Functionality of Jerusalem Artichoke Derived Inulin in Vietnamese Coffee and Developing a Novel Food Grade Microemulsion System

by

Xuhao Zhao

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Ottawa, Ontario

Abstract

Jerusalem artichoke (JA) is an important source of inulin, a dietary fiber with many health benefits. To investigate the potential of JA on antioxidant activity of coffee, JA coffee was made by adding roasted JA tuber to Vietnamese coffee at a ratio 1:1 (w/w). The TPC (total phenolic content) increased by 13.6% in JA-coffee effecting higher antioxidant activity by 48.0% and 26.9% in ORAC and DPPH assays, respectively. Additionally, JA improved such coffee characteristics as flavor and foam. Mass spectrometric analysis showed that the majority of inulin contents were DP 3 and DP 4. Furthermore, to utilize coffee waste, phenolic compounds were extracted from spent coffee. A food-grade microemulsion system was composed of grape seed oil, soya lecithin, ethanol, and water. The feasibility of this novel microemulsion system for the delivery of phenolic acids was studied by applying it to spent coffee phenolic extracts. Finally, the uniformity of microemulsions was improved by the LiposoFast which confirmed by Cryo-SEM.

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List of Abbreviations

AAPH	2, 2'-azobis(2-methylpropionamidine) dihydrochloride
CGA	Chlorogenic acid
DP	Degree of polymerization
DPPH	2,2-diphenyl-1-picryhydrazyl
FD	Freeze-dried fresh Jerusalem artichoke tuber
GAE	Gallic acid equivalent
GSO	Grape seed oil
HLB	Hydrophile-lipophile balance
HPLC	High performance liquid chromatography
IN	Inulin
INC	Inulin coffee
JA	Jerusalem artichoke
JAC	Jerusalem artichoke coffee
L/E	Lecithin/ethanol ratio
MW	Molecular weight
O/W	Oil in water
ORAC	Oxygen radical absorbance capacity
PC	Plain coffee
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RST	Roasted Jerusalem artichoke tuber
SEM	Scanning electron microscopy

TE	Trolox equivalent
TOF-MS	Time-of-flight mass spectrometer
TPC	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
W/O	Water in oil

1 Chapter: Literature Review

1.1 Botanical fact, chemistry, and application of Jerusalem artichoke

Jerusalem artichoke (JA) (*Helianthus tuberosus* L.) is native to North America, and it is also known as topinambour and a species of sunflower[1]. It is found from eastern Canada to North Dakota, and south to Florida and Texas. JA is cultivated widely since it can be tolerant of arid saline soils, plant diseases, and pests. JA is considered as a healthy vegetable since it is rich in fibers and minerals and contains minor amount of fat (0.01 g total lipid/100 g) as shown in Table 1-1. JA has the potential to be an alternative economic plant with a worldwide yield of 17,843 kg/ha which is higher than that of corn (4,472 kg/ha) and potato (16,448 kg/ha) [2], but the disadvantage of JA is that it contains less energy than that of traditional economic crops, for example, the energy per kg of corn is 3490 kcal, potato (1000 kcal), and JA (760 kcal) [2]. What is more, JA has the capacity to remove Cd, Pd, Ni and Cu from heavy metal-contaminated soils which could be applied in environment protection [3].

JA tuber is an important source of inulin, containing 15% to 20 % (wet weight) [4] and 63% to 75.5% (dry weight) [5]. Besides, JA tubers have many other health benefits. According to the United States Department of Agriculture (USDA) (Table 1-1), the content of potassium in JA tubers is 429 mg/100 g which is significantly higher than that of bananas (358 mg/100 g)[6]. As JA tubers contain low amounts of polyamines, they added to diets of patients under chemotherapy and patients with diabetes [7].

JA tubers have potentials with ethanol production and reduction of flour consumption [8]. It is demonstrated that with the combination of *Aspergillus niger* SL-09 and *Saccharomyces cerevisiae* Z-06 in the fermentation of JA tubers, 98% of inulin in JA

tubers were utilized, and the efficiency of ethanol production was 90% of theoretical yield [8]. Additionally, diterpenes with anti-cancer properties have been successfully extracted from JA which indicates that JA is a healthy ingredient as well as a material of drugs [9].

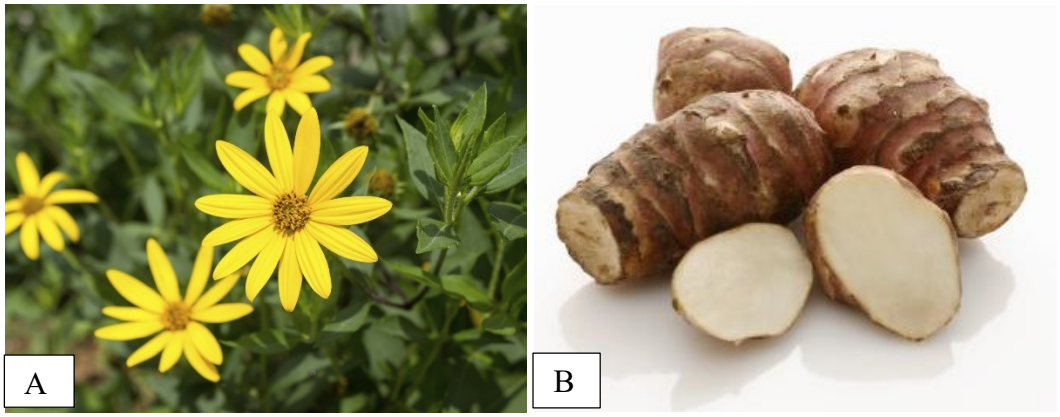


Figure 1-1 JA plant (A) and JA tuber (B)

Table 1-1 Nutrients of Jerusalem artichoke (Tuber) [6b], wet basis

Nutrient	Value per 100g
Water (g)	78.01
Total lipid (g)	0.01
Carbohydrate (g)	17.44
Inulin (g)	~15
Fiber, total dietary (g)	1.6
Potassium (mg)	429
Phosphorus/mg	78
Magnesium (mg)	17
Calcium (mg)	14
Iron (mg)	3.40

1.2 Inulin and health benefits

Inulin is a natural oligo- and/or polysaccharides in which D-fructose units are linked together β-(2→1)-D-fructosyl-fructose bonds (labelled in Figure 1-2), and the chain of D-fructose normally ends with a α-D-glucose unit [10]. The degree of polymerization (DP) of D-fructose in inulin ranges from 2 to 60, which varies depending on plant sources, harvest times, storage conditions and production processes [11]. The roots of chicory, artichoke, and Jerusalem artichoke are main plant sources of inulin, and a comparison of the physicochemical characteristics of inulin derived from them is summarized in Table 1-2. Low-DP inulin is more soluble in water than high-DP inulin. Short-chain inulin is a high potential raw material for ethanol production [8]. High molecular weight inulin is able to form microcrystals in water contributing to a smooth creamy texture. Thus, inulin has a potential to replace cream in food processing since it can form a fat-like mouth sensation [12].

An early study conducted by Coudray *et al.* reported that intake of inulin could increase the absorption of calcium, which was beneficial to the balance of calcium in the human body[13]. Inulin is a fructan soluble dietary fiber, and a low-calorie healthy food ingredient. Inulin cannot be digested or absorbed by the small intestine, so it can be used to control blood glucose and diabetics [14]. Moreover, inulin is fermented in the colon by beneficial bacteria, and this fermentation selectively promotes the growth of bifidobacteria species [15].The prebiotics effect and properties have wide applications in the food industry and inulin can be a replacement of artificial sweeteners [12]. A recent study suggests that prebiotics effect of inulin is associated with the chain length (and hence DP). Short-chain inulin has the ability to enhance the viability of *L. rhamnosus* in

prebiotics yogurt, and it has better performance in the taste and odor than long-chain inulin [16].

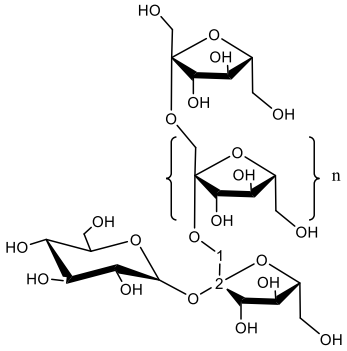


Figure 1-2 Chemical structure of inulin, by ChemBioDraw Office.

Table 1-2 Comparisons of physicochemical characteristics of chicory, artichoke and Jerisalem artichoke inulin [12]

Characteristics	**Standard Chicory Inulin**	**Artichoke Inulin**	**Jerusalem Artichoke Inulin**
Average degree of polymerization	12	46	<40
Inulin/oligofructose content (% fresh.)	16.2 (mean)	2~7	15~20
pH	5~7	5~7	5~7
Sulphated ash (% on dry matter basis)	<0.02	<0.02	NA
Heavy metals	<0.02	<0.02	NA
Appearance	White powder	White powder	White powder
Taste	Neutral	Neutral	Neutral

1.3 Vietnamese coffee and health benefits

Coffee is a brewed drink prepared from roasted coffee beans, which are the seeds of berries from the Coffea plant. It is one of the most popular beverages all over the world with an annual production of 6.7 million tons in the years 1998 to 2000 [17]. Based on statistical data from FAOSTAT (Food and Agriculture Organization of the United Nations, Statistics Division), Vietnam is the second major coffee manufacturing country with an average annual production of 0.8 million tons which is only after Brazil (2.1 million tons)[18]. It is noted that the gap between Vietnam and Brazil in coffee production is narrowing [18].

Coffee has numerous health benefits as a widely consumed pharmacologically active beverage. It is reported that coffee drinking reduces the risk of cardiovascular diseases with the caffeine (80-175 mg) contained in a cup of coffee, enhancing the microvascular functions in healthy individuals [19]. Epidemiological studies have demonstrated that coffee consumption has an association with the reduction of risk in bladder, breast, buccal, pharyngeal and colorectal cancers [20]. Coffee brew contains 0.47 to 0.75 g/100 mL soluble dietary fiber, which is a significantly higher level than other common beverages, and the predominant components are galactomannans and arabinogalactans. It is suggested that coffee consumption may contribute to the prevention of chronic diseases such as type 2 diabetes mellitus, arteriosclerosis, and neurodegenerative diseases [21]. Additionally, a moderate coffee intake has a protective effect against nonalcoholic fatty liver disease due to the antioxidant effect of coffee [22].

Coffee as a main source of antioxidants in diets contains a large amount of phenolic compounds which contribute to the majority of antioxidant activity [23].

1.4 Phenolic compounds as antioxidants

Phenolic compounds are ubiquitously generated as secondary metabolites in plants during adaptation to environmental stress conditions [23]. Phenolic compounds not only provide protection against pathogens and predators in plant growth, but also contribute to the appearance (mostly color) and the sensory characteristics [24].

1.4.1 Chemical structure of phenolic compounds

The chemical structure of phenolic compounds in plants is typically composed of an aromatic ring and hydroxyl or methoxyl substituents, as classified in Table 1-3 [25].

Phenolic acids can be classified into two subgroups, hydroxybenzoic acids (Figure 1-3-A) and hydroxycinnamic acids (Figure 1-3-B), and the substituents can be hydroxyls, methoxyls, etc. Typical hydroxybenzoic acids include gallic, vanillic and syringic acids etc. Hydroxycinnamic acids include caffeic, ferulic, *p*-coumaric and sinapic acids [26].

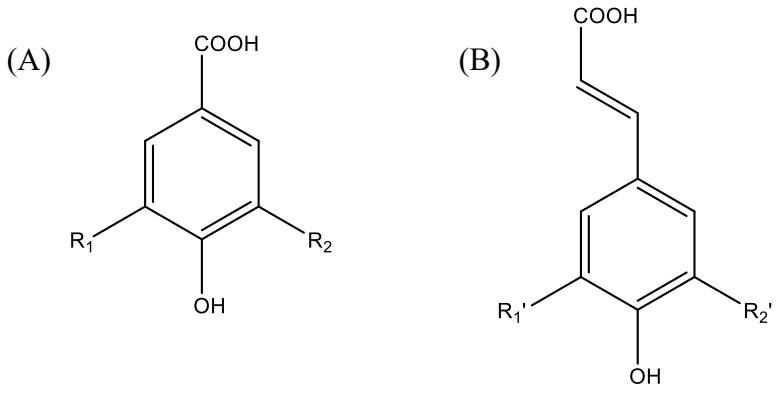


Figure 1-3 Structure of phenolic acids

Flavonoids are another important species of phenolic compounds that widely exist in plants. As shown in Table 1-1, flavonoids have a configuration C_6-C_3-C_6 composed of two aromatic rings joined by a heterocyclic ring. The general chemical structure of flavonoids is shown in Figure 1-4. Substituents to the hexa-heterocycle, R_1 and R_2 give

rise to flavonoid compounds, including oxygenation, alkylation, glycosylation, and sulfation [27].

