Thesis of the Degree of Master of Food Engineering

Characterization and Encapsulation of Oils Extracted from Citrus (*Citrus junos*) By-products using Supercritical Carbon Dioxide

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Abstract

The processing of citrus fruits into juice and other products is one of the world's largest processing industries. The by-products (citrus peels and seeds) of this industry are about 50 % of the raw processed fruit. This not only wastes a resource of potential value, but also causes disposal problems. These by-products could be turned into an asset since they contain a wide range of healthy bioactive compounds. For these purposes, the oils extracted from those citrus by-products using supercritical carbon dioxide were characterized and encapsulated in order to add value to those by-products and hence the possible application of the resulting oils in many areas such as in food, pharmaceuticals, perfumery and cosmetic industries.

In the first study, the characteristics of oils extracted from a mixture (MX) of citrus seeds (CS) and citrus peels (CP) using hexane and supercritical carbon dioxide (SC-CO_2) were studied. The SC-CO_2 extraction conditions were 45 °C and 60 °C for temperature, 200 and 250 bar for pressure while for hexane extraction was 70 °C. Hexane extraction showed significantly ($p < 0.05$) higher oil yield than SC-CO_2 extraction. The chemical composition was analyzed by GC-MS and phytosterols, monoterpenes, sesquiterpenes and oxygenated monoterpenes were the main compounds of the oils. The fatty acid composition was determined by GC and linoleic acid was the major fatty acid. The oxidative

stability (OS) analysis was performed by Rancimat and the hexane extracted oils showed higher OS. The antioxidant activity was tested with DPPH and ABTS assay and SC-CO_2 extracted oils showed higher scavenging activity.

The aim of the second study was to investigate the impact of combining the CS and CP on the bioactive compounds, antioxidant and antimicrobial activities of resulting oil obtained using a modified SC-CO_2. The extraction conditions were pressure of 200 and 300 bar at temperature of 45 °C for neat SC-CO_2 and SC-CO_2+ethanol. The yield showed to increase significantly ($p < 0.05$) by increasing pressure. The total phenolic and total flavonoid content were determined, and CP oils showed higher total phenolic content, whereas CS oils showed the higher total flavonoid content. The tocopherol and phytosterol content were analyzed using HPLC, and α-tocopherol and sitosterol were respectively the main compounds of the extracted oils. The antioxidant activity was determined by DPPH and ABTS assay and the oils extracted by SC-CO_2+ethanol at 200 bar showed higher activity with IC_{50} values of 0.52 and 0.53 mg/ml for CP and MX, respectively, for DPPH assay. For antimicrobial activity, the MX oils showed higher activity and the oils were more susceptible for gram-positive than gram-negative bacteria.

The third study dealt with the formation, characterization and release behaviors of citrus oil-polymer micro-particles using PGSS process. Citrus oil was encapsulated in poly-ethyl glycol (PEG) by means of the particles from gas saturated solutions (PGSS) process. The influence of process conditions, i.e., pressure, temperature, and pre-expansion temperature, and oil/polymer mixing ratio, on the characteristics of particles and the efficiency of encapsulation has been analyzed. Again, the oxidative stability and release behaviors of encapsulated citrus oil were investigated. Spherical particles with particle sizes of 190.56–373.32 μm were obtained. The efficiency of encapsulation ranged between 43.95-83.87 % and it was dependent on process parameters. The oxidative stability and *in vitro* release significantly changed depend on storage temperature and pH of the incubation media, respectively and the oxidative stability was significantly improved by encapsulation.

Chapter 1

General Introduction

1.1. Background

Citrus, also known as *agrumes* (which means sour fruits), is considered to be of one of the world's major fruit crops with worldwide popularity and availability which contributes to human being diets. Because of large and unclear numbers of citrus species and large cultivation areas, the most well-known citrus fruits with commercial purposes are lemons, oranges, limes, tangerines, and grapefruit. Even though citrus fruits are cultivated worldwide in <140 countries, the biggest amount of citrus fruits are mostly grown on subtropical and tropical regions [1].

The origin and history of citrus plant is full of interesting and controversy legends. Some researchers do believe that citrus is a native of the tropical and subtropical regions of Asia, originating in some parts of Southeast Asia like the Malay Archipelago, India, and China [2]. Originally lemons were grown in India and mandarins and sweet oranges originated from China. Recent research proposes that, while some commercial species such as lemons, mandarins and oranges came originally from Southeast Asia, the true origins of citrus fruit are New Caledonia, New Guinea and Australia [3]. The spread of citrus to other parts of the world like southern Europe and northern Africa was slow. The Portuguese and Spanish explorers are the first to introduce the citrus to America and around 1655 and 1769, orchards first appeared in Florida and California, respectively. The citrus production, industrial processing, and global have considerably augmented since then, showing citrus as the world most important fruit [4].

1.1.2. Citrus fruit description and worldwide production

Citrus plants are in general the evergreen shrubs or small trees, bearing flowers, which give a strong scent. The fruits can be in different forms (for instance, elongated, oblong and round) and their size varies from 3.8 to 14.5 cm in diameter [3]. Generally, the citrus fruits consist of an

external skin made up of an epidermis (a leathery and waxy-like layer), the flavedo (a sub-epidermal part that encloses color and oil sacks producing essential oils), the albedo (a spongy-like part below the flavedo, a good source of flavonoids), and vascular bundles (a set of thin threads along the flesh; Figure 1.1). The internal flesh is composed of segments, often situated and aligned around the central core of the fruit and covered by a thin segment membrane called the septum. In most varieties, the sacs containing seeds and juice fill those segments, and the various acids (mainly citric acid) together with a complex mixture of sugars and oils give the flavor characteristic [4].

The global annual production of citrus fruit has increased significantly in last decades, from about 58 million metric tons in the late 1990s to a total approximate of >121 million metric tons between 2014 and 2015, with oranges contributing more than a half of global production [5] (FAO, 2015). China, Mediterranean region, Brazil, USA, and Spain are the world's most citrus-producing countries, where they represent 60 % of overall global production [5] (FAO, 2015).

1.1.3. Bioactive compounds of citrus peels and seeds

Even though many citrus fruits, such as grapefruits, oranges and tangerines can be consumed fresh, a huge proportion of citrus fruits produce is processed into juice and other products, which as a result leaves a huge amount of citrus peels and seeds as by-products. These citrus by-products can be valorized since they contain a wide range of healthy bioactive compounds. The following are some bioactive compounds in citrus peels and seeds.

Phenolic compounds are essential for the nutritional and sensory qualities that give the flavors, tastes and colors of many plants. While phenolics are abundant elsewhere in the plant kingdom, the citrus peels and seeds have a uniqueness of having several compounds (like polymethoxylated flavones, flavanone glycosides and flavanones) which cannot be found in other plants [6].

Carotenoids are the main pigments in ripe citrus fruit peels of most cultivars, which contribute to their various colors ranging from red yellow to golden. The occurrence of carotenoids in citrus peels makes citrus peels important sources of dietary carotenoids [7].

