized monodisperse PLGA microsphere cores. In comparison with Figure 5.6A-b which consisted of ac-Rb₁ encapsulated PLGA microspheres, the initial burst effect and the releasing rate decreased.
Our results (Figure 5.6B) indicate that increasing the crosslinker amount decreased the ac-Rb\(_1\) release rate. This is attributed to the cross-linking reaction hardening the gelatin matrix, as shown by FTIR, DSC and TGA, which would enhance the resistance of ac-Rb\(_1\) release. As a result, increasing the amount of crosslinker decreased the ac-Rb\(_1\) diffusivity through the delivery system. Thus, the total cumulative ac-Rb\(_1\) release from the cross-linked gelatin hydrogels containing ac-Rb\(_1\)-PLGA microspheres was adjustable by controlling the amount of crosslinker, resulting in 24 - 35% release of ac-Rb\(_1\) after 400 h. Therefore, ac-Rb\(_1\)-PLGA microspheres-gelatin hydrogels can provide a powerful system for controlling the release for longer periods of time, which may have application for cancer treatment using implantable devices or for transdermal patches.
Figure 5.6. Release profiles of ac-Rb₁ from various delivery carriers; A) a)* gelatin hydrogel without crosslinker; b) PLGA microsphere obtained by microfluidic method; c) ▲ PLGA microsphere produced by microfluidic method / gelatin hydrogel crosslinked by 50µL GA; B) a) ■ PLGA microsphere/ gelatin hydrogel crosslinked by 10µL GA; b) ▲ PLGA microsphere/ gelatin hydrogel crosslinked by 50µL GA; c) ▼ PLGA microsphere/ gelatin hydrogel crosslinked by 100µL GA. Releasing medium was PBS (pH=7.4) at 37°C.

5.3.3 Bioactivity of released ac-Rb₁

Our results previously showed that ac-Rb₁ ginsenoside has potent immunosuppressive effects [16]. For the current study, the immunosuppressive activity of released ac-Rb₁ was examined by bioassay analysis using macrophages in vitro. The effect of ac-Rb₁ treatment on LPS-stimulated NO and TNF-α production in macrophages can be seen in Figure 5.7. This figure shows that ac-Rb₁ released from the PLGA microspheres/gelatin hydrogel carrier significantly inhibits the production of NO and TNF-α, in LPS-induced RAW 264.7 macrophages. This inhibition occurs in a dose-dependent manner within the dose range of 5-150 µg/mL released ac-Rb₁ extract. The immunosuppressive activity of ac-Rb₁ did not change significantly before encapsulation within carrier and after release from the delivery carrier. This indicates that the combination delivery system did not
significantly affect the immunosuppressive activity of ac-Rb₁. Figure 5.7 also represents that the influence of ac-Rb₁ is inflammatory mediator-specific i.e. the magnitude of inhibition by ac-Rb₁ before encapsulation and after release from the combination delivery carrier was much greater with respect to NO production. The ac-Rb₁ released from the delivery system of monodisperse PLGA microspheres combined with gelatin hydrogel after 400 h, inhibited the LPS-induced NO production more significantly with the lowest level of NO production around 7 µM in presence of 150 µg/mL released ac-Rb₁. In fact, the combination delivery system presents a promising tool of immunosuppressive ac-Rb₁ product delivery for next-generation biomedical agents in drug-release devices.

Figure 5.7. Inhibitory effect of ac-Rb₁ on LPS-stimulated 24 hr macrophage production of a) NO and b) TNF-α. Murine macrophages (RAW 264.7 cells) were pre-treated with the ac-Rb₁ ginsenoside and dexamethasone (DEX) for two hours after which LPS (1 µg/mL)
µg/mL) was added, and the culture supernatants were analyzed for NO and TNF-α by Griess reaction assay and ELISA, respectively. Dexamethasone (DEX) at 5 µM was used as a positive control. Three independent experiments were performed and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. #Values p<0.05 Significantly different from LPS-induced group.