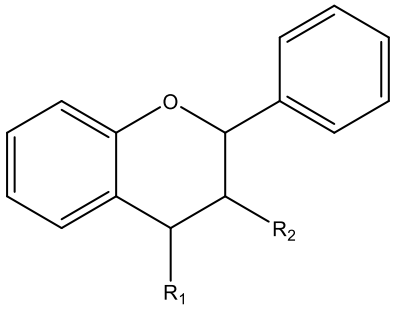


Figure 1-4 Structure of flavonoids

Table 1-3 Classes of phenolic compounds in plants

Class	Structure
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	C_6-C_1
Acetophenones, phenylacetic acids	C_6-C_2
Hydroxycinnamic acids, phenylpropanoids	C_6-C_3
Naphthoquinones	C_6-C_4
Xanthones	C_6-C_1-C_6
Stilbenes, anthraquinones	C_6-C_2-C_6
Flavonoids, isoflavonoids	C_6-C_3-C_6
Lignans, neolignans	$(C_6$-$C_3)_2$
Bioflavonoids	$(C_6$-C_3-$C_6)_2$
Lignins	$(C_6$-$C_3)_n$
Condensed tannins (proanthocyanidins or flavolans)	$(C_6$-C_3-$C_6)_n$

***Adapted from studies by Blasundram *et al*, 2006**

1.4.2 Phenolic compounds in coffee and biosynthesis

Phenolic compounds have a major contribution to the antioxidant activity of coffee. According to Farah *et al* 2005, chlorogenic acids (CGA) are the main components of the phenolic fraction of green coffee beans, reaching levels up to 14 % (dry matter basis) [23]. The chemical structure of CGA is shown in Figure 1-5. Additionally, simple phenolic acids have effects on the flavor of coffee such as acidity, bitterness, and astringency [28].

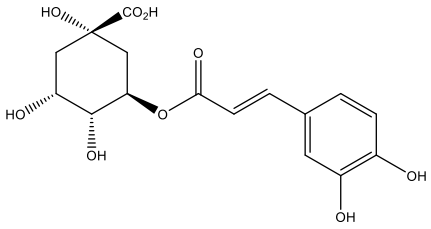


Figure 1-5 Chemical structure of chlorogenic acid

Phenolic acids in the coffee plant are biosynthesized through the shikimic acid pathway with phenylalanine and tyrosine, as shown in Figure 1-6. It is reported that CGA mainly exists in the surface of coffee seeds, in conjunction with the cuticular wax, and in the cytoplasm adjacent to the cell walls of the endosperm parenchyma [29]. Most of the phenolic compounds in coffee bind with melanoidins during the coffee processing, but a small amount of them may be degraded. Although CGA may be entirely degraded into phenol derivatives in the process due to its thermal instability, the amount of melanoidin-bound phenolic acids significantly increases as a result of the roasting process, and the bound phenolic acids in coffee add up to 25~47 % of the total antioxidant activity. There are three possible degradation pathways, including 1) CGA is transformed to isomers; 2) CGA is dehydrated and transformed to quinolactones; 3) CGA is hydrolyzed and transformed into smaller molecules [30].

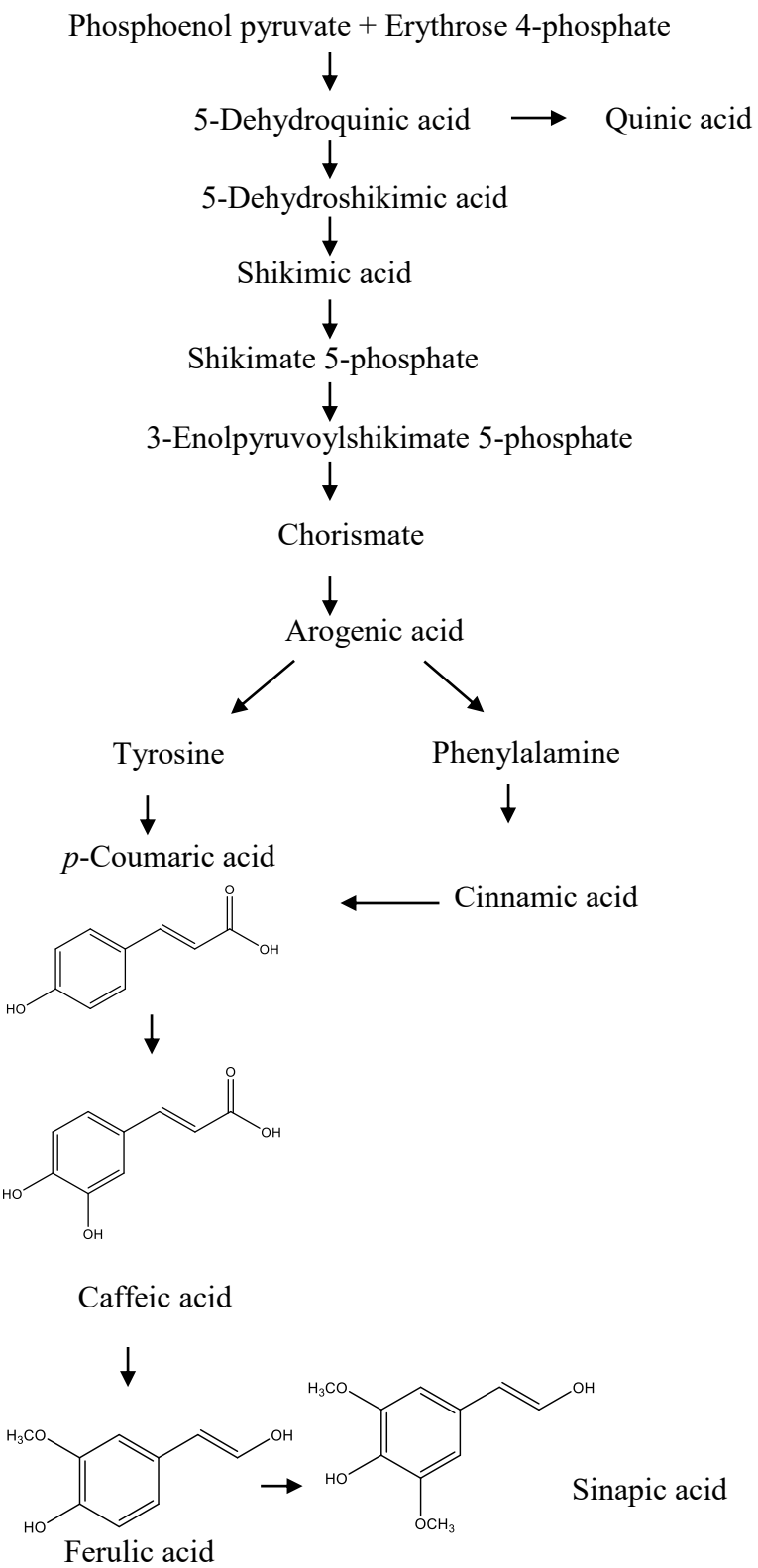


Figure 1-6 Biosynthesis pathway of hydroxycinnamic acids and quinic acid with phenolic acid structures, adapted from Farah A *et al.* 2005.

1.5 Free radicals and antioxidant activity assays

Reactive oxygen species (ROS) are free radicals that contain unpaired electrons which make them extremely unstable, even though they are byproducts of normal aerobic metabolism [31]. ROS include superoxide anion, perhydroxyl radical, hydrogen peroxide and hydroxyl radical. Another type of free radical is reactive nitrogen species (RNS) derived from nitrogen, such as nitric oxide, nitrogen dioxide, and peroxynitrite radicals [32]. The imbalance between ROS effects and the detoxification capacity of a biological system is the predominant factor of oxidative stress [33]. Oxidative stress is involved in many human diseases such as Asperger syndrome, Alzheimer's disease, aging, and age-related diseases. Moreover, it is confirmed that oxidative stress plays a crucial role in the pathology of cancer, neurodegenerative diseases, and inflammatory diseases [34]. Thus, ROS, oxidative stress and antioxidants are of great interest to many chemists and biochemists. Numerous assays have been developed for the measurement of antioxidant activity.

1.5.1 Oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) of phenolic extracts was first reported by Cao *et al*. 1993 [35]. The ORAC assay is a widely used assay to determine the *in vitro* antioxidant activity of many compounds in the field of scientific research and nutraceutical industries. Huang *et al*. modified the ORAC assay by the development of a high-throughput instrument composed of a robotic eight-channel liquid handling system and a microplate fluorescence reader. This modification significantly increases the efficiency of ORAC assay [36].

The ORAC assay is based on an oxidation reaction of peroxyl radicals generated from 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and the fluorescein probe. The products of this reaction are non-fluorescent compounds, so the changes in fluorescence intensity indicate the antioxidant capacity of the sample against free peroxyl radicals. An example of fluorescence decay curve is shown in Figure 1-7. The fluorescence intensity was read during a period of 60 minutes at 1-minute intervals. The ORAC value calculations were based on Net AUC (area under the curve) of fluorescence decay reactions. The Net AUC was calculated as follows:

AUC = [0.5 + (R1/R0 + R2/R0 + R3/R0 +.....+ Rn/R0)] × CT,

in which R0 represents the initial fluorescence reading, Rn is the last fluorescence reading, and CT is the cycle time in minutes.

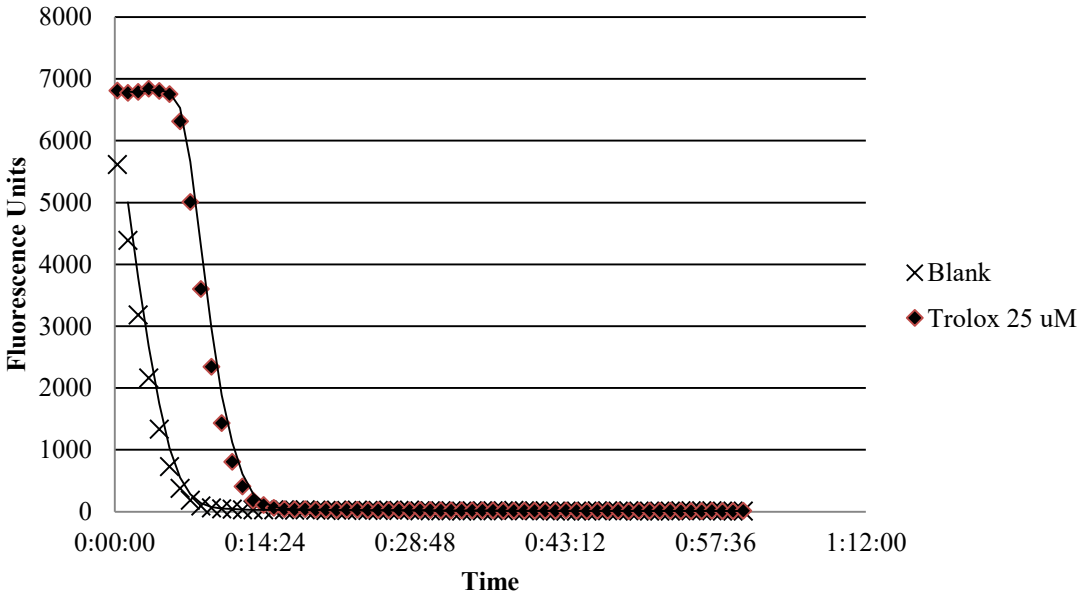


Figure 1-7 Fluorescence decay curve.

1.5.2 DPPH radical scavenging assay

The DPPH (2, 2-diphenyl-1-picryhydrazyl) radical scavenging assay was first reported by Brand-Williams *et al* in 1995. This simple, efficient and sensitive method has been widely applied in the antioxidant activity research. DPPH is an organic RNS that has a color of deep purple. It reacts with antioxidants to form a pale yellow hydrazine (Figure 1-8). The capacity of antioxidants to react with DPPH radicals is determined by measuring the decrease in absorbance at 519 nm since DPPH radicals absorb at 515-525 nm [37]. The antioxidant content is proportional to % DPPH scavenging activity (or % DPPH discoloration) which is calculated using the following equation:

% DPPH scavenging activity = 100% x (1- (absorbance of sample/absorbance of control))[37]

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet}$$

$$DPPH^{\bullet} + R^{\bullet} \longrightarrow DPPH\text{-}R$$

Figure 1-8 Reactions of DPPH radical and an antioxidant (AH) or radical species ($R^{\bullet}$)

1.5.3 Relationship between total phenolic content (TPC) and antioxidant capacity

Since phenolics have a free radical scavenging capacity, TPC can be used to show a positive correlation between TPC and antioxidant activity.[38] Total phenolic content is also known as the Folin-Ciocalteu method. Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate. This method is convenient, simple, and performed on a UV-visible spectrophotometer. TPC measures all compounds that are readily oxidized under experimental conditions, and it normally works as a predictable analysis towards phenolic content [38]. The reaction of antioxidants and Folin-Ciocalteu reagent in an alkalescent environment forms a solution with a bluish violet color. The

total phenolic content is positively correlated to the darkness of the solution measured as the absorbance at 725 nm.

1.6 Microemulsions and applications

1.6.1 Microemulsion systems, definition, characteristics and formations

The concept of microemulsions was first introduced by Hoar and Schulman in 1943. Oil-alkali-metal soap-water systems were studied, and transparent oil-continuous systems were successfully generated with high soap/water ratios [39]. With the advance in studies of microemulsions, a widely accepted definition of a microemulsion was put forward by Danielsson and Lindman in 1981[40] "a microemulsion is a system of water, oil and an amphiphile which is a single optically isotropic and thermodynamically stable liquid solution". Compared with normal emulsions, microemulsions have many advantages in physicochemical properties, such as thermodynamic stability, transparent appearance, low viscosity, and small droplet size. The differences between normal emulsions and microemulsions are summarized in Table 1-4.