The volatile compounds are the other important components in the citrus peels. The active volatile fraction of the peel oils confers the flavor and aroma to the citrus oils. There are just over 300 citrus volatiles and oils, and other chemical components including terpene hydrocarbons (like monoterpenes hydrocarbons and sesquiterpenes), aldehydes esters, volatile organic acids, alcohols and ketones [8].

Citrus seeds are key sources of oils for pharmaceutical, industrial and nutritional applications.

The fatty acids are essential elements of citrus oils and the unsaturated fatty acids constitute a high proportion, where linoleic acid and oleic acid are the most abundant [9]. Moreover, citrus seeds are good source of phytosterols. They are of nutritional interest since they can play a great role in lowering the LDL cholesterol and total serum cholesterol in humans by dietary cholesterol absorption inhibition [10].

In addition to phytosterols and fatty acids, citrus seed oils are good sources of tocopherols (Vitamin E). Tocopherols are natural antioxidant agents with biological activities. It is believed that the major function of tocopherols in the oils is to protect the polyunsaturated fatty acids and other unsaturated components against peroxidation [10].

1.1.4. Sample of study (*Citrus junos*)

The *Citrus junos*, also called Yuza in Korea or Yuzu in Japan is a citrus crop that originates in East Asia with a classification of family mentioned in Table 1.1. It is known to be a hybrid of Ichang papeda and sour mandarin [11]. The fruit looks somewhat like a small grapefruit with a rough skin and can be either green or yellow depends on the degree of ripeness. Yuzu fruit typically ranges from 5.5 to 7.5 cm (in diameter) but can be as large as a regular grapefruit

(Figure 1.2). The average weight of fruit ranges from 114.1 to 231.8 g/fruit and the peels and seeds occupy about 43 % and 9 %, respectively, of the whole fruit [12].

Table 1.13 . Classification of *Citrus junos*

Kingdom:	Plantae
Phylum:	Angiosperms
Class:	Rosids
Order:	Sapindales
Family:	Rutaceae
Genius:	*Citrus*
Species:	*C. junos*

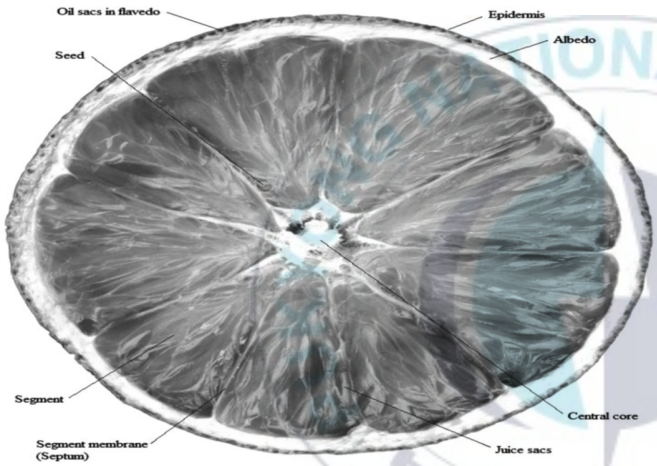

Figure 1.10. The schematic section of a citrus fruit depicting various parts

(A) (B) (C)

Figure 1.11. Whole fruit of *Citrus junos* (A), peels (B) and seeds (C)

1.2. Supercritical fluids (SFs)

Supercritical fluid (SF) is the condition of a fluid at a pressure and temperature beyond its critical point. Above this critical point, the substance is neither at its liquid nor at its gas phases. It can act both like a gas and a liquid. In other words, it can dissolve components like a liquid and diffuse through solids like a gas. Also, closer to the critical point, minor changes in temperature and/or pressure yield in huge changes in viscosity, density, and diffusivity.

Traditionally, bioactive compounds are extracted by hazardous organic solvent. Many works describing the extraction methods have been done and reported for extraction of bioactive compounds from plant matrices. Such extraction by conventional methods have the drawbacks including the use of toxic solvents that have undesirable health effects or using the high temperature that may cause the degradation of the thermally unstable compounds [13]. In recent years, the use of supercritical fluid extraction (SFE) for the removal of biomaterials from various kinds of plant and animal matrices has been drawn much attention due to increasing awareness of environmental problems. This technique has some benefits over conventional methods; mostly because of its uniqueness of its physical properties. SF is appropriate as an alternate for conventional solvents in a range of laboratory and industrial processes. Carbon dioxide is the common used SF, being used for extraction of biomaterials from animal and plant sources containing thermo-unstable compounds and it has been also used in particle formation.

1.2.1. Properties of SFs

The SF properties can be elucidated by considering the density, viscosity, diffusivity, and solvating power. The SF density is very sensitive to small changes in pressure and temperature around its critical point. The physical phase of the substance of constant composition can be explained by a phase diagram depicted in Figure 1.3. In the diagram, there are three lines which describe the melting, boiling, and sublimation process which identify the regions corresponding

to the solid, liquid, and gas states. The vapor pressure begins at the triple point and finish at the critical point. The critical region starts at the critical point and there is one phase only and it has both gas and liquid properties [14].

The SF Solvating power is greatly reliant on its pressure and temperature. At minimum pressure, the SF solvating power drastically diminishes with increasing temperature, while at higher pressures it rises with augmenting the temperature. If the parameter 'pressure' is replaced by the parameter 'density', the solubility-temperature relationship becomes much easier. This abnormal behavior happens since the density reduces significantly with an increase in temperature at lower pressure; while at higher pressure, change in temperature causes lesser effect on density. Therefore, density is the first consideration regarding to the solvent power of SFs.