5.3.4 Analysis of release data

To better understand the release mechanism from the microspheres and core-shell structures, we fit the ac-Rb₁ release curves from Figure 5.6 in Matlab™ to determine if the release behavior is controlled by Fickian diffusion, erosion or a combination of both. Four well known empirical models were examined, i.e. the Hixon-Crowell, Korsmeyer-Peppas, Weibull, and first-order models [4, 33-35]. According to the r values shown in Table 5.1, the Weibull model provides the best fit for the ac-Rb₁ release experimental data for all the examined delivery systems. The curves of the best fitting and the linear regression lines from the Weibull model are shown in Figure 5.8.

Table 5.1. The release model parameters of different carriers for ac-Rb₁ pH=7.4 polydisperse microsphere= Poly-MS; monodisperse microsphere= Mono-MS; glutaraldehyde= GA.
Almost comparable fitting with the Weibull model was observed in the case of using the Korsmeyer-Peppas model for ac-Rb\textsubscript{1} release from the core-shell combination system; however the fit was not as good for the ac-Rb\textsubscript{1} release from Poly-MS and gelatin hydrogel without crosslinker. A poor fitting was obtained by using the first order and Hixson-Crowell equations, which showed that they are not proper models for the systems of this study. From Table 5.1 and Figure 5.8, the slope of the regression line of the Weibull model is the constant $b$. According to the literature [43], for values of $b$ lower than 0.75, the release follows Fickian diffusion whereas for $b > 0.75$, other mechanisms such as erosion better describe the release. In this study, the results indicate that this value for the Poly-MS, Mono-MS, the gelatin hydrogel without crosslinker system and PLGA- gelatin hydrogel combination system is 0.31, 0.38, 0.15 and 0.54, respectively as
can be seen in Table 5.1. Therefore, the diffusion mechanism controlled the release of ac-Rb1 within the studied time-frame as best described using the Weibull and Peppas models [35, 44] and can be used for a long term drug delivery applications.
**Figure 5.8.** Release profiles (left) and fitting regression lines (right) by Weibull kinetic model for a, b) ac-Rb\textsubscript{1} release from PLGA microsphere produced by conventional method, c, d) ac-Rb\textsubscript{1} release from PLGA microsphere produced by microfluidic method, and e, f) ac-Rb\textsubscript{1} release from PLGA microsphere crosslinked gelatin hydrogel combination system.

### 5.4 Conclusions

The ac-Rb\textsubscript{1} loaded PLGA microspheres with the narrowest size distribution were incorporated into gelatin hydrogels for the development of bioactive natural extract ginsenoside ac-Rb\textsubscript{1} delivery system. The crosslink reaction of gelatin and glutaraldehyde was confirmed by FTIR, DSC and TGA. The slower and more sustained release profile is represented by the combination of microsphere crosslinked hydrogel delivery systems in comparison with the non-crosslinked system. The *in vitro* release kinetics data followed a Fickian trend with the best fit observed with Weibull model. Furthermore, the released ac-Rb\textsubscript{1} from PLGA/gelatin system showed immunosuppressive effect on LPS-induced macrophages which confirmed that the combination delivery system could retain the immunosuppressive activity of ac-Rb\textsubscript{1}. This novel system could be a promising tool for bioactive plant extract ac-Rb\textsubscript{1} delivery with immunosuppressive activity.
5.5 References


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Chapter 6

6 Conclusions and Recommendations

6.1 Conclusions

Ginseng root has been used for thousands of years in Eastern medicine and is one of the best-selling medicinal plants in the world [1]. *Panax ginseng* (Korean or Asian), *Panax quinquefolius* (American) are the two most well-known ginseng varieties around the world [2]. Although significant research is available on Asian ginseng [1, 2], the study of American ginseng and its components is not well reported. Like Asian ginseng, American ginseng is reported to have a variety of pharmacological effects, including effects on the central nervous system, cardiovascular system, immune system and cancer treatment [1, 3]. The qualitative and quantitative studies of bioactive compounds from medicinal plants such as ginseng mostly depend on the choosing of the appropriate extraction method [4, 5]. Extraction is the first step of any medicinal plant study that has an important role on the final result. Moreover, different chromatographic and spectrometric methods have been reported as a significant aid to the analysis and characterization of bioactive compounds obtained from extraction methods [6-10]. In recent years, different ginseng extracts are being used as nutraceutical and supplements world-wide. Also novel formulations have been reported for delivery of bioactive compounds extracted from medicinal herbal plants in order to deliver the bioactive plant extract to specific sites of action [11, 12].