The formation of a microemulsion can be explained by the following equation, $\Delta G_f = \gamma \Delta A - T\Delta S$, where ΔG_f is the free energy of the formation, γ is the surface tension of the interface between two phases, ΔA is the change of interfacial area, ΔS is the change of entropy of the system, and T is the temperature. The presence of surfactants (amphiphiles) lowers the surface tension between two phases, and the $\gamma \Delta A$ term would be relatively small when the reduction of surface tension is large enough. When a negative free energy of the formation is achieved, spontaneous microemulsification will occur [41].

There are three different types of microemulsions: water-in-oil (W/O), oil-in-water (O/W), and bicontinuous (Figure 1-9)[42]. The structure of W/O microemulsion is similar

to the reverse micelle in which each water droplet is surrounded by polar heads of surfactant molecules with the non-polar tails pointing outwards. O/W microemulsion is similar to a micelle, and it solubilizes oil in the hydrocarbon core of surfactant molecules. Bicontinuous microemulsion has a sponge-like structure in which both oil and water are continuous phases.

The hydrophile-lipophile balance (HLB) is an empirical expression for the relationship of hydrophilic and hydrophobic fragments in a surfactant molecule. Surfactants with high HLBs are more soluble in water than those with low HLBs. Generally speaking, W/O microemulsions requires surfactants with low HLB (3-6) while O/W microemulsions prefer high HLB surfactants (8-18). Surfactants with HLBs greater than 20 tend to need a co-surfactant to form a microemulsion [41].

Table 1-4 Comparison of microemulsions and emulsions

Property	Microemulsions	Emulsions
Appearance	Transparent	Cloudy, milky
Droplet size	< 200nm	> 500 nm
Formation	Spontaneously occurred, low energy required	High input of energy required
Stability	Thermodynamically stable, monophasic	Separate to two phases under gravitational force
Interfacial tension	Low	High
Viscosity	Low	High

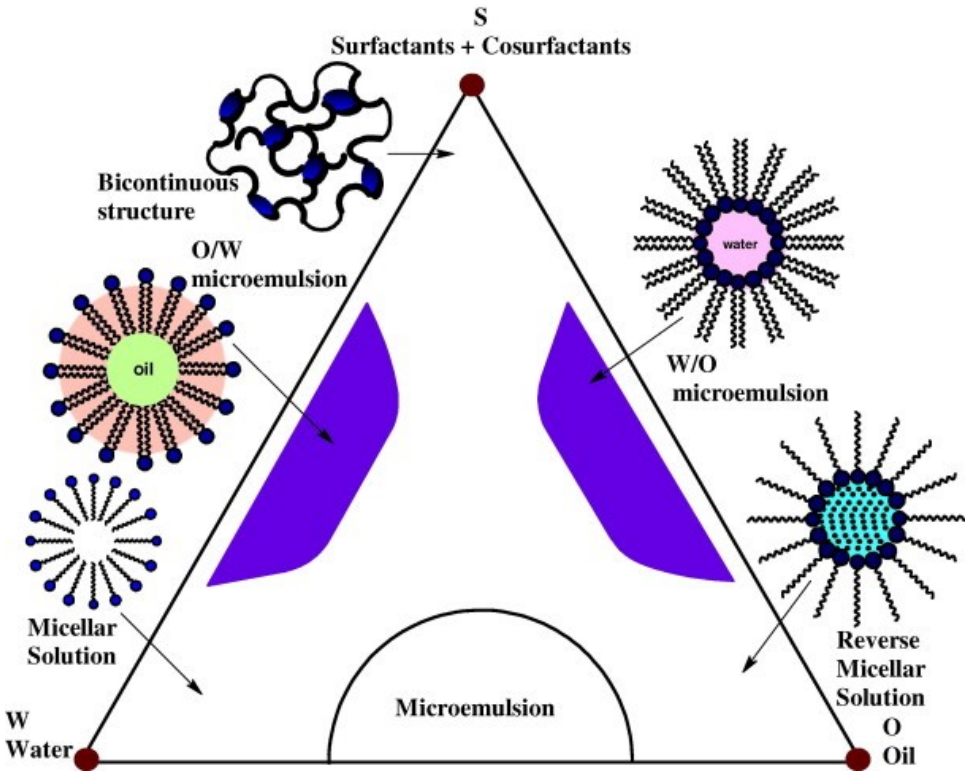


Figure 1-9 Hypothetical phase regions of microemulsion systems. Malik MA *et al*.[42]

1.6.2 Applications of microemulsions

Microemulsions can be applied to many fields such as the food industry, cosmetics, drug delivery, chemical synthesis, and analytical chemistry. It is demonstrated that microemulsions provide a novel efficient route to synthesize various inorganic and organic nanoparticles [42]. Numerous inorganic nanoparticles such as copper, cobalt, nickel, gold and silver nanoparticles could be synthesized with the reduction method through microemulsions, and the sizes of particles are controllable [43]. The main organic nanoparticles synthesized from microemulsions are nanosized polymer particles. According to Candau *et al*, the polymerization reaction is conducted in O/W

microemulsions, and the resultant hydrophobic nanoparticles are dispersed in the water phase [42].

Microemulsions can be used as a solubilization capacity enhancer for drugs with poor solubility. In this area, not only the enhancement of solubilization but also the different physicochemical characteristics of drugs in microemulsions and free systems should be investigated [44]. Microemulsions contain both oil and water in a single phase. They have the ability to dissolve materials with different polarities at the same time. Thus they are playing important roles in drug delivery. The applications of microemulsions in drug delivery depend on the type of microemulsions. Lipophilic drugs can be dispersed in the oil phase or hydrophobic surfactant tails of an O/W microemulsion system, while hydrophilic materials tend to disperse in the water phase of a W/O microemulsion system [41 45]. As the polar heads of surfactants coat the surface of an O/W microemulsion droplets, they tend to be retained upon dilution in biological aqueous phases in oral or parenteral drug delivery [41]. In contrast, W/O microemulsions are good for the delivery of labile hydrophilic drugs such as peptides and oligonucleotides that can be degraded by enzymes [46]. W/O microemulsions can provide protection for those drugs from the proteolysis in the gastrointestinal system [47].

2 Chapter: The potential of inulin in Jerusalem Artichoke to affect the bioactivity of Vietnamese coffee

2.1 Abstract

A novel coffee composed of Vietnamese coffee and roasted JA was created in this study. Phenolic compounds were extracted from coffee samples and determined by HPLC, and the HPLC results showed that chlorogenic acid was the predominant composition in coffee. The contribution of JA derived inulin on antioxidant activity of coffee was investigated through multiple assays, and the TPC increased by 13.6% in JA-coffee affecting higher antioxidant activity by 48.0% and 26.9% in ORAC and DPPH assays, respectively. Analysis of components of oligosaccharides in JA coffee was conducted on MS. Results showed that the main sugar components observed were D-fructose, sucrose, and inulin with degree of polymerization 3 and 4.

2.2 Introduction

Coffee has the highest *in vitro* antioxidant activity among common beverages. Antioxidants such as chlorogenic acids (CGA) and melanoidins in coffee, have been a research topic of great interest for decades. Melanoidins are macromolecular nitrogenous materials with a brown color formed in Maillard reactions [48]. Coffee infusion melanoidins are composed of polysaccharides, proteins, and phenolic compounds. Phenolic acids can bind with melanoidins non-covalently [49], and melanoidin-bound phenolic compounds contribute to the antioxidant activity of coffee along with free phenolic acids [30]. It indicates a possibility that inulin may have potentials on the production of melanoidin-bound phenolic compounds.

As demonstrated previously, inulin is a dietary fiber that has numerous health benefits, but inulin is not a common nutrient in common diet, while coffee is one of the most common diets. As a result, the combination of inulin and coffee is of interest, as well as the effect of inulin on antioxidant activity of coffee. Roasted JA tuber (Figure 2-1) contains approximately 75% inulin and has a taste of natural sweetness which makes it an ideal material to replace artificial sweeteners.

Figure 2-1 Roasted JA tuber sample

The objectives of this study were to a) create a new blend of coffee by adding roasted JA tuber into Vietnamese coffee, b) extract and characterize phenolic compounds in JA coffee, c) analyze the oligosaccharides composition in JA coffee, and d) evaluate antioxidant activities of JA-coffee by multiple assays.

2.3 Materials and methods

2.3.1 Sample preparation

The Vietnamese coffee, roasted and fresh Jerusalem artichoke root (JA) samples were purchased from local commercial. Inulin standard was purchased from Sigma-Aldrich (Oakville, ON, Canada).

Fresh JA was sliced and freeze-dried. All samples were gound into powder by a grinder and mixed in different ratios. There were five samples, plain coffee (100% Vietnamese coffee powder), inulin coffee (10% inulin standard with 90% Vietnamese coffee powder), JA coffee (50% ground roasted JA and 50% Vietnamese coffee powder), roasted JA (100% ground roasted JA), freeze-dried JA (100% ground freeze-dried JA)

2.3.2 Materials and instruments

HPLC grade Methanol and analytical grade acetic acid were purchased from Caledon Laboratories LTC (Georgetown, ON, Canada). HPLC grade formic acid and acetonitrile were purchased from Sigma-Aldrich Canada (Oakville ON, Canada). Sodium carbonate was obtained from Church and Dwight Canada Corp (Mississauga, ON, Canada). The phenolic acid standards, gallic, protocatechuic, p-hydroxybenzoic, chlorogenic, caffeic, vanillic, syringic, p-coumaric, sinapic, ferulic, o-coumaric; flavonoid standards, pyrogallol, catechin, epicatechin, rutin, quercetin-3-beta- glucoside, epicatechin gallate, myricetin, quercetin, apigenin and kaempferol, were analytical grade and purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Fluorescein, Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), 2, 2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), rutin, Folin-Ciocalteu reagent, and 2, 2-diphenyl-1-picryhydrazyl radical (DPPH), were analytical grade and obtained from Sigma-Aldrich (Oakville, ON, Canada). Water was purified by Milli-Q Integral Water Purification System (EMD Millipore Corp.).

The high performance liquid chromatography (Waters e2695 HPLC system) was equipped with a 2998 photodiode array (PDA) detector and Empower workstation on computer (Waters, Milford, MA, USA). FLx800TM Multi-Detection Microplate Reader

with Gen5TM software (BioTek Instruments, Ottawa, Canada). Pierce Reacti-Therm I #18821 Heating/Stirring Module (Thermo Scientific, MA, USA), Proxeon Nanoelectrospray Emitter (Thermo Scientific, Odense, Denmark), QStar XL hybrid quadrupole time-of-flight mass spectrometer (TOF-MS) with a nanoelectrospray ionization source (AB Sciex, Framingham, MA, USA).

2.3.3 Extraction of phenolic compounds

Each sample was weighed 1 g and then mixed with 20 mL of acidified (1% acetic acid) methanol solution (80%). The mixture was stirred for 3 hours at room temperature (23 °C) and centrifuged at 4000 RPM for 15 minutes at 23 °C. The supernatant was collected and stored at -20 °C in the freezer. Each sample extraction was done triplicated.

2.3.4 Characterization and determination of phenolic compounds

Phenolic compounds extracts were diluted with methanol and filtered on a 45 μm membrane filter. Phenolic acid and flavonoid standards were prepared and diluted in methanol. Phenolic compounds were separated by a Synergi-Max-RP (reversed phase) column (250 x 4.6 mm, 5 μm) with column temperature 35 °C. The mobile phases were solvent A (0.01% formic acid) and solvent B (100% acetonitrile). The gradient program was as follows: 0-35 minutes, 90% solvent A; 35-40 minutes, 50% solvent A; 40-50 minutes, 90% solvent A. The injection volume was 10 μL, and the flow rate was 1 mL/min with a total run time of 50 minutes. The wavelength for identification of phenolic acids was 280 nm, and for flavonoids were both 280 nm and 320nm. Spectra of all samples and standards at a range of 200-700 nm were recorded. For the preparation of standard curve, the mixture of all phenolic acids and flavonoids standards were prepared in five different concentrations, and the standard curve was plotted based on the peak

areas of each concentration. The concentrations of phenolic compounds in samples were calculated based on the standard curve equations.