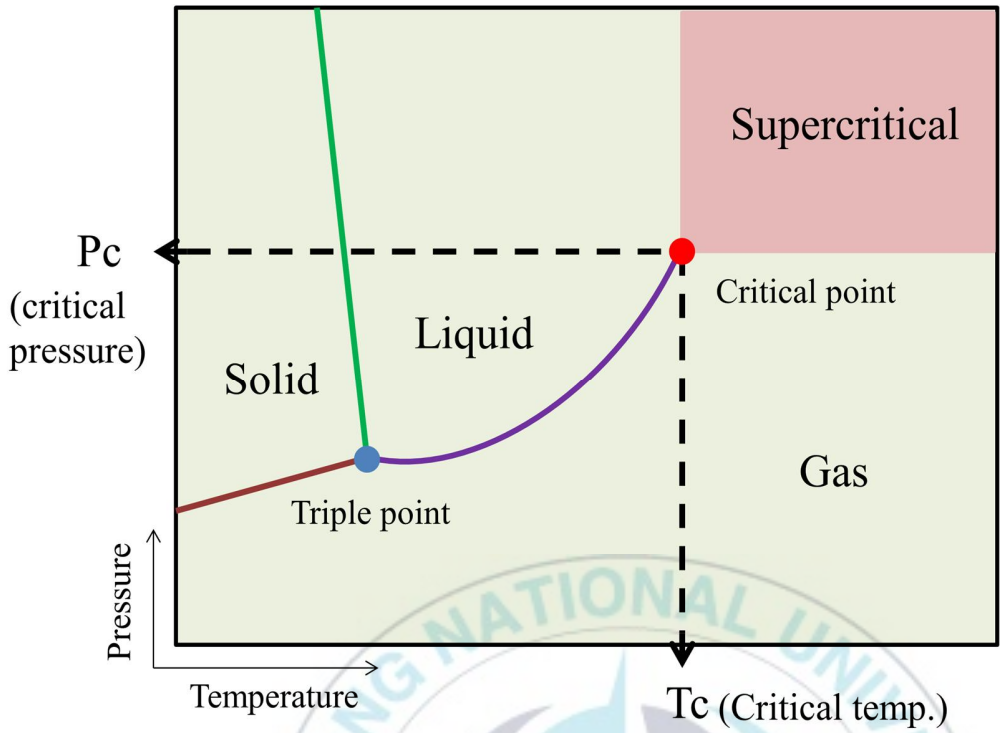

Figure 1.12. Pressure-temperature phase diagram of a substance with critical temperature (Tc) and pressure (Pc)

Table 1.14. Physical properties of gases, supercritical fluids, and liquids

	Density (g/mL)	Dynamic viscosity (g/cm-sec)	Diffusion coefficient (cm^2/sec)
Gas (ambient)	0.0006-0.002	0.0001-0.003	0.1-0.4
Supercritical fluid (critical temperature and pressure)	0.2-0.5	0.0001-0.0003	0.0007
Liquid (ambient)	0.6-1.6	0.002-0.03	0.000002-0.00002

SF shows the physicochemical properties intermediate between those of a gas and a liquid. SF has diffusivities and viscosities similar to those of a gas, whereas the densities are like those of liquids (Table 1.2). Therefore, a SF can effuse fast compared to a liquid in a solid matrix, yet has a solvent-like strength to extract the solute from the matrix. Additionally, SF has no surface tension, as there is no liquid/gas phase boundary. By varying the temperature and pressure of the fluid, the properties can be turned to be more gas or more liquid-like.

SF has good density, high diffusivity and low viscosity which show high solvating strength. This is the main advantages of SF in which their physical properties are similar to those of both liquids and gases. Additionally, the combination of high diffusion and low viscosities coefficients in SF is a most important advantage as lower viscosity helps the good infiltration of the solutes, good mass transfer, a small pressure drop, and hence an improved phase separation.

1.2.2. Supercritical carbon dioxide (SC-CO_2)

The SC-CO_2 is a fluid state of carbon dioxide (CO_2) where it is at or above 73.8 bar (critical pressure) and 31.1^oC (critical temperature). The SC-CO_2 separation provides more benefits over the other SFs due to the non-flammability, non-toxicity, inexpensive, inertness to most materials of CO_2. Again, it can be used under mild operational conditions. Moreover, high diffusivity, liquid-like density, low viscosity and high compressibility make the SC-CO_2 an excellent solvent [15]. In addition, the possible mild operational conditions of SC-CO_2 process make the SC-CO_2 a good candidate for extraction of heat labile compounds [16, 17].

1.2.2.1. SC-CO_2 extraction

SC-CO_2 extraction can be described as separation of chemicals, flavors and other bioactive compounds from the products (plant, animal, algae, etc) which are together with SC-CO_2 to form a same mobile phase. In this process, the subjection of that mobile phase to above or near the

critical temperatures and pressures enhances the solvating power of the mobile phase. The process starts with CO_2 in vapor form, then compressed into a liquid form prior to become supercritical and when it attains its supercritical conditions, the extraction takes place [18].

In some cases, the addition of modifiers or co-solvents to the supercritical phase is needed when the solute extraction kinetics is slow or when the solubility of analyte in SC-CO_2 is low. In other words, the modifiers or co-solvents are added to SC-CO_2 to improve the extraction efficiency. Therefore, the addition co-solvents (like solar solvents) to CO_2 widens its extraction range to extract even more polar compounds. For instance, when the SC-CO_2 extraction with 20 % of ethanol was carried out, above 80 % of the phospholipids were obtained from plant matrix [19].

1.2.2.2. Comparison between SC-CO_2 and organic solvent extraction

Solvent extraction is a conventional method for extraction. The benefits of SC-CO_2 over other conventional methods like solvents extraction and distillation are reduction in operational steps, automation, safe operations due to using the inorganic solvents and the use of modest temperature in the critical range favorable for thermally labile compounds, hence the exceptional quality of the resulting product [20]. Moreover, due to the manipulation of extraction conditions, the higher selectivity can be easily achieved in SC-CO_2 extraction. However, the main drawback is the cost of supercritical extraction equipment and requires the high sensitive process control. In addition, the phase transition of the combination of solvents and solutes should be predicted or measured with higher accuracy, which is complex and difficult [21].

1.2.3. SC-CO_2 for polymer processing

CO_2 is a good solvent for many non-polar and some polar molecules with low molecular weight. It is a very poor solvent for most high molecular weight polymers (due to their high polarity). Very few polymers (like certain amorphous fluoropolymers and silicones) have shown a good

solubility in pure CO_2 under mild conditions [22]. Though the solubility of most polymers in pure CO_2 is extremely low, the solubility of polymers in SC- CO_2 is higher.

The concentration of dissolved CO_2 in polymer mainly depends on the processing temperature and pressure. The dissolved CO_2 causes a considerable reduction in viscosity due to increase in a free volume of polymer. Thus, less energy is consumed during the process. The dissolved CO_2 also alters the other physical properties such as reduction in density and increase in diffusion coefficient. Therefore, it has a tremendous potential as a plasticizer in polymer processing.

1.2.3.1. Solubility and viscosity of SC-CO_2 in polymer processing

The knowledge of SC-CO_2 solubility in molten polymers is crucial for the commercial success of supercritical-polymer processes. The dissolved SC-CO_2 in molten polymers alters most physical properties of the polymers like the viscosity, density, diffusivity and swollen volume. A lot of attention has been paid to the situations where polymers are dissolved in SC-CO_2. Only a limited part of this concerns molten polymers, the most likely form for processing.

In general, an increase in pressure increases the solubility of SC-CO_2. The same law is applicable also to a polymer and SC-CO_2. The density of SC-CO_2, which is a strong function of temperature and pressure, plays a vital role in deciding its solubility in a polymer. However, the quantity of SC-CO_2 dissolved in different polymers also differs depending on the available chemical groups. A difference in the solubility can be explained with the specific intermolecular interaction between SC-CO_2 and the chemical groups available in the polymers.

The processing of high molecular weight polymers is not an easy task. High viscosity is a major obstacle in processing a high molecular weight polymer. An option is the processing of a polymer at elevated temperatures since viscosity decreases with increasing temperature. But at elevated temperatures, degradation of polymers is an important concern. In addition, it consumes

a large amount of energy. The use of organic solvents can avoid this problem by reducing the viscosity of polymers at low temperatures. Nevertheless, emissions of the solvents into the environment, the separation of the solvents, and the reactive nature of the solvents are the major problems.