In this study, the ultrasound assisted extraction method was used for extraction of ginsenosides from North American ginseng with DMSO and methanol as solvents at ambient temperatures from 25-33°C. Ginsenosides are believed to be thermally unstable compounds, therefore using a proper extraction method is important to obtain
ginsenosides which are bioactive. Our results showed that the choice of solvent has a significant effect on extraction of specific bioactive ginsenosides. Both DMSO and methanol were effective solvents for ultrasonic extraction of ginsenosides. However, DMSO showed higher extraction and better selectivity with a total ginsenoside yield of almost 10 (%, w/w) compared to a total ginsenoside yield of almost 8 (%, w/w) when using methanol as solvent. Moreover, aqueous solvents were found to increase the extraction efficiency in comparison with the pure solvents, with the best extraction efficiency obtained using 80/20 \( \frac{v_{\text{solvent}}}{v_{\text{water}}} \). Furthermore, large quantities of acetyl ginsenoside \( R_b_1 \) were found using ultrasonic extraction with DMSO, which was negligible in the case of using methanol and was not obtained using Soxhlet methanol extraction. Therefore, the presence of acetyl ginsenoside \( R_b_1 \) is related to the effect of extracting solvent and attributed to the high solubility parameter and temperature stability of DMSO. Although the solubility of acetyl ginsenoside \( R_b_1 \) is a complex phenomenon, the individual components of the solubility parameter of DMSO provide insight into how DMSO is a more effective solvent for extraction of acetyl ginsenoside \( R_b_1 \).

The large quantity of acetyl ginsenoside \( R_b_1 \) that were found using ultrasonic extraction with DMSO was fractionated and isolated using a preparative HPLC column and subsequently identified by MS, FTIR, and NMR as \( 6''-O \)-acetylginsenoside \( R_b_1 \). This compound was then tested for immunomodulation and anti-angiogenesis effects. Both 80% DMSO ginseng extracts and 80% methanol ginseng extracts were found to inhibit the production of NO and TNF-\( \alpha \) in LPS-induced RAW 264.7 macrophages in a dose-dependent manner with higher dose resulting in better inhibition. However, the DMSO extract caused more inhibition than the methanol extract for the production of NO and TNF-\( \alpha \) in LPS-induced RAW 264.7 macrophages. This was attributed to the presence of more acetyl ginsenoside \( R_b_1 \) in the DMSO extract which significantly inhibited the production of NO and TNF-\( \alpha \) in LPS-induced RAW 264.7 macrophage cells in a dose-dependent manner within the dose range of 5-150 \( \mu \)g/mL. Acetyl ginsenoside \( R_b_1 \) was also shown to have anti-angiogenic properties and exhibited potency towards inhibiting tube-like structure formation in endothelial cells. These findings suggest that the presence of the acetyl moiety has influence on biological activity.
Moreover, supercritical fluid chromatography coupled with an evaporative light scattering detector was employed in the separation of the SFE extracts of North American ginseng with 15-31% of methanol in supercritical CO$_2$ as the mobile phase. Pure CO$_2$ was a poor mobile phase for separation of the polar components of North American ginseng extract, particularly for the ginsenoside components of interest. This is attributed to the low solubility of the polar ginsenosides in pure CO$_2$. The effect of temperature and pressure on the separation of mixture of ginsenoside standard was tested with the best condition being found at T=50°C and P=150 bar. The retention time of the ginsenosides was altered by tuning the pressure and temperature. Under a constant pressure, the density of the mobile phase decreased with increasing temperature, leading to a lower solvating power of the mobile phase. The lower solubility of the ginsenosides in the mobile phase resulted in longer retention times. The retention time of all the ginsenosides decreased with increasing pressure from 100 to 200 bar at a constant temperature of 50°C. However, under a constant temperature, increasing pressure resulted in increased density and solving power of the mobile phase, leading to a shorter retention time. The effect of three types of additives including acidic TFA, basic DIPA, and ionic AA aqueous solution on the separation efficiency was examined, with the best selectivity being found in the case of the addition of 0.05% v/v TFA in methanol. By adding varying amount of additives, the retention time changed indicating the varying solubility of the extract fractions. A high-concentration fraction in the SFE extracts of North American ginseng was isolated with the SFC system and characterized with NMR, HPLC-RID, and ESI-MS. The identity of this unknown peak was confirmed to be the disaccharide sucrose, which has not been reported.