Table 2-1 Phenolic acid and flavonoids standards and retention times

Phenolic acid	Retention time (min)	Flavonoids	Retention time (min)
Gallic acid	4.73	**Pyrogallol**	5.97
Protocatechuic	7.51	**Catechin**	9.13
P-oh-benzoic	10.76	**Epicatechin**	10.79
Chlorogenic	11.10	**Rutin**	14.00
Caffeic	11.56	**Quercetin 3 beta- glucoside**	15.17
Vanillic	11.76	**Epicatechin gallate**	15.40
Syringic	11.89	**Myricetin**	19.71
P-coumaric	15.60	**Quercetin**	24.44
Sinapic	16.38	**Apigenin**	28.45
Ferulic	16.75	**Kaempherol**	29.14
O-coumaric	19.99		

2.3.5 Oxygen radical antioxidant capacity (ORAC) assay

The ORAC method was modified by Gunenc *et al* [50]. In ORAC assay, all reagents were prepared with ORAC working buffer (potassium phosphate buffer pH 7.4), including different concentrations of Trolox standard solutions (100, 50, 25, 12.5 and 6.25 mM), fluorescein working solution (0.068 μM), a solution of rutin control (10 μM), and 153 mM AAPH. 5 mL phenolic extracts were dried, dissolved and diluted by ORAC working buffer. Trolox standards 20 μL, rutin control, 120 μL of fluorescein working solution and sample dilutions were added into wells of a 96 micro-well plate respectively, and the plate was inserted into the fluorescence reader. After a 20 minutes' incubation at 37 °C, 60 μL of 153 mM AAPH was added to each well, and the total volume of each well was 200 μL. ORAC working buffer 200 μL was used as blank. The experimental results of samples were recorded as x μM TE (Trolox equivalent)/g of a sample, since they were calculated from the standard curve of Trolox standards.

2.3.6 DPPH (2-diphenyl-1-picryhydrazyl) radical scavenging assay

Gallic acid was used as standards in DPPH radical scavenging assay, and 7 different concentrations of standards ranging from 1, 10, 20, 30, 40, 50, 60 μg/mL were prepared in 80% methanol. The phenolic extracts of samples were dried and then dissolved in 80% methanol. Gallic acid standard solutions 20 μL or sample dilutions 20 μL was added to wells of a 96-well microplate respectively, then 180 μL 50 μM DPPH solution was loaded to each well and gently mixed, which made the total volume of each well was 200 μL. The 50 μM DPPH solution was used as the control. The microplate was sealed and placed in dark and incubated for 60 minutes. After incubation, the absorbance under 519 nm was read and recorded by the Multi-Detection Microplate Reader.

The % DPPH discolorations of samples and standards were calculated by the equation as followed:

$$\text{DPPH scavenging activity (\%)} = (1 - \left(\frac{\text{sample absorbance}}{control\ absorbance}\right)) \times 100$$

The gallic acid standard curve was obtained from % discolorations vs. concentrations, and the same as sample DPPH discoloration curve. Five different dilutions of sample were used to measure IC50 which was known as the concentration of antioxidant to cause a 50% decrease in the initial amount of DPPH. IC50s represented for the antioxidant activity of samples, and they were expressed into mg GAE/g of a sample based on the standard curves.

2.3.7 Total Phenolic Content

In TPC assay, gallic acid was used as the standard, and 5 concentrations (0.5, 0.25, 0.125, 0.0625, and 0.0312 mg/mL) were prepared with Milli-Q water. The 10-fold diluted Folin-Ciocalteu solution and 60 g/L sodium carbonate solution were prepared with Milli-Q water for further uses. Phenolic extracts were dried by nitrogen flow and re-dissolved by Milli-Q water under a 5-minute ultrasound treatment.

Gallic acid standard solution or sample phenolic extract dilutions was measured of 50 μL, and 475 μL of 10-fold Folin-Ciocalteu solution were added to an opaque Eppendorf tube and mixed well. 475 μL of 60 g/L sodium carbonate solution was added to the mixture and gently mixed after 5 minutes, so the total volume was 1 mL. Milli-Q water 50 μL with 475 μL of Folin-Ciocalteu solution and sodium carbonate were used as blank. Each mixture was measured 200 μL and added to a 96-well microplate respectively, then the microplate was sealed and incubated in darkness for 2 hours. After incubation, the absorbance at 725 nm was read and recorded by the Multi-Detection

Microplate Reader. The standard curve was plotted by absorbance vs. concentration of gallic acid, and the TPC (total phenolic content) values of samples were calculated based on the regression equation of the standard curve, so they were demonstrated as mg GAE (gallic acid equivalent)/ 100 g.

2.3.8 Inulin extraction

Milli-Q water was heated to 85 °C, and 20 mL of it with 1 g of sample were added to a 50 mL beaker and covered with aluminum foil in order to prevent evaporation, then the mixture was stirred by a magnetic stirrer at 85 °C for 3 hours. After cooling down to room temperature, the mixture was centrifuged at a speed of 12,000 rpm at 23 °C for 15 minutes. The surfactant was filtered by a 45 μm filter for further uses.

2.3.9 Mass Spectrometry (MS) sugar component analysis

The MS analysis of inulin extracts of samples was conducted in Carleton Mass Spectrometry Center. A mixture of glucose, fructose, sucrose and maltose with a concentration of 0.5 mg/ml was used as standards. Inulin extracts of samples were placed into the Proxeon nanoelectrospray emitter and detected by a hybrid quadrupole TOF MS. The spectra of samples were recorded with the voltage of ESI (electrospray ionization), and the data was analyzed by ChemStation software.

2.3.10 Statistical analysis

Phenolic compounds extractions were performed three times individually. HPLC, TPC, ORAC and DPPH results were obtained from three individual experiments. Experimental results are expressed in a form of mean value ± SEM (standard error of the mean). One-way analysis of variance (ANOVA) and correlations were performed by Excel software to determine the statistical significance and relationships of data. $\alpha = 0.05$ was used in ANOVA, so values were considered significantly different if p value is less than 0.05.

2.4 Results

2.4.1 Determination and characterization of phenolic compounds in coffee and JA samples

Phenolic compounds in samples (plain coffee, inulin coffee, JA coffee, roasted JA, freeze-dried JA) were extracted by 80% acidified methanol and detected by HPLC-UV. Phenolic acid standards and flavonoids standards were prepared in 5 concentrations to make standard curves. Figure 2-2 showed that the maximum total phenolic compound content was PC (62.0 mg/g), following by INC (57.66 mg/g) and JAC (33.0 mg/g). Comparing to coffee samples, JA contained relatively less phenolic compounds, and phenolic compounds concentration in FD (7.44 mg/g) was significantly higher than that of RST (3.1 mg/g).

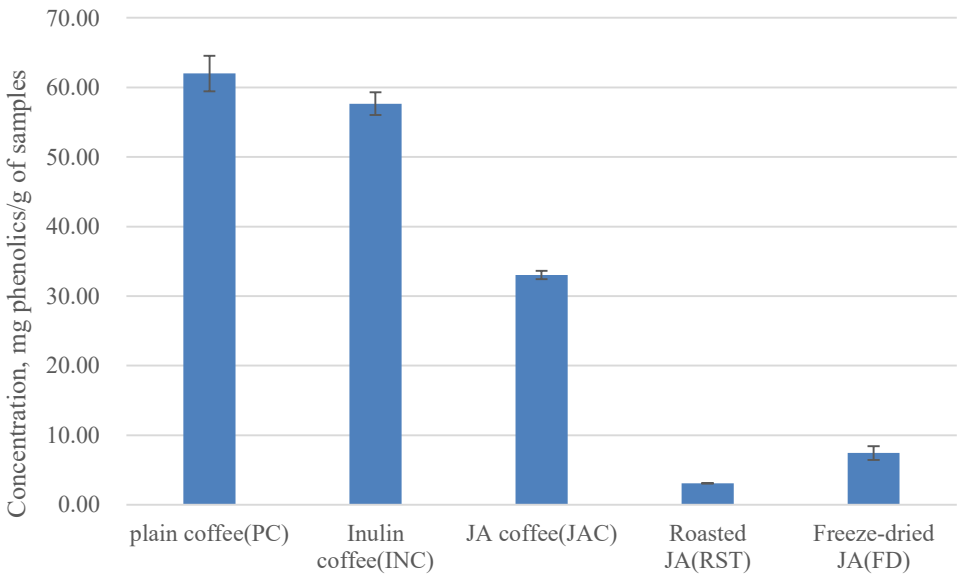


Figure 2-2 Total phenolic compounds content in samples determined in HPLC analysis, mg/g of sample, $p<0.05$. Results are expressed as mean values ± SEM.

Table 2-2 Phenolic composition in samples, mg/g

Compound	Plain coffee (PC)	Inulin coffee (INC)	JA coffee (JAC)	Roasted JA (RST)	Freeze-dried JA (FD)
Gallic acid	nd	nd	0.40	0.23	nd
p-oh-benzoic	5.68	4.80	2.55	0.10	0.12
Caffeic	1.44	1.34	0.74	0.06	0.06
Syringic	nd	nd	nd	nd	nd
p-coumaric	3.07	2.81	1.53	nd	nd
Ferulic	nd	nd	nd	nd	nd
Pyrogallol	2.63	2.47	2.62	2.61	7.26
Chlorogenic acid	44.55	41.92	23.22	nd	nd
Rutin	4.63	4.33	1.98	0.09	nd
Total	62.00 *	57.66 *	33.04	3.10 **	7.44 **

***nd: not detected. *, ** p<0.05.**

2.4.2 Antioxidant activity analysis

The antioxidant activities of phenolic compound extracts of five samples were determined by multiple assays including TPC, ORAC, and DPPH. The correlation between results of TPC and the two antioxidant activity assays were analyzed. Because of that chicory inulin powder is a light and fluffy material, positive control INC was prepared in 10% standard chicory inulin with 90% PC instead of 50:50. Antioxidant activity of JA coffee was compared to plain coffee (PC) as a negative control and inulin coffee (INC) as a positive control. Antioxidant activities of roasted JA (RST) and freeze-

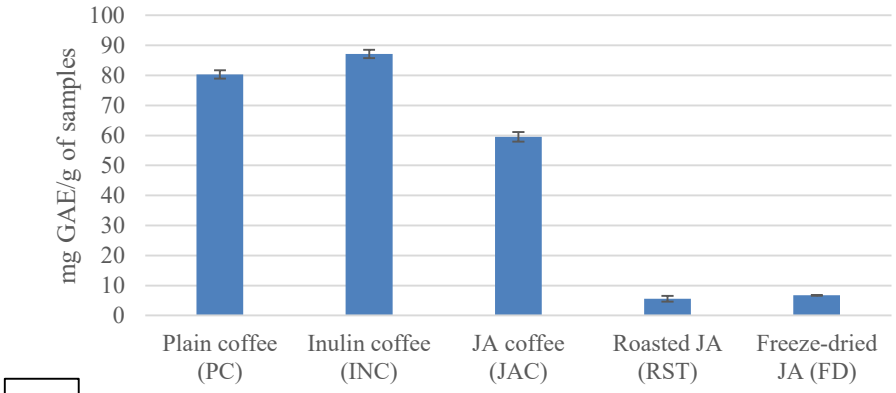
dried JA were compared to each other to investigate the effect of roasting, and they were blanks to JA coffee.

The results are listed in Table 2-3. As shown in Figure 2-3, a strong correlation among the results of three assays was shown. In the three assays, INC showed the highest antioxidant activity (87.1 mg GAE/g in TPC, 671μmol TE/g in ORAC, 27.9 IC50 mg GAE/g), following PC in the second place (80.4 mg GAE/g in TPC, 561μmol TE/g in ORAC, 23.8 IC50 mg GAE/g) and JAC (59.6 mg GAE/g in TPC, 319 μmol TE/g in ORAC, 15.1 IC50 mg GAE/g). Antioxidant activity of FD was higher than that of RST in three assays.

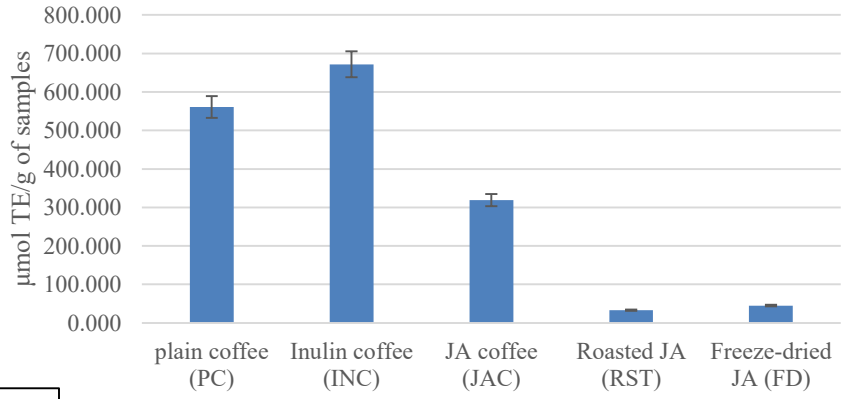
Table 2-3 Summary of results from multiple antioxidant activity assays.