An alternative to this option is the use of supercritical CO_2 as a plasticizer or a solvent for viscosity reduction in various processing. The dissolved SC-CO_2 causes plasticization at a low temperature. This plasticization is due to the reduction in the glass transition or melting point of a polymer [23]. Plasticization is generally referred to the reduction in viscosity due to the dissolved gas [24]. In this way, the processing of polymers can be carried out at low temperatures and hence, the degradation of polymers can be avoided.

1.2.3.2. Application of SC-CO_2 in the production of micro-particles

Milling, grinding, crystallization and spray drying are the particle formation methods commonly used in the coating, toner and drug delivery industries. Narrow particle size distribution, solvent recovery and avoiding the emissions of VOCs are the major challenges associated with these methods. In addition, milling and grinding are not suitable for thermo instable and low glass transition temperature or melting point compounds due to the frictional heat dissipated during processes. Therefore, the industries have been looking for new technologies, which would provide micro-sized particles with a narrow particle size distribution using as small as possible quantity of VOC. This has motivated chemical engineers as well as chemists to apply a SC-CO_2 rather than classical methods.

In the last decade, the research on particle production using SC-CO_2 has rapidly been growing. Various methods already exist that use SC-CO_2 as a solvent or anti-solvent. Those methods include rapid expansion of supercritical solutions (RESS), gas anti-solvent crystallization (GAS), supercritical anti-solvent precipitation (SAS), and precipitation by compressed anti-solvent

(PCA), solution enhanced dispersion by supercritical fluid (SEDS) and particles from gas saturated solutions (PGSS)

1.2.3.2.1. Rapid expansion of supercritical solution

The rapid expansion of supercritical solution (RESS) method utilizes a dramatic change in the dissolving power of a solvent, when it is rapidly expanded from a supercritical pressure to a low pressure. After expansion, the solvent exist as a gas that makes the collection of the resulting particles (solute) much easier.

RESS is based on crystallization or precipitation of a solute in order to facilitate the powder production. The method can generally be used if the solubility of a solute (polymer) in SC-CO_2 or another appropriate fluid is high. A fluid is pressurized and heated to have the supercritical conditions needed for the process and passed through an extractor containing a solute in order to form single phase solution. Following this, the solution is depressurized over a nozzle to atmospheric pressure. The rapid depressurization leads to nucleation of the solute caused by the lowering of the solvating power and therefore particles are formed. After the depressurization, SC-CO_2 turns into the gas phase and is purged out of the collecting device.

1.2.3.2.2. Supercritical anti-solvent methods

Supercritical anti-solvents methods are applicable to materials whose solubility in a supercritical fluid is very low. In these methods, a supercritical fluid is used as an anti-solvent. The operating principle is the same for all supercritical anti-solvent methods.

A solute is first dissolved in a solvent and then, exposed to a supercritical fluid in order to generate particles. The selected solvent has a good affinity for the supercritical fluid. The solvating power of the solvent is reduced and the solution becomes supersaturated with the solute. Consequently, the precipitation of the solute takes place and micro-sized particles are formed. The nozzle through which the supercritical fluid is added is an important factor in order to

control the morphology and size of the particles. At the end of the process, the precipitator is washed with the anti-solvent to remove the solvent completely.

1.2.3.2.3. Particles from gas saturated solution

Unlike RESS, a gas is dissolved in a solute under sub- or supercritical conditions in the particles from gas saturation (PGSS) method. A solution is formed by saturating a solute with a gas. The gas-saturated solution possesses a low viscosity due to an increase in free volume. Moreover, the interfacial tension between the gas and the liquid phase is lowered as the surface tension of the gas in the supercritical state is zero. These properties ease the expansion of the solution. The solution is then expanded over a nozzle from a supercritical pressure to ambient pressure. It causes a super saturation of the gas and an intense expansion of the nucleated gas bubbles leads to explosion of the molten material into fine particles. The particles are solidified due to the cooling effect of an expanded gas.

The PGSS method can be performed either using batch or continuous mode. In a batch mode a solution is formed using a stirrer while in a continuous mode a static mixer is used to saturate a molten polymer with a gas. The batch process has been applied for the generation of powder of poly (ethylene glycol). Conventional coating systems like acrylic coatings, polyester-epoxy systems and low melting polyester coatings have been produced using a continuous process.

1.3. Objectives of the thesis

The processing of citrus fruits leaves massive by-products. This not only wastes a resource of potential value, but also causes disposal problems. This work was designed to look for possible ways to valorize those by-products. In addition, due to all advantages of SC-CO_2 over conventional methods, the SC-CO_2 was used as a solvent and its implication was discussed broadly in this study.

Therefore, to accomplish all of the following tasks have been performed:

1. Comparison of characteristics of oils extracted from a mixture of citrus seeds and peels using hexane and supercritical carbon dioxide (Chapter 2).
2. Bioactive compounds, antioxidant and antimicrobial activity of oils obtained from a mixture of citrus peels and seeds using a modified supercritical carbon dioxide (Chapter 3).
3. Formation, characterization and release behaviors of citrus oil-polymer micro-particles using PGSS Process (Chapter 4).

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Chapter 2

Comparison of Characteristics of Oils Extracted from a Mixture of Citrus Seeds and Peels using Hexane and Supercritical Carbon Dioxide

2.1. Introduction

The citrus fruit of the family Rutaceae is one of the most grown fruit worldwide. It is broadly grown in the tropical and subtropical parts of the world, and many other regions, with a yearly production of about 102 million tons [1]. Besides their huge size consumption as fresh fruits, the citrus fruits are primarily processed into juice. The by-products of this industry including seeds, peels and pulps are around 50 % of the unprocessed fruits [2]. Consequently, this not only wastes useful resources, but also poses a problem for management, pollution, and environmental issues, due to microbial spoilage [3]. These by-products could be turned into an asset since some studies successively showed their potentiality in many areas such as in food, pharmaceuticals, perfumery and cosmetic industries due to the biomaterials they contain [4, 5].

Citrus peels (CP) contain a high concentration of bioactive compounds. These compounds have been reported to have high antioxidant activity and exert antimicrobial effects against food borne pathogens [6, 7] due to their high contents of terpenoids, coumarins, phenolic acids and flavonoids. One product among a variety of products which can be obtained from CP is essential oils. Citrus essential oils have extensive applications. Principally, they serve as aroma in various food products such as beverages, dairy products, soft drinks, etc [8]. The pharmaceutical industries use those essential oils as flavoring agents to cover unlikable tastes of drugs.

Furthermore, the low volatile essential oil components play a key role in perfumes and cosmetics [8].