Research on ginsenoside delivery and release is of interest due to their unique biological activity. A new approach for a bioactive ginseng extract of acetyl ginsenoside Rb$_1$ delivery is presented. Encapsulation of acetyl ginsenoside Rb$_1$ within PLGA microspheres was performed with both a conventional double emulsion method and a microfluidic method. SEM and light microscopy analysis showed that all microspheres have similar spherical morphology, although the microfluidic approach produced more uniform size spheres (50-65 µm) compared to the more polydisperse microspheres from the w/o/w emulsion method (10-70 µm). The monodisperse microspheres produced by
the microfluidic method (50-65 µm) were found to exhibit a slower release rate and a smaller burst effect than polydisperse microspheres formed by the double emulsion method (10-70 µm). These microspheres can be used for drug delivery applications to maintain a therapeutic agent concentration for an extended time period. DSC was used to study of the thermal behavior of ac-Rb₁-loaded PLGA microspheres. Also, the XRD of ac-Rb₁-loaded PLGA showed that ac-Rb₁ is entrapped within PLGA microspheres in an amorphous state. DSC and XRD of the microspheres confirmed the molecular-level uniform distribution of ac-Rb₁ within the PLGA polymer. Moreover, a new delivery system consisting of PLGA microspheres embedded within gelatin hydrogels was developed for natural extract ginsenoside ac-Rb₁ delivery in vitro. The chemical interactions between gelatin hydrogel and crosslinker (glutaraldehyde) was confirmed by FTIR. The combination of microsphere crosslinked hydrogel delivery systems gave a lower burst effect and more sustained release than gelatin hydrogel without crosslinker. Also, in comparison with our previous system which consists of ac-Rb₁ encapsulated PLGA microspheres, the initial burst effect decreased. Moreover, the combination delivery systems' swelling behavior and release profiles were effected by the pH of the medium. At lower pH values i.e 1.2, gelatin's acidic groups remain un-ionized so gelatin shrinks gradually and the amount of cumulative release is decreased. However, by increasing the pH to 7.4, the weakly acidic gels become ionized leading to network swelling, increasing the release of ac-Rb₁. Also, Four empirical models, Hixon-Crowell, Korsmeyer-Peppas, Weibull, and first-order models [13-16] were used to fit the ac-Rb₁ release curves. The in vitro release kinetics data followed a Fickian trend with the best fit observed using the Weibull model. This indicates that the release mechanism is governed by diffusion during ac-Rb₁ release. Acetyl ginsenoside Rb₁ released from the combination delivery system was shown to give potency towards inhibition of NO and TNF-α production in LPS-induced macrophages. Hence, the combination delivery system presents a promising tool of immunosuppressive ac-Rb₁ product delivery for next-generation biomedical agents in drug-release devices.
6.2 Recommendations

The following recommendations will be useful in the future investigations of this study:

i) As the acetyl ginsenoside Rb₁ solubility in DMSO and methanol is a complex phenomenon, further investigation such as using vibrational circular dichroism (VCD) spectroscopy, in combination with density functional theory (DFT) calculations is required to consider other physicochemical properties of solvents and solute.

ii) Acetyl ginsenoside Rb₁ shows immunosuppressive and anti-angiogenesis activities on macrophages and human cell lines, respectively. Further \textit{in vivo} study of acetyl ginsenoside Rb₁ can be evaluated.

iii) Different SFC columns such as Ethylpyridine (2-Ethylpyridine-bonded silica), Diol (Propanediol-bonded silica), and Amino (Aminopropyl-bonded silica) can be used in comparison with Cyano (cyanopropyl-bonded silica) column to evaluate separation of polar and non-polar components of North American ginseng extract.

iv) Different SFC columns can be used in series to evaluate ginseng extracts separation.

v) The online mass identification of ginseng extracts can be evaluated by coupling SFC system with mass spectroscopy detector.

vi) The supercritical fluid chromatography of ginsenosides and other ginseng extract can be investigated quantitatively.

vii) PLGA with different molecular weight and different ratios of monomer can be studied to examine the release behavior to target release for specific disease treatments.

viii) Detailed study on microfluidic droplet formation and the effects of microchip geometry on microsphere size can be simulated with computational fluid dynamics (CFD).
ix) Rather than empirical mathematical models for studying the mechanism of acetyl ginsenoside Rb₁ release, mechanistic mathematical modeling which are based on real phenomenon such as diffusion, degradation, and erosion can be used to understand the release process.
6.3 References


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Appendices

Appendix A. Purity (%) of 6”-O-acetylginensoside Rb1.