Sample	TPC, mg GAE/g	ORAC, μmol TE/g	DPPH, IC50 mg GAE/g
PC	80.4 ± 1.40	561 ± 3.88	23.8 ± 0.179
INC	87.1 ± 1.37	671 ± 6.31	27.9 ± 0.536
JAC	59.6 ± 1.59	319 ± 2.32	15.1 ± 1.73
RST	5.57 ± 0.951	32.6 ± 0.445	1.10 ± 0.0228
FD	6.70 ± 0.0959	44.9 ± 0.175	1.11 ±0.0242

***Values are means ± SEM, PC and INC, RST and FD, p <0.05.**



A



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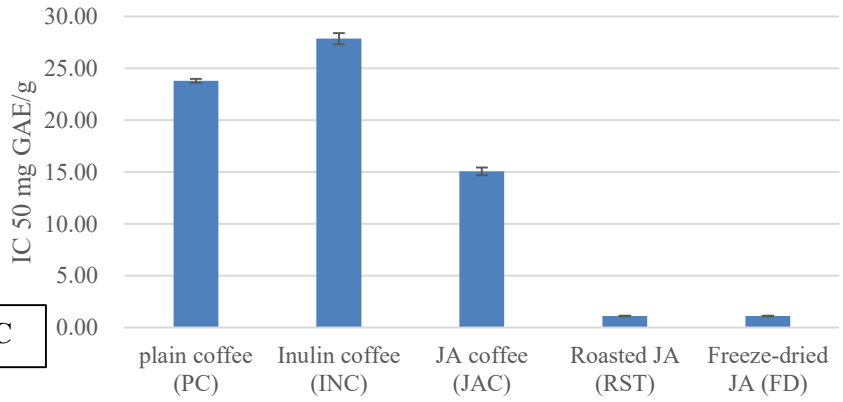
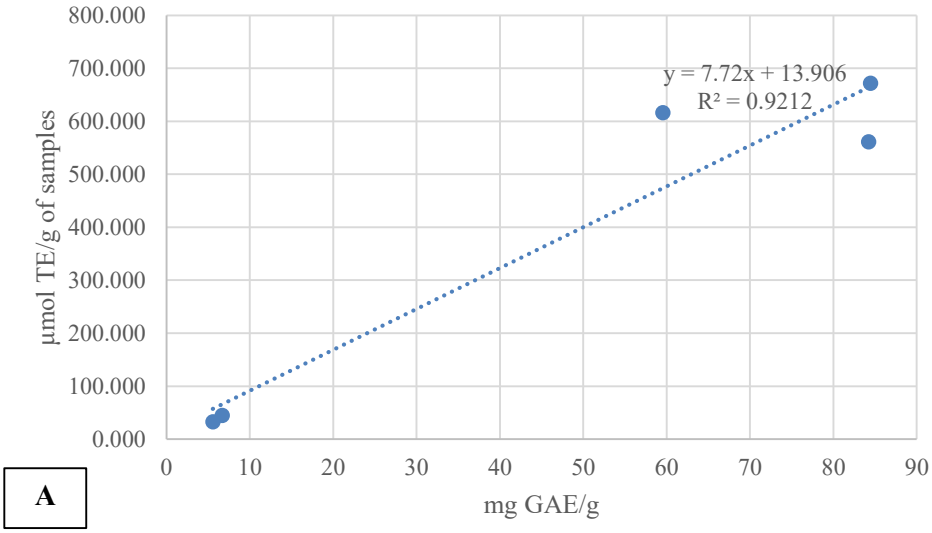
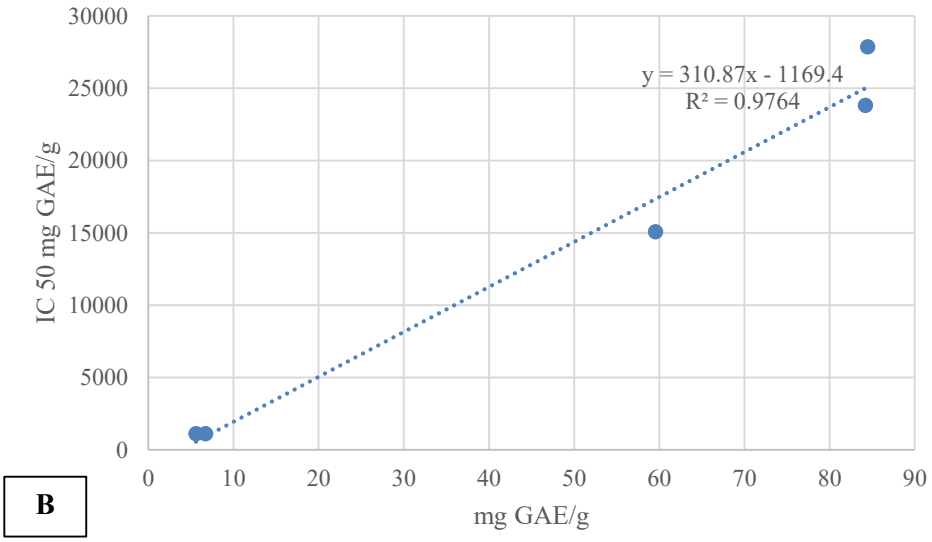


C

Figure 2-3 Antioxidant activity of phenolic extracts from PC, INC, JAC, RST and FD determined by A) TPC, mg GAE/g of sample, B) ORAC, µmol TE/g of sample, and C) DPPH, IC50 mg GAE/g. $p<0.05$. Values are means ± SE



A



B

Figure 2-4 The linear correlation of results of (A) ORAC vs. TPC, (B) DPPH vs. TPC. R^2>0.9.

2.4.3 MS analysis

MS analysis of inulin extracts of JA coffee and plain coffee were conducted to confirm the presence of sugar composition import from JA in JA coffee. A mixture of D-glucose, D-fructose and sucrose was used as monosaccharides standards, and the spectrum of standards was shown in Figure 2-6. The peaks of three monosaccharides were singly charged under negative mode. D-glucose and D-fructose existed the same peak at 179 Da since they are isomers with MW 180. The peak at 341 Da represented sucrose (MW=342). Figure 2-5 showed the spectra of inulin extracts from JA coffee. Strong singly charged peaks (a and b in Figure 2-5) appeared at 179 and 342, and it indicated the presence of fructose and sucrose in JA coffee. In Figure 2-5, singly charged peak c (503 Da) and peak d (665 Da) indicated the presence of inulin-type oligosaccharides (DP= 3, 4 respectively). No peaks related to inulin compounds could be observed in the spectrum of plain coffee (Figure 2-7), while a peak at 179 Da is the evidence of monosaccharides which might be glucose or fructose in plain coffee.

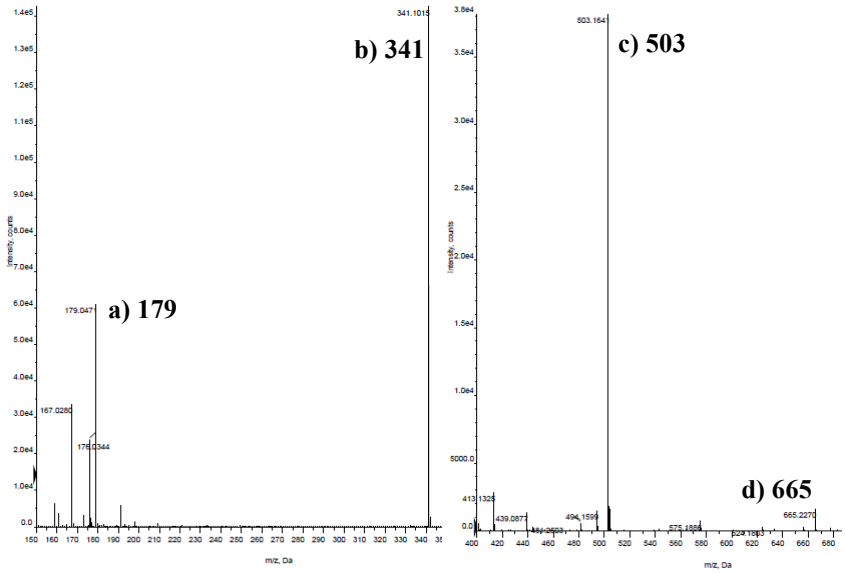


Figure 2-5 MS spectra of JA coffee inulin extracts. (A) 150 to 350 Da. (B) 400 to 680 Da

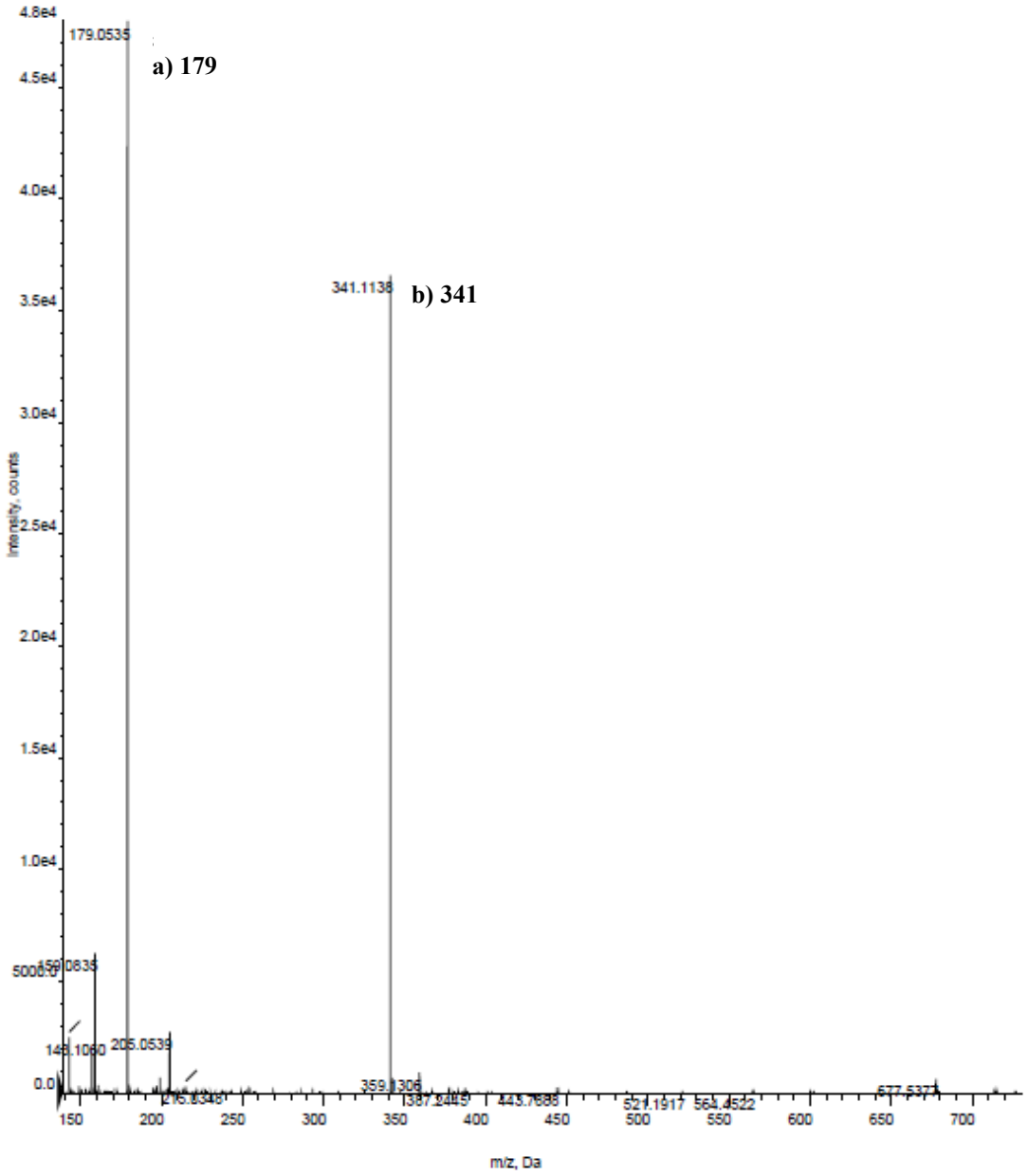


Figure 2-6 MS spectrum of single sugar standards, negative mode, singly charged. Peak a) D-glucose, D-fructose, peak b) sucrose.