Citrus seeds (CS) is another by-product of citrus fruits processing, even though many researchers have drawn much attention to the CP and their essential oils, the importance of CS has been also studied due to the presence of diverse compounds that can be useful for adding value to many products [9]. The chemical composition, the characteristics and structure specifics of CS oils lend to a fascinating features such as a semi-siccative property [10].

Among the different techniques that have been employed to extract the oils from citrus by-products, distillation and solvent extractions are common [11]. However those techniques present some disadvantages including long extraction time, volatile compounds loss, residues of toxic substances and degradation of unsaturated compounds due to high temperature [12, 13]. The supercritical carbon dioxide (SC-CO_2) extraction of natural products has recently drawn many researchers. The SC-CO_2 extraction is not only the environment friendly extraction, but also the minimum degradation of bioactive compounds (since CO_2 has a close-room critical temperature: 31 °C), and the prospect of getting solvent-free products [14, 15]. Also, in SC-CO_2 extraction, the solvating power of SC-CO_2 fluid can be increased or decreased by manipulating pressure and/or temperature, giving a really high selectivity. More importantly, the separation of dissolved solutes and SC-CO_2 could be simply done by depressurization [16].Therefore the use of SC-CO_2 extraction can not only result the extract of high quality but also it can be an arsenal for elimination or notably decrease the necessity for eco- unfriendly organic solvents [17].

Although many studies have been dedicated to the study of CP and CS [18-20], there is still scarcity of information on the possible combination of CP and CS either by using solvent or SC-CO_2 extraction. This combination of CP and CS may not only lead to the increase of the bioactivity of resulting oils due to synergistic activity among the compounds they contain but also it may enhance the availability of the active compounds [21].

Therefore, the purpose of the present work was threefold: First, to extract the oils from CS, CP and mixture (MX) of CS and CP by SC-CO_2 and hexane. Second, to study the characteristics (chemical and fatty acids composition, physical properties, oxidative stability and antioxidant activity) of the extracted oils and to assess whether the oil from MX has a potentiality so that it can be used for different applications. Moreover, we compared the characteristics of oils extracted by hexane and those extracted by SC-CO_2.

2.2. Materials and methods

2.2.1. Chemicals

Hexane and ethanol were bought from SK CHEMICALS (Busan, South Korea). 1,1 diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid (trolox) were purchased from Sigma Aldrich (Busan, South Korea). Potassium persulfate ($K_2S_2O_8$) was purchased from Samchun Company (Busan, South Korea). Carbon dioxide (99.99 % purity) was obtained from KOSEM Company (Busan, South Korea). All the chemicals and reagents used were of HPLC and analytical grade.

2.2.2. Plant material

The citrus fruits (*Citrus junos*), common name: Yuza, provenance: Nam He (Busan, South Korea), season: Nov–Jan (2014) were bought and given kindly by Y.G, Co. All the fruits were of eating quality and without blemishes or damage.

2.2.3. Sample preparation

Citrus fruits were cleaned using tap water, peeled off and the peels and seeds were collected. The CP were cut and dried in a freeze dryer at -50 °C for four days. The CS were cleaned using tap water and oven dried at 103 °C until the weight become constant. Prior to extraction, the dried

plant materials (namely CP and CS) were ground with a blender and sieved using a 710 μm metal sieve.

2.2.4. Extraction procedure

2.2.4.1. Solvent extraction

The powders of CP, CS and MX (mixing ratio: 60:40, for CP and CS, respectively) were extracted using hexane (10 g of powder sample/200 ml of 95 % hexane) in a Soxhlet apparatus at 70 °C for 20 h. The oil was further recovered by evaporating off the solvent under vacuum at 45 °C using rotary evaporator (Model N-1100, Eyela, Japan) and remaining solvent was removed off by oven drying at 50 °C for 1 h.

The yield of extracted oil was calculated with the following formula:

$$\text{Yield (\%)} = \frac{\text{weight of extracted oil}}{\text{weight of sample}} \times 100$$

2.2.4.2. Supercritical carbon dioxide extraction

The flow diagram of the equipment used for SC-CO_2 extraction is shown in Figure 2.1. 100 g of powder sample of CP, CS and MX (mixing ratio: 60:40, for CP and CS, respectively) was placed in the extractor vessel and cotton piece was placed at bottom and top end of the extraction vessel to avoid any possible carryover of sample materials. The extraction vessel was a stainless steel high pressure cylinder with a water heating jacket and thermostat was used to measure the temperature. The back-pressure regulator (BPR) was used to measure the pressure.

The CO_2 was pumped up by a high pressure pump of a maximum capacity of 8.328 l/h with a cooling head at constant flow rate, passed through heat exchanger and directed to the bottom of the extraction vessel in the form of upflow configuration. The CO_2 and oil in the SC state left the extractor and reached the valve, where the pressure was decreased. The oil was received in glass tube connected to the valve which was kept in ice packed column whereas CO_2 left through the

gas meter. SC-CO_2 extraction was done at temperatures of 45 and 60 °C, pressure of 200 and 250 bar and CO_2 flow rate of 27 g/min and the extraction time of 2 h.