The purity of 6”-O-acetylginensoside Rb1 was measured by HPLC system, using semi-preparative Atlantis T3 Waters column (10 x 250 mm with 5µm packing) at room temperature. A full loop injection was employed (100 µL) with the flow rate set at 4 mL/min and 6”-O-acetylginensoside Rb1 peak was detected by a UV detector at 203 nm. The mobile phase was a binary gradient of acetonitrile (A) and HPLC grade filtered water (B) at a constant composition of 35% (A) for 2 min followed by a linear gradient to 45% from 2–12 min. The column was flushed and equilibrated for 35 min before each analysis. Fractions obtained were evaporated under vacuum to isolate the unknown extract, 6”-O-acetylginensoside Rb1. Then, 6”-O-acetylginensoside Rb1 was re-injected to the system to calculate the purity based on the peak areas:

\[
\text{Purity} \% = \frac{A_{6”-O-acetylginensoside \ Rb1}}{\sum A} \quad (A.1)
\]

6”-O-acetylginensoside Rb1 (purity, >99%): White powder, mp 54 °C, ES-MS (m/z) 1149.66.

Note: Retention time shifted slightly by 0.28% - 10% for all individual ginsenoside standard and ginsenoside in ginseng extract.
Appendix B. Effect of methanol concentration on the retention of ginsenosides standard mixture at 50°C, 150 bar and a flow rate of 1.5 mL/min.

5% methanol

15% methanol

31% methanol

gradient 15-31%
Appendix C. Dimensionless number’s calculations.

The Reynolds number is defined as

\[ Re = \frac{\rho ud}{\mu} , \]  

(C.1)

where \( \rho \) denotes the density of the continuous phase, \( u \) is the average velocity of the continuous phase, \( d \) is the hydraulic diameter and \( \mu \) is the viscosity of the continuous phase. The hydraulic diameter is defined as

\[ d = \frac{4A}{P} , \]  

(C.2)

where \( A \) and \( P \) denote the cross sectional area and the wetted perimeter of the cross-section, respectively. The definitions for \( A \) and \( P \) take the form

\[ A = h \times w , \quad P = 2 (h + w) , \]  

(C.3a,b)

where \( h \) and \( w \) denote the height and width of the cross-section, respectively. The average velocity of the continuous phase can be calculated as follows

\[ u = \frac{Q}{A} , \]  

(C.4)

where \( Q \) is the flow rate of the continuous phase. For 2% PVA aqueous solution the density and viscosity are \( \rho = 997.05 \) (kg/m\(^3\)), \( \mu = 8.957 \times 10^{-4} \) (kg/(m.s)). For instance, if \( Q = 0.1 \) (mL/min), \( h = 0.1 \) (mm) and \( w = 0.2 \) (mm), the values for the average velocity and the Reynolds number can be calculated in the form

\[ u = \frac{0.1 \times 10^{-6} / 60 (m^3 / s)}{0.1 \times 0.2 \times 10^{-6} (m^2)} = 0.0833 (m / s) , \]  

(C.5)

\[ Re = \frac{997.05 (kg / m^3) \times 0.083 (m / s) \times 0.1333 \times 10^{-3} (m)}{8.957 \times 10^{-4} (kg / (m.s))} = 12.37 . \]  

(C.6)

The capillary number is defined as

\[ Ca = \frac{\mu u}{\gamma} , \]  

(C.7)
where $\gamma$ is the interfacial tension between the oil and water phases. The value of the interfacial tension for the materials experimented in this dissertation is $\gamma = 0.022$ (N/m).

A sample calculation for the capillary number for the above parameters takes the form

$$Ca = \frac{8.957 \times 10^{-4} \text{ (kg/ms)} \times 0.083 \text{ (m/s)}}{0.022 \text{ (N/m)}} = 0.0034 . \quad (C.8)$$
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