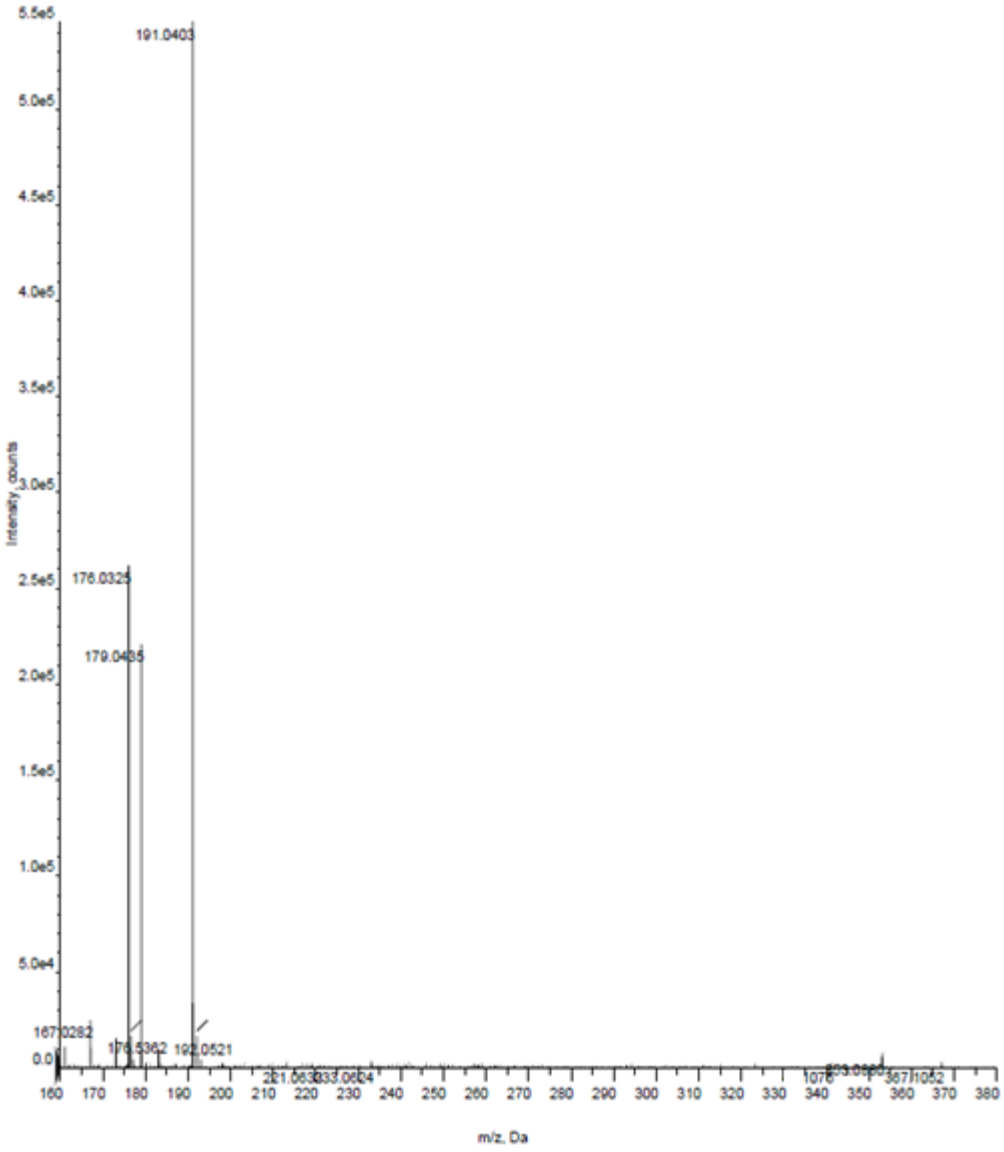


Figure 2-7 MS spectra of plain coffee inulin extracts

2.5 Discussion

A novel JA coffee was created by blending roasted JA tuber and Vietnamese coffee beans with a ratio of 1:1. In results of HPLC analysis, phenolic content in plain coffee was the highest, 62.0 mg/g. According to Table 2-2, chlorogenic acid was the predominant composition of phenolic extracts of plain coffee (44.55 mg/g), inulin coffee (41.92 mg/g) and JA coffee (23.22 mg/g). Quantitative results in this study showed correlation with literature values. As reported by Farah *et al* 2005, chlorogenic acid is the main phenolic acid in coffee, and the concentration of chlorogenic acid in commercial roasted coffee has a wide range from 8.1 to 52.8 mg/g [23].

The total phenolic content (TPC) and antioxidant activity values of samples were shown in Figure 2-3, and the relationship between TPC and antioxidant activity results were analyzed by correlation equations which were shown in Figure 2-4. The R^2 value of the correlation between ORAC and TPC was 0.9212, and that of the correlation between DPPH and TPC was 0.9764. The R^2 values were near to 1 which indicated that TPC had a strong correlation between the antioxidant activities.

In order to make the antioxidant activity of plain coffee, inulin coffee and JA coffee more comparable, the experimental results of inulin coffee and JA coffee were converted to the form Antioxidant Activity/ 1 g coffee with additives as shown in Figure 2-8. Overall, the antioxidant activities of coffee with additives were significantly higher than that of coffee only, and it indicated that the addition of inulin significantly increased the antioxidant activity of coffee. As shown in Figure 2-8-A, JA additive coffee had a significantly higher TPC value (119.11 mg GAE in 1g coffee with 1g JA) than inulin control (96.83 mg GAE in 1g coffee with 0.11g inulin). However, it showed an opposite

result in ORAC that ORAC value of inulin control (746.22 μmol TE) was considerably higher than that of JA coffee (637.55 μmol TE), and they were not significantly different from each other in DPPH assay. This difference might be due to the different principles of assays. In comparison, inulin standard additive increased antioxidant activity of coffee by 20.5%, 32.9% and 30.1% in TPC, ORAC and DPPH assay respectively, while the values for roasted JA tuber were 48%, 13.6%, and 26.9% respectively. According to the studies by Saengkanuk *et al.* 2010, the JA tuber contains 63~75.5% of inulin (dry matter basis) [5], so the inulin content of roasted JA tuber used in this study was approach to 0.63~0.75 g in 1 g of roasted JA tuber which was more than the mass of inulin standard additive (0.11g). This might indicate that the purified inulin standard was more efficient than raw JA derived inulin.

The phenolic compounds in roasted JA and freeze-dried fresh JA were extracted and determined by HPLC to investigate the effect of roasting process on phenolic compounds content. As shown in Figure 2-3, comparing free phenolic compounds content of roasted JA tuber and freeze-dried fresh JA tuber from three assays, the latter contains more free phenolics than the former, and it could due to the loss of phenolic acids during roasting process [25]. It indicated that the roasting process could cause degradation of phenolic compounds or the combination of free phenolics and other compounds. As a result, the JA additive increased the bioactivity of plain Vietnamese coffee might because of the bioactive melanoidins formed from inulin and phenolic compounds, as well as the phytochemicals in roasted JA.

As shown in Figure 2-5, 6, 7, the main components of oligosaccharides in JA coffee were fructose, sucrose, oligosaccharides with DP 3 and 4. The spectrum of inulin extracts

in sample showed a correlation to the literature for strong peaks at 503 and 665 Da [51]. The MS analysis of JA coffee confirmed the existence of inulin in it, and the DPs of JA derived inulin were relatively lower comparing to Artichoke inulin (DP=46), chicory inulin (DP=12) [12]. The addition of roasted JA in coffee may enhance the flavor while provide many health benefits. Besides prebiotics functions as demonstrated before, inulin-type fructans were proved to be beneficial to calcium absorption in adults especially older women since they cannot be hydrolyzed by mammalian enzymes and the production of short-chain acids in the fermentation process of inulin in the human intestinal tract which can enhance the solubility of calcium [52]. Additionally, the fermentation of carbohydrates in colon can stimulate its motility, thus ameliorate constipation problems which especially bother elders [53]. It was reported that the addition of oligofructose in the diet of rats decreased the triacylglycerols in very-low-density lipoproteins, and inulin has the ability to modify lipid metabolism in rats [54]. As a result, the introduction of inulin to daily diets is of interest. JA tubers had been successfully prepared in dried chips and they were suggested to be widely utilized in food processing as a functional modifier [55].

In this study, roasted JA tuber powder was used as additive in coffee to modify the characteristics. Overall, the results of this study showed the remarkable functionality of roasted JA tuber in Vietnamese coffee with increases of antioxidant activity by 48.0%, 13.6%, 26.9% in TPC, ORAC and DPPH assays respectively, and it also showed potential to improve characteristics of coffee such as smoothness, color, taste and flavor. Thus, roasted JA tuber was a possible replacement of artificial sweetener in coffee as

well as a functional additive, and JA coffee had the potential to be introduced to common human diets for its characteristic flavor and health benefits.

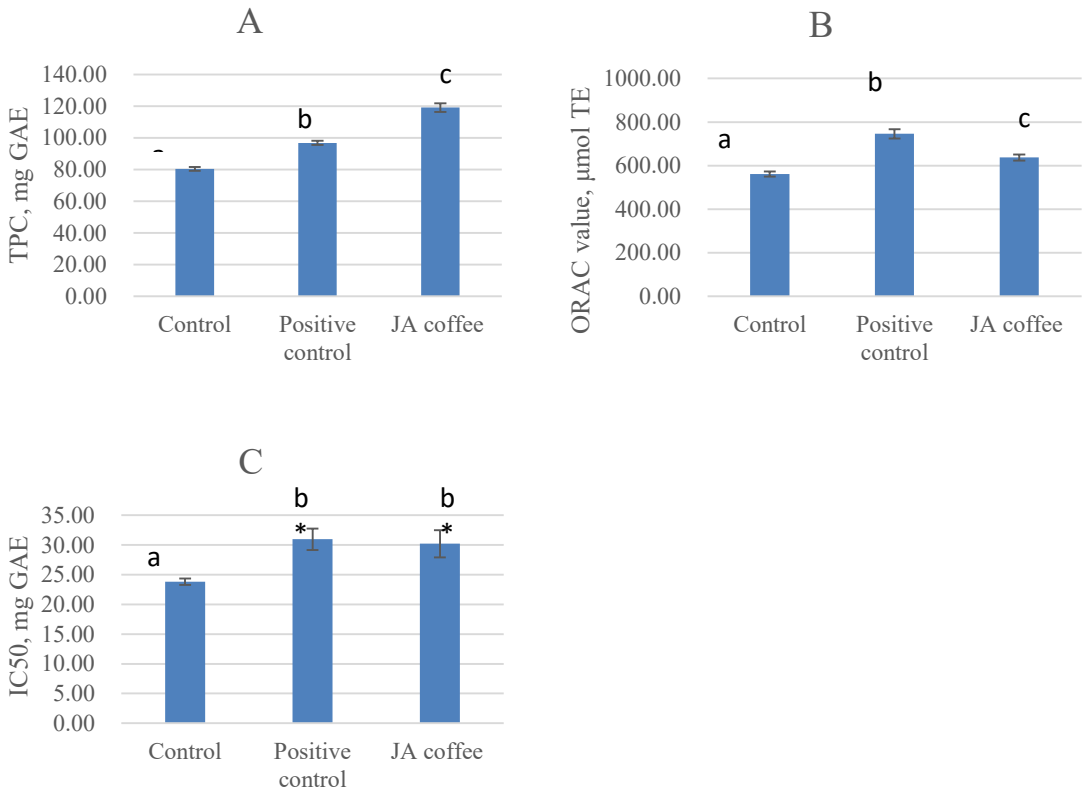


Figure 2-7 Antioxidant activity of the control (1g coffee), positive control (1g coffee with 0.11g inulin standard) and JA coffee (1g coffee with 1g JA). A) TPC, mg GAE, B) ORAC, µmol TE C) DPPH, IC50 mg GAE. Values with different letters are significantly different ($p<0.05$). Values are means ± SEM

2.6 Conclusions

The phenolic compounds from plants are important antioxidants that can be more commonly used in daily diets. In the present study, the functionality of JA derived inulin on Vietnamese coffee was investigated. The antioxidant activity of JA coffee was significantly higher than that of Vietnamese coffee. The reason behind this could be the interaction of inulin with phenolic compounds in coffee. The success of JA in increasing

antioxidant activity of coffee makes JA coffee a fine source of natural antioxidants as well as a possible diet for people with diabetes or cardiovascular diseases.

Future studies should aim to investigate the mechanisms behind the functionality of inulin on increasing the antioxidant activity of Vietnamese and other brands of coffee. Furthermore, evaluation of the *in vivo* antioxidant activity of phenolic extracts from coffee will be of great interest.

2.7 Connecting statement to Chapter 3

In Chapter 2, the phenolic content in coffee was analyzed by HPLC, and the addition of inulin and JA was proved to have the ability to increase the antioxidant activity of Vietnamese coffee. The results in Chapter 2 indicates that the JA coffee product not only contains the healthy ingredient JA, but also provides an enhanced antioxidant activity. Coffee brews are common diets in daily life, and there is a large amount of coffee waste produced which can be a resource and should not be ignored.

In Chapter 3, the phenolic compounds in coffee waste were extracted and determined by HPLC. To utilize the phenolics in coffee waste, a lecithin-based food grade microemulsion system was developed. The study aims to apply phenolic extracts from coffee waste to the food grade microemulsion system, and the stabilization area was expressed in pseudoternary phase diagram.

3 Chapter: Development of a novel food grade microemulsion system

3.1 Abstract

In this study, a novel food grade microemulsion system composed of grape seed oil, lecithin, ethanol, and water was developed and applied on phenolic compounds extracts of spent coffee ground. Nine different formulations were prepared among which A2 (19.61% Lecithin/Ethanol, 78.43% Grapeseed oil, 2.00% water) and B2 (19.51% Lecithin/Ethanol, 78.05% Grapeseed acid, 2.44% phenolic solution) were considered to be worth studying due to their low levels of surfactants, high solubilization of grape seed oil, and relatively lower viscosity. The phenolic compounds in spent coffee ground were extracted and determined by HPLC, and the concentration was 0.39 mg/mL. Phenolic compositions in microemulsions were in a range from 5.85 to 58.5 μg/g. Effects of the extrusion technique, LiposoFast device on microemulsions were investigated through Cryo-SEM observation. Results indicated that the extrusion technique was able to make microemulsions more uniform in structure.

3.2 Introduction

As illustrated previously, microemulsions have been investigated in a wide range of fields including food, pharmaceutical and cosmetic products, nanoparticles synthesis routes, chromatography, and extraction techniques. Nevertheless, the large amount of surfactants required in microemulsions for sufficient formulation stability is one of the significant disadvantages of microemulsions. It is reported that high surfactant contents in consumer products may cause unwanted residues, and high surfactant levels in synthesis processes may cause undesired byproducts or contaminants [56]. Another drawback of microemulsion systems is that many of them have a poor oil solubilization capacity. In

this case, mixed surfactants and co-surfactants have been studied to reduce the interfacial tension which improves the stability of microemulsions formulations [57].