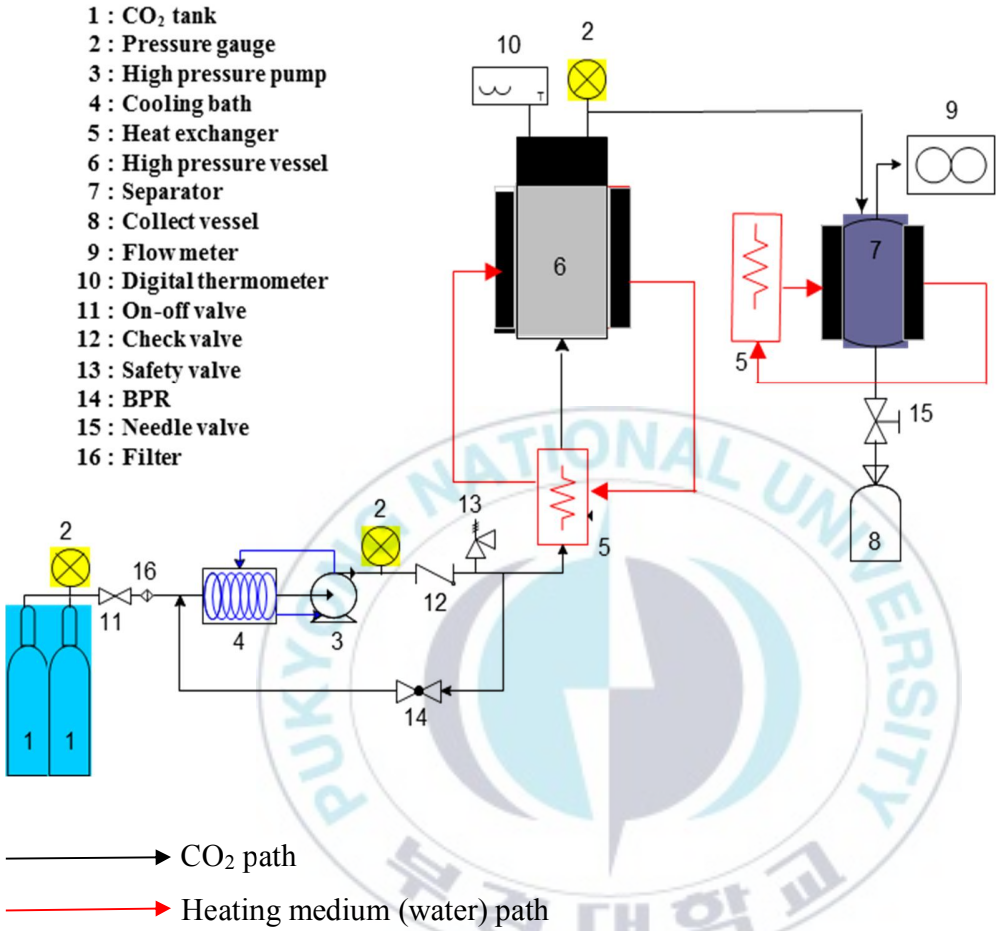


Figure 2.13. Schematic diagram of SC-CO_2 extraction process

2.2.5. Fatty acids composition determination by Gas chromatography

The fatty acid methyl esters (FAMEs) were prepared by following the method developed by Metcalf et al. [22]. The FAMEs were then analyzed using a gas chromatograph (Agilent Technologies model 6890, Wilmington, USA) coupled with a flame ionization detector. The column used was a fused silica BPX70 column, 100 m length x 0.25 mm i.d. 0.2 µm of film thickness (Supelco, Bellefonte, USA). The oven temperature was held at 130 ºC during separation; both detector and injector were held at 250 ºC. The nitrogen was used as carrier gas with a flow rate of 1.0 ml/min. 1.0 µl of methyl esters of free fatty acids were injected by an auto injector. The compounds were identified by comparison of retention time with standard fatty acid methyl esters mixture (Supelco™, USA).

2.2.6. The chemical composition of extracted oils by GC-MS

GC-MS analyses of extracted oils were performed on a gas chromatograph coupled to a mass spectrometer (GCMS-QP2010 Ultra, Shimadzu, Japan) with electron impact ionization (70 eV). A DB-5MS UI capillary column (30 m×0.25 mm, 0.25 µm film thickness; Agilent Technologies, Hewlett-Packard, CA, USA) was used. The temperature program used was: 60 °C for 2 min, 60–200 °C at 10 °C/min, 200–325 °C at 5 °C/10.5 min and 325-340 °C at 10 °C/min. The helium was used as carrier gas with a flow rate of 1.0 ml/min; split ratio was 100: 1; mass range was 25–600 m/z.

2.2.7. Physical properties

2.2.7.1. Color

The Color of the extracted oils was determined by a reflectance tintometer (Lovibond RT Series, model SP60, UK). The values were expressed as L* value as being the lightness of a sample; a* value represents green (−) to red (+); the b* value describes blue (−) to yellow (+).

2.2.7.2. Viscosity

Viscosity measurements of extracted oils were done by a viscometer (model LV DV-II+P—Brookfield, Middleboro, MA, USA), spindle (25) and only 20 ml of oil was used. It was performed at a temperature of 20 °C with 12 rpm. The viscosity was expressed in cP (centipoise).

2.2.8. Oil quality and stability

Peroxide and acid values were determined according to official AOCS methods [23]. Oxidative stability index (OSI) was determined at 120 °C with an air flow of 20 l/h using a 743 Rancimat (Metrohm, Herisau, Switzerland). The sample (3 g) of extracted oils was weighed out in the reaction vessel and then placed in an electric heating block. A flow of air was supplied to the oil samples and volatile organic acids contained in a current of air from the oil sample were gathered in a measuring vessel containing distilled water (60 ml). As oxidation was going on, the conductivity of the water was automatically measured and the results were expressed as induction time (h).

2.2.9. Antioxidant activity determination

2.2.9.1. DPPH Radical Scavenging Assay

The DPPH assay was determined in accordance with a method developed by Brand-Williams et al. [24] with some modifications. 0.1 ml of extracted oil was added to 2.9 ml of a 0.2 mM ethanol DPPH radical solution. After 30 min at ambient temperature (in dark), the absorbance was read at 517 nm using UV-vis spectrophotometer (UVmini-1240, SHIMADZU, JAPAN). The percent of scavenging activity was calculated using the following formula:

$$\text{Radical scavenging activity (\%)} = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100$$

Where $A_{control}$ and A_{sample} are absorbance of control and sample respectively.

2.2.9.2. ABTS·+ Radical Scavenging Assay

This was determined by using the ABTS free radical decolorization assay described by Re et al. [25] with some modifications. In brief, the pre-formed radical monocation of ABTS was obtained by reacting ABTS solution (7 mM) with 2.45 mM potassium persulfate. After 14 h in the dark at ambient temperature, the solution was diluted with ethanol to obtain the absorbance of 0.7 ± 0.2 at 734 nm. The extracted oils and trolox were separately dissolved in ethanol at different dilutions. An aliquot of 0.2 ml of undiluted oil and/or of each dilution was added to 1.8 ml of ABTS free radical cation solution. After 1 h the absorbance was measured at 734 nm using UV-vis spectrophotometer. The percent of scavenging activity was calculated using the previous formula.

2.2.10. Statistical analysis

The results were reported as mean ± standard deviation of three replicates. The analysis of variance (ANOVA) was performed to compare the results using SPSS for Windows (version 20.0.0, SPSS Inc.). Turkey's multiple-range tests were used to compare the significant differences ($P < 0.05$) of the mean values.

2.3. Results and discussion

2.3.1. Yield of extracted oils

The results for the yield (%) of extracted oils are shown in Figure 2.2. The yield of extracted oils from CS, CP and MX using hexane was 24.94 %, 2.07 % and 10.02 %, respectively. Whereas the yield of extracted oils of CS, CP and MX using SC-CO_2 extraction at 200 bar and 45 °C was 15.20 %, 1.61 % and 8.85 %, respectively. Again the SC-CO_2 extraction at 250 bar and 60 °C showed the yield of 19.6 %, 1.78 %, 11.3 % for CS, CP and MX, respectively. For all samples, the yield was significantly higher ($P < 0.05$) for hexane than SC-CO_2 extraction except for MX where the SC-CO_2 extraction at 250 bar and 60 °C showed the highest yield. This is due to the