Lecithin as a biological amphiphile is reported to be an available surfactant in the preparation of non-toxic microemulsion systems. It is noteworthy that lecithin-based microemulsions need the presence of a short-chain alcohol working as a co-surfactant to balance the hydrophilic and hydrophobic effects [58]. Lecithin-based microemulsions have been reported numerous applications in drug delivery. According to Dreher *et al*, a soybean lecithin microemulsion gel composed of soybean phosphatidylcholine, isopropyl palmitate and water could successfully work as a penetration enhancer of anti-inflammatory drugs for transdermal drug delivery without significant skin irritation [59]. Curcumin microemulsions with soybean oil/lecithin were introduced by Lin *et al*, and they showed exclusively prominent cytotoxic effects on human hepatoma HepG2 cell line [60]. In this study, food grade soy lecithin was used as the surfactant with ethanol as the co-surfactant.

Grape seed oil is a byproduct of winemaking and grape juice processing. It contains about 90% of fatty acids in which 70% of linoleic acid [61]. It has a wide range of applications in food, pharmaceutical, and cosmetics products. It is reported that grape seed oil can be a composition of microemulsions as the oil phase. Davidov-Pardo *et al* successfully developed a method of resveratrol encapsulation with grape seed oil microemulsions which contained 10% oil phase (grape seed oil: orange oil 1:1, w/w), 10% Tween 80 as surfactants, and 80% water phase [62]. Grape seed oil was used as oil continuous phase in microemulsions in this study.

In the daily coffee beverage consumption, a massive amount of spent coffee ground is generated. Recently, possible applications of the coffee waste are of interests for resource saving and environmental protection purposes. It is demonstrated that byproducts in coffee processing is a source of important ingredients such as fibers and antioxidants, and spent coffee grounds have a total phenolic content of 20 mg GAE/g approximately [63]. The feasibility of applying phenolic compounds extracts on the lecithin-based microemulsions was studied in the present research.

In this study, the third phase titration method was used to prepare microemulsions. The objectives of this study were to: a) develop a novel food grade microemulsion system with lecithin, ethanol, grapeseed oil, and water, b) apply the microemulsion system on phenolic compound extracts from spent coffee ground.

3.3 Materials and methods

3.3.1 Materials and instruments

Soya lecithin was obtained from a local nutrition store. HPLC grade methanol, anhydrous ethanol, and acetic acid were purchased from VWR (Mississauga, ON, Canada). Grape seed oil was purchased from Mother Earth (Ottawa, ON, Canada). The coffee sample was obtained from CAFÉ CIMO inc. (Montreal, QC, Canada).

Instruments included the HPLC-UV-PDA (e2695, Waters, Milford, MA, USA), a Vega-II XMU SEM with a cryo-stage (TESCAN, Brno, Czech Republic), LiposoFast-Basic device and 100 nm polycarbonate membranes (AVESTIN Inc, Germany).

3.3.2 Optimization of surfactant: co-surfactant ratios

Lecithin was used as the surfactant, and anhydrous ethanol was the co-surfactant. Four ratios were tested for seeking an optimized ratio, and they were lecithin: ethanol

(L/E) 90:10, 80:20, 75:25, and 70:30 (w/w). Certain ratios of lecithin and ethanol were mixed well for further study.

L/E 90:10 had a significantly higher viscosity than other ratios. L/E 80:20 performed a fine, homogeneous and transparent mixture, and it stayed stable after 24 hours standing. However, the L/E 75:25 and 70:30 were opaque mixture at the moment prepared, and they were separated into two phases after 24 hours standing. Generally speaking, L/E 80:20 was considered to be the optimized ratio of surfactant: co-surfactant, and it was prepared freshly before experiments.

3.3.3 Formation of microemulsions and the pseudoternary phase diagram

The L/E (80:20) was mixed with the second phase, grape seed oil (GSO), in 9 different ratios (10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10) in screw-capped glass tubes. The third phase, Milli-Q water, was titrated into each ratio with 5 μL per time as the mixture was stirred by a magnetic stirrer at 1000rpm. The titration ended until the system was non-transparent, so the borders of the one-phase microemulsion region could be calculated based on the percentage of the three phases in the system. Each sample was prepared in triplicates. The temperature was kept at room temperature, 23 ± 0.2 °C. Samples in which water was the third phase belonged to Group A, and the compositions of samples in Group A were listed in Table 3-1.

Based on the percentage of each phase, pseudoternary phase diagram of Group A was conducted by software SigmaPlot (version 10.0).

Table 3-1 Ratios of L/E to grape seed oil in Group A

Sample No.	L/E, %	Grape seed oil, %
A1	10	90
A2	20	80
A3	30	70
A4	40	60
A5	50	50
A6	60	40
A7	70	30
A8	80	20
A9	90	10

3.3.4 Preparation of spent coffee ground and phenolic compounds extraction

Coffee was brewed by a coffee maker with the proportion 5 grams of coffee: 20 mL of Milli-Q water. The coffee waste (wet) was freeze-dried and stored in a -20 °C freezer.

1 gram of dried spent coffee ground was mixed with 20mL of acidified (1% acetic acid) methanol solution (80%), and the mixture was stirred for 3 hours at room temperature (23°C), and then centrifuged at 4000 RPM for 15 minutes at room temperature (23 °C). The supernatant was collected and stored in a -20°C freezer for further uses.

3.3.5 Determination of phenolic compounds in extracts from spent coffee ground

Phenolic extracts from spent coffee ground was filtered, and 10 μL of it was injected to HPLC-UV-PAD equipped with a Synergi-Max-RP (reversed phase) column

(250 x 4.6 mm, 5 μm) with column temperature 35 °C. The mobile phases were solvent A (0.01% formic acid) and solvent B (100% acetonitrile). The gradient program was as follows: 0-35 minutes, 90% solvent A; 35-40 minutes, 50% solvent A; 40-50 minutes, 90% solvent A. The flow rate was 1 mL/min. The phenolic standards were the same as in Chapter 2. The concentrations of phenolic compounds in the extracts were calculated according to standard curves.

3.3.6 Application of the microemulsion system on phenolic compounds extracts

Phenolic compounds extracts of coffee waste were measure 5 mL and dried by nitrogen flow and re-dissolved by 5 mL Milli-Q water with the assistance of ultrasound treatment. This solution replaced Milli-Q water in microemulsions formation procedures. Samples in which phenolic extracts water solution was the third phase belonged to Group B, and the compositions of samples in Group B were listed in table 3-2 (p 45). The pseudoternary phase diagram was conducted by software SigmaPlot (version 10.0), as well.

3.3.7 Mini-extruder extrusion technique by the LiposoFast device

The mini-extruder (LiposoFast) device was equipped with a 100 nm carbonate membrane. A8 and B8 opaque solution was taken 400 μL and added into a syringe of the device respectively and then extruded 20 times through the membrane in the middle. Final extruded samples (EA2, EB2) were collected into a sealed vial respectively and stored at room temperature (23 °C) overnight to remove air bubbles formed in the process.

3.3.8 Cryo-SEM (cryogenic scanning electron microscope)

A2 and B2 were chosen to perform Cryo-SEM. Microemulsions were added on a sample holder and placed into the cryo-stage with -65 °C. The samples were viewed at 20.00 kV. Extruded samples of A8 and B8 were also viewed. This part was conducted at Nano Imaging Facility laboratory (Carleton University, Ottawa, ON.).

Table 3-2 Ratios of L/E to grape seed oil in Group B

Sample No.	L/E, %	Grape seed oil, %
B1	10	90
B2	20	80
B3	30	70
B4	40	60
B5	50	50
B6	60	40
B7	70	30
B8	80	20
B9	90	10

3.4 Results

3.4.1 Formations of microemulsions

As demonstrated in 3.3.2, the optimal ratio of L/E was 80:20 since lower ratios caused separation of phases (Figure 3-1).

The results of the maximum of aqueous phase capacity of samples were listed in Table 3-3. The pseudoternary phase diagram of the microemulsion systems was shown in

Figure 3-2. Since a relative lower level of surfactants, a lower viscosity, and a relatively stronger capacity of aqueous phase of microemulsions were desired, A2 with a formulation of 19.61% of L/E 80:20, 78.43% of grape seed oil (GSO), and 2% of water was considered to be the best formulation and proceeded to further studies.

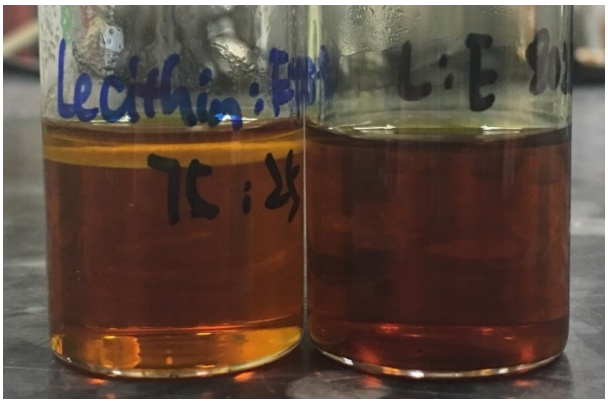


Figure 3-1 Comparison of L/E 75:25 (left) and L/E 80:20 (right)

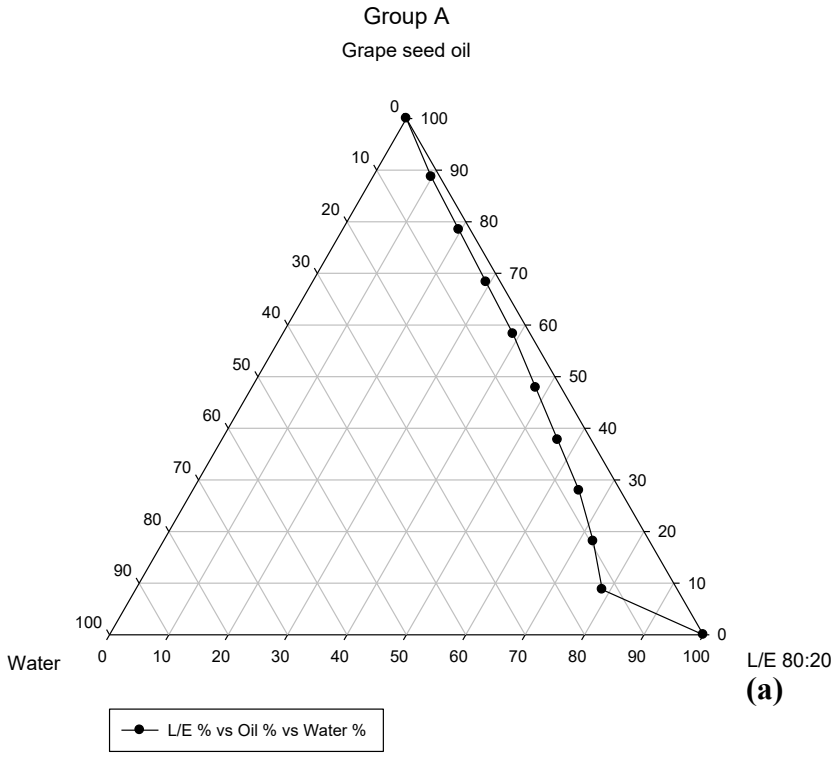


Figure 3-2 Pseudoternary phase diagram of lecithin: ethanol (80:20)/grape seed oil/water microemulsions, (a) microemulsion area. (L/E, lecithin/ethanol)

Table 3-3 Compositions of microemulsions in Group A

Sample No.	% L/E	% Oil	% Water
A1	9.85	88.67	1.48
A2	19.61	78.43	1.96
A3	29.27	68.29	2.44
A4	38.83	58.25	2.91
A5	47.85	47.85	4.31
A6	56.60	37.74	5.66
A7	65.12	27.91	6.98
A8	72.40	18.10	9.50
A9	78.60	8.73	12.66

3.4.2 Determination of phenolic compounds in extracts from spent coffee ground

Phenolic compounds in spent coffee ground (freeze-dried) was extracted with 80% acidified methanol and determined by HPLC-UV. The average concentration of total phenolic compounds in the extracts was 0.39 ± 0.01 mg/mL.

3.4.3 Application of the microemulsion system on phenolic compounds extracts from spent coffee ground

The aqueous dispersions with phenolic extracts from spent coffee ground were titrated to L/E and oil mixtures as the third phase, and transparent microemulsions were formed, while they became opaque when the critical amount of aqueous phase was titrated. The proportions of three phases in microemulsions were listed in Figure 3-3. As

demonstrated previously, sample B2 (19.51% L/E, 78.05% GSO, 2.44% aqua) was selected to perform further experiments.

3.4.4 Mini-extruder extrusion technique and Cryo-SEM

Two microemulsions were prepared with the compositions 20% lecithin/ethanol, 80% grape seed oil, and 15 µL water titrated in sample A, and 20% lecithin/ethanol, 80% grape seed oil, and 15 µL phenolic extracts solution titrated in sample B. The two microemulsions were treated with the LiposoFast micro extrusion device. The images of samples before and after extrusion were obtained by a Cryo-SEM. The differences between them were shown in Figure 3-4.