fact that unlike carbon dioxide, hexane is non-selective, extracting more phospholipids, unsaponifiable matters and other substances. Therefore the amount of matter yielded by using hexane was probably increased by those matters. Under SC-CO_2 extraction at 200 bar and 45 °C, due to the selectivity of SC-CO_2, the solubility of some compounds in SC-CO_2 is lower than hexane, furthermore some compounds can be easily soluble in hexane than SC-CO_2 under those conditions, for example the solubility of phospholipids was found to be almost impossible in SC-CO_2 regardless of conditions that can be used [10, 26]. However, the yield showed an increase when the pressure and temperature were raised up to 250 bar and 60 °C for SC-CO_2 extraction. This was also shown well for MX where the yield for SC-CO_2 extraction at 250 bar and 60 °C was even higher than hexane. This increase of yield resulting on the increase of pressure and temperature for SC-CO_2 extraction might be explained by considering the two mechanisms of temperature on solubility. In fact the vapor pressure of the solute increases always with temperature, whereas the density (or solvent power) of SC-CO_2 decreases. In order to elucidate the effect of these mechanisms on the solubility, the crossover pressure can be taken into consideration. This is a pressure around which the convergence of isotherms at different near-critical temperatures occurs. Below the crossover pressure, the density effect dominates, and in this case the solubility diminishes with augmenting the temperature. Contrary at pressures above the crossover pressure, the vapor pressure effect dominates; thus the solubility increases with temperature [27-29]. Therefore matching this with our results it might be worth to say that at that condition (250 bar and 60 °C) the vapor pressure of the solutes might dominate the effect of solvent density decrease hence the solubility of solutes increases which consequently might increase the yield. However since the extraction system was composed of multi-solutes, it is somehow difficult to figure out the exact crossover pressure for individual solute and/or the whole system.

In addition, the non selectivity of hexane extraction also reflects how the refining processes necessary for SC-CO_2 extracted oils would be even considerably lesser than those required for hexane extracted oils.

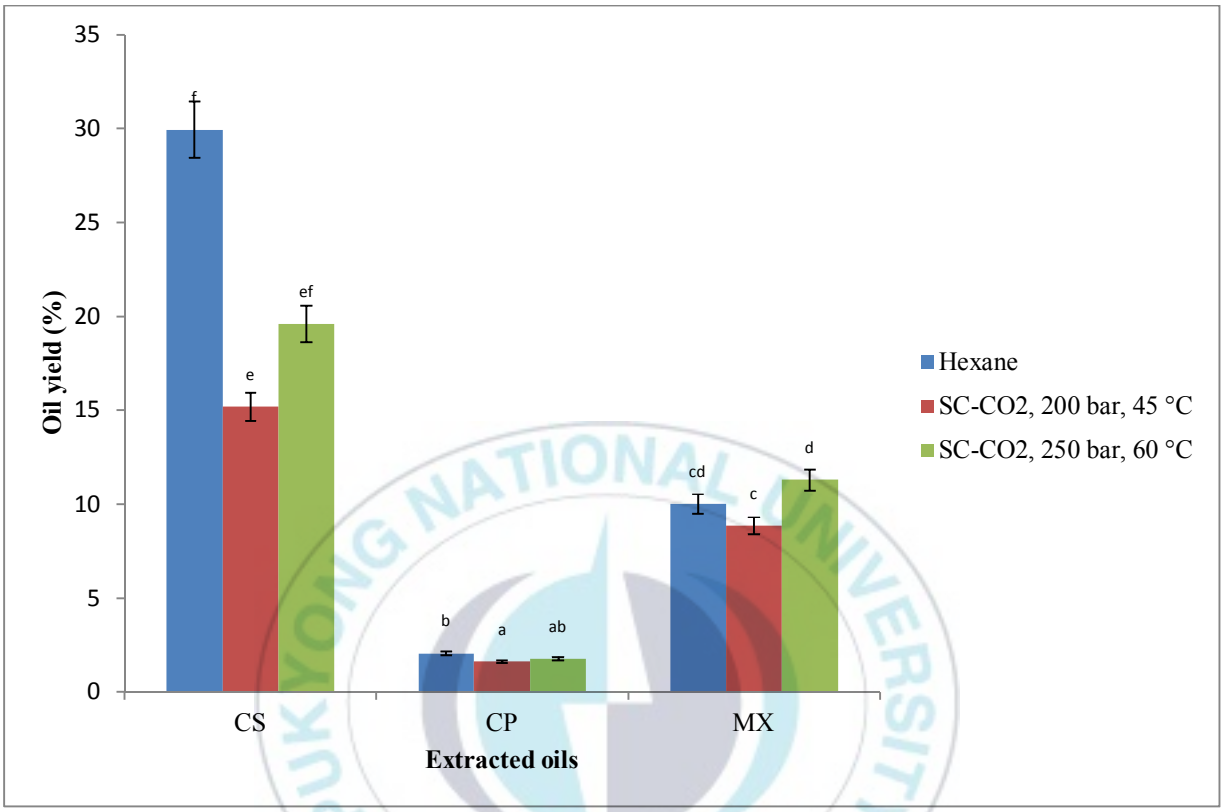


Figure 2.14. The yield of extracted oils

Values are presented as mean ± standard deviation (n=3).

Different letters on the histogram imply the significant difference ($P < 0.05$).

CS: citrus seeds

CP: citrus peels

MX: mixture of citrus peels and citrus seeds (mixing ratio: 60:40 for CP and CS, respectively)

2.3.2. Fatty acid composition

Table 2.1 shows the fatty acids composition of extracted oils both with SC-CO_2 and hexane. Generally, hexane extracted oils showed high composition of saturated fatty acids, while the SC-CO_2 extracted oils showed high composition of unsaturated fatty acids. Among saturated fatty acids, palmitic acid, stearic acid and undecanoic acid were identified. For all extracted oils palmitic acid was predominant with 21.72 % and 26.93 % for CS and MX oil extracted by hexane, respectively. On the other hand, palmitic acid was 17.67 % and 18.38 % for CS and MX, respectively for the oils extracted by SC-CO_2 at 200 bar and 45 °C. Whereas for SC-CO_2 at 250 bar and 60 °C, the palmitic acid was shown to be 20.01 % and 19.77 % for CS and MX, respectively. The unsaturated fatty acids viz. oleic acid, linoleic acid, elaidic acid and linolenic were identified and linoleic acid showed highest composition. These differences could be due to the operating temperatures of the two extractions (45 °C for SC-CO_2 and 70 °C for hexane extraction), which could result the minimal degradation and higher recovery of unsaturated fatty acids with SC-CO_2 than hexane extraction[30]. However even though there was a slight difference between SC-CO_2 at 200 bar and 45 °C and SC-CO_2 at 250 bar and 60 °C in terms of fatty acid composition, the difference was not pretty remarkable unlike hexane extraction which showed that these conditions (250 bar and 60 °C) did not affect so much the fatty acid composition.

Table 2.15. Fatty acid composition of extracted oils determined by GC

Fatty acids	Hexane			SC-CO_2, 200 bar, 45 °C			SC-CO_2, 250 bar, 60 °C		
	CS	CP	MX	CS	CP	MX	CS	CP	MX
Palmitic acid (16:0)	21.72	na	26.93	17.68	na	18.38	20.01	na	19.77
Stearic acid (18:0)	6.23	na	4.18	3.4	na	3.56	2.36	na	2.67
Undecanoic acid(11:0)	5.15	na	3.23	nd	na	nd	2.66	na	1.94
Oleic acid (18:1)	26.00	na	24.99	29.51	na	29.25	30.67	na	29.27
Elaidic acid(18:1)	4.28	na	5.95	5.05	na	5.44	5.78	na	4.83
Linoleic acid(18:2)	34.32	na	32.27	39.44	na	38.7	36.00	na	37.73
Linolenic acid(18:3)	2.30	na	2.45	4.92	na	4.67	2.52	na	3.79
ΣSFA	33.1	na	34.34	21.08	na	21.94	25.03	na	24.38
ΣMUFA	30.28	na	30.94	34.56	na	34.69	36.45	na	34.10
ΣPUFA	36.62	na	34.72	44.36	na	43.37	38.52	na	41.52

SFA: saturated fatty acid; MUFA: mono unsaturated fatty acid; PUFA: poly unsaturated fatty acid

nd: not detected

na: not analyzed

2.3.3. The chemical composition of extracted oils

The results of chemical composition of extracted oils are given in Table 2.2. The total number of compounds identified in the oils from CS, CP and MX extracted by hexane were 11, 12 and 14 representing 82.11 %, 93.16 % and 98.66 % of the total peak area respectively. On the other hand, the total number of compounds identified in the oil from CS, CP and MX extracted by SC-CO_2 at 200 bar and 45 °C were 8, 13 and 14 representing 82.48 %, 97.94 % and 98.08 % of the total peak area respectively. Moreover, the SC-CO_2 extraction at 250 bar and 60 °C showed a total number of 16, 18, 22 compounds for CS, CP and MX respectively. Generally, CS oils were particularly rich in phytosterols, while the oils from CP and MX were rich in monoterpenes hydrocarbons, sesquiterpenes hydrocarbons and oxygenated monoterpenes hydrocarbons. The main phytosterols identified in the oil extracted from CS were β-sitosterol, campesterol and Δ-avenasterol regardless of extraction method.

As far as the composition of the extracted oils of CP is concerned, monoterpene hydrocarbons were the main compounds for both extraction methods and conditions with limonene and *β*-myrcene as the major components. Sesquiterpene hydrocarbons constituted the second major portion of the oils with γ-Terpinene as the major component, while the oxygenated monoterpenes hydrocarbons were only linalool and terpineol. For the oil extracted from MX, it was mainly composed of monoterpene hydrocarbons, sesquiterpenes, oxygenated monoterpene hydrocarbons and some phytosterols as well. As mentioned the total number of compounds was increased for the oils extracted by SC-CO_2 extraction at 250 bar and 60 °C compared to SC-CO_2 at 200 bar and 45 °C). As discussed above, once again this increase might be a result of the vapor pressure of the solutes which might predominate the effect of solvent density decrease hence the increase of solubility of the solutes which as consequence might allow the larger number of compounds to

be extracted. For instance, as can be seen on table 2.2, the psoralens (like auraptene) which constitute the non volatile part of citrus essential oils were shown in the oils extracted by SC-CO_2 at 250 bar and 60 °C while they were not present in the oils of SC-CO_2 extraction at 250 bar and 60 °C . Thus indicates how the SC-CO_2 at 250 bar and 60 °C might even allow the extraction of dense molecules [31].

	Peak area (%)									
	Hexane				SC-CO_2, 200 bar, 45 °C			SC-CO_2, 250 bar, 60 °C		
Compounds	RT	CS	CP	MX	CS	CP	MX	CS	CP	MX
Limonene	7.271	5.1	59.17	51.68	5.73	57.18	48.56	6.39	63.48	53.07
Cymene	7.189	nd	nd	0.80	nd	1.80	nd	nd	0.41	0.52
β-Myrcene	6.495	nd	3.04	1.56	nd	2.38	1.34	0.20	1.47	1.08
β-phellandrene	7.325	nd	1.25	3.16	nd	2.73	0.67	nd	0.98	0.39
Germacrene	14.525	nd	1.22	nd	nd	1.14	nd	nd	1.33	1.42
Bicyclogermacrene	14.72	nd	4.81	1.95	nd	4.73	5.27	0.12	5.15	1.07
γ-Terpinene	7.78	nd	11.27	8.29	nd	13.59	9.96	nd	7.73	5.59
Cadinene	14.975	nd	nd	nd	nd	nd	nd	nd	0.51	0.18
β-farnesene	13.945	nd	2.83	1.56	nd	3.28	3.43	nd	2.34	2.86
Terpineol	8.805	nd	0.9	nd	nd	1.38	1.02	nd	0.10	nd
Linalool	8.46	nd	2.80	2.53	nd	3.94	3.72	nd	1.80	1.04
Stigmasterol	40.31	2.18	0.8	1.74	1.6	0.48	nd	1.80	0.14	1.00
β-sitosterol	41.1	32.25	2.63	12.9	26.63	2.93	5.85	28.37	3.01	8.80
Δ-Avenasterol	41.088	5.69	nd	4.18	5.06	nd	3.01	3.19	nd	1.03
Campesterol	39.97	7.55	nd	2.00	4.23	nd	3.5	5.05	nd	2.09
Tocopherol	38.569	2.2	nd	1.81	3.52	nd	2.7	2.08	0.10	1.44
Tocopheryl acetate	36.006	nd	nd	nd	nd	nd	nd	2.04	1.34	1.84
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	29.92	18.86	2.44	4.50	30.34	2.38	9.05	26.55	3.12	8.68
Auraptene	28.473	nd	nd	nd	nd	nd	nd	0.53	3.09	1.86
7H-Furo(3,2-g)(1)benzopyran-7-one	25.245	1.29	nd	nd	nd	nd	nd	0.93	3.39	2.00
Palmitic acid methyl ester	20.34	1.56	nd	nd	nd	nd	nd	0.11	nd	0.16
Stearyl aldehyde	32.34	nd	nd	nd	nd	nd	nd	3.43	nd	1.17
9,12-Octadecadienoic acid (Z,Z)	32.37	4.12	nd	nd	5.37	nd	nd	4.19	nd	1.36
Methyl N-methyl anthranilate	13.425	1.31	nd	nd	nd	nd	nd	0.38	nd	nd
Total identified (%)		**82.11**	**93.16**	**98.66**	**82.48**	**97.94**	**98.08**	**85.36**	**99.49**	**98.65**

Table 2.16. Chemical composition of extracted oils determined by GC-MS

nd: not detected

2.3.4. Physical properties

The results for color and viscosity measurements are presented in Table 2.3. In general, there was no substantial difference in viscosity of extracted oils. The viscosity of extracted oils ranged between 78 cP to 80 cP and there was no significance difference among them. Regarding the color, for both extraction methods the oils obtained from CS and MX showed higher L* and b* values compared to CP. In addition the L* value reflecting the lightness was found to be higher in SC-CO_2 extracted oils than hexane extracted ones regardless of raw material. For instance, the L* value for CS and MX oil was