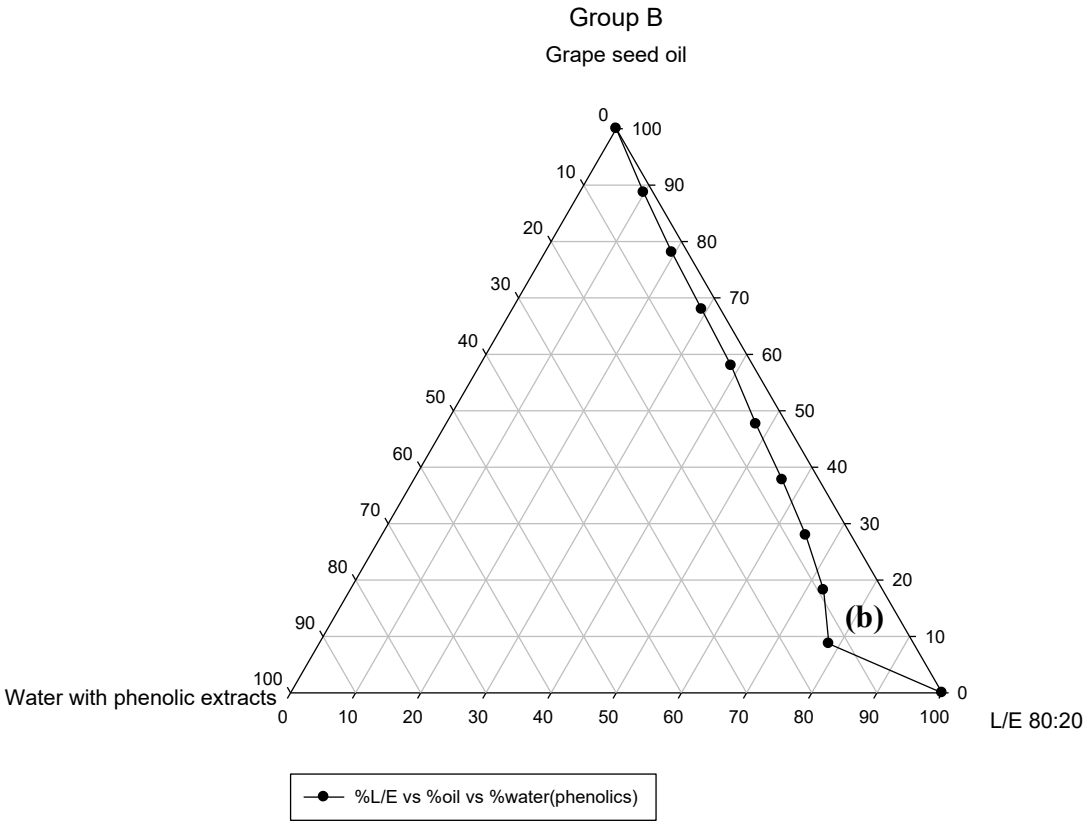


Figure 3-3 Pseudoternary phase diagram of lecithin: ethanol (80:20)/grape seed oil/water with phenolic extracts microemulsions, (b) microemulsion area. (L/E, lecithin/ethanol)

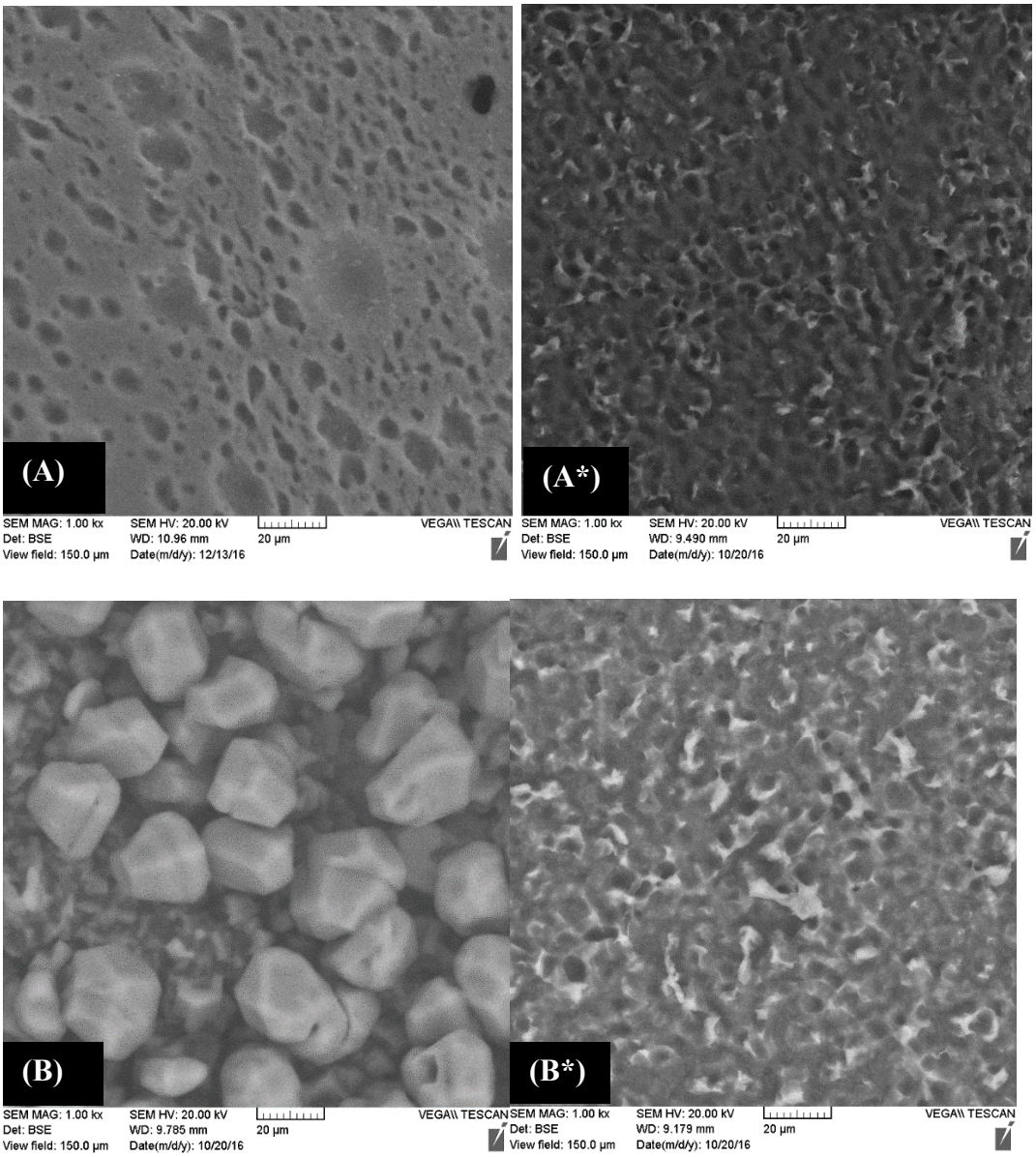


Figure 3-4 Structures of microemulsions under Cryo-SEM. (A) contains 20% lecithin/ethanol, 80% grape seed oil, and 15 µL water. The appearance of A is transparent. (A*) is A after the treatment of LiposoFast device and transparent. (B) is a phenolic-based microemulsion with 20% lecithin/ethanol, 80% grape seed oil, and 15 µL phenolic extracts solution. (B*) is B after the treatment of LiposoFast device and transparent.

3.5 Discussion

In the present study, formulations of food grade microemulsion systems composed of grape seed oil, lecithin: ethanol (80:20), and water were investigated. Based on the pseudoternary phase diagram (Figure 3-2), the phase behavior of each formulation could be read. The one-phase microemulsion area (area (a) and (b) in Figure 3-2, 3-3) was the area enclosed by the phase-behavior line and the grape seed oil axis. Abbasi et al introduced lecithin-based microemulsion systems with n-propanol as the co-surfactant. [64] It is reported that lecithin-based microemulsions are stable with a wide range of ionic strength (0~6M) and pH (1~10.5), and the microemulsion areas increase as the temperature rises which may indicate a higher solubilization capacity of triglycerides. [64] Comparing with n-propanol, ethanol could not be soluble with lecithin at any proportion, but ethanol as a co-surfactant was able to lead to microemulsions with a microscale of water added (Table 3-3).

The phenolic compounds extracts from spent coffee ground were introduced to microemulsion systems, and the total phenolic content of each formulation was listed in Table 3-4. Sample B9 showed the highest concentration of phenolic compounds 58.5 μg/g, and B2 had a concentration 9.75 μg/g. According to the pseudoternary phase diagram of phenolic-additive microemulsion systems, the phase behavior of phenolic-additive microemulsions was similar to that of original lecithin-based microemulsions, and it indicated that the lecithin-based microemulsion systems were stable with addition of phytochemicals such as phenolic acids. Based on Edris *et al*, five different microemulsion systems were used to investigate the solubilization behavior of essential oils including clove buds, thyme, and oregano essential oils, and results showed that

microemulsions as solubilization enhancing systems improved the solubilization of all essential oils, what's more, different phenolic compounds complex had different effects on the phase behavior of microemulsions.[65] Results of this study showed that the lecithin-based microemulsions could enhance solubilization of grape seed oil as the literature. Furthermore, the effects of isolated phenolic acids on the lecithin-based microemulsions would be the further research aim.

According to the SEM photos of samples (Figure 3-4), the structures of both A and B were sponge-like texture which indicated that the microemulsion type of A (19.61% L/E, 78.43% GSO, 2% water) and B (19.51% L/E, 78.05% GSO, 2.44% aqua phase) were the bicontinuous microemulsions. Comparing A and B with the extruded samples (A*, B*), more uniformed microemulsions structures were formed with the treatment of extrusions by the LiposoFast device with a 100 nm pore-sized membrane. This indicated that microemulsions could be size-controllable.

Liposomes are of interest in drug delivery fields because of their bilayer cytomembrane-like structures. They are vehicles formed by phospholipids, and water-soluble drugs can be enclosed by the phospholipids membrane, while hydrophobic substances can be retained in the bilayer wall.[66] Lecithin is a natural material for preparation of liposomes. However, the preparation of lecithin liposomes calls for high-cost purified lecithins, organic solvents (detergents), and a process of solvent evaporation which is an extra cost.[66b] In contrast, lecithin-based microemulsions obtained from this study were easier to prepare, low-cost, and environmental friendly since no toxic solvents were involved in the preparation process. Moreover, microemulsions may be more effective than liposomes in transdermal delivery of hydrophobic drugs. As Mura *et al.*

reported, the transdermal delivery of clonazepam, a poorly water-soluble benzodiazepine, through liposomes and microemulsions was investigated. Drug permeability studies showed that microemulsion gels were considerably more effective than liposomal formulations.[67]

Overall, the grape seed oil/lecithin: ethanol (80:20)/water microemulsion systems were established, and they were stable with the presence of phenolic acids. Extrusion technique was proved to be capable to uniform the microemulsions.

3.6 Conclusions

Soya lecithin, a food grade surfactant, was proved to have the ability to form microemulsions with grape seed oil and ethanol as the co-surfactant. The optimal surfactant: co-surfactant ratio was 80:20. Microemulsions in the novel system were stable with the addition of phenolic compounds extracted from spent coffee ground, and the amount of phenolic compounds in microemulsions had a range from 5.85 to 58.5 μg/g of microemulsion. Cryo-SEM showed that the extrusion technique improved the appearance and homogenization of the lecithin-based microemulsions. This food grade microemulsion system could be used in many areas such as drug delivery, dietary supplements, and cosmetics.

4 Chapter: General conclusions and future directions

In the present study, the JA derived inulin significantly increased the antioxidant activity of coffee as indicated by TPC, ORAC, and DPPH assays. Phenolic compounds in coffee were extracted with acidified methanol for quantitative and qualitative analysis by HPLC. Results showed that the predominant antioxidant component in coffee was chlorogenic acid, and in Jerusalem artichoke tuber was pyrogallol. MS analysis confirmed that the inulin in the JA coffee were from the roasted JA tuber additive. As a result, the JA coffee could be introduced to healthy diets for its high antioxidant activity and inulin compositions. Meanwhile, the contribution of inulin to *in vivo* antioxidant activity of coffee and the biochemical mechanism of that to *in vitro* antioxidant activity of coffee would be of interest in future studies.

In this study, novel food grade microemulsions were successfully prepared by the combination of grape seed oil, lecithin, ethanol, and water, among which lecithin was the surfactant and ethanol was the co-surfactant. The lecithin-based microemulsions showed oil solubilization enhancing effects. The lecithin-based microemulsions were stable with the addition of phenolic compounds from spent coffee ground. The mini-extrusion technique (LiposoFast) was proved to be an effective improvement for the preparation of microemulsions of uniform size. Additionally, grape seed oil is rich in unsaturated fatty acids and antioxidants, and it has many potential health benefits such as cardio-protective, neuroprotective, anti-inflammatory, anti-carcinogenic and anti-obesity effects[62]. Thus, the microemulsion system has potential applications not only in the food industry, but also for pharmaceutics and cosmetics. Future directions would be focused

on the stability of the microemulsion system, such as the effects of temperature, pH, ionic strength, and different antioxidants.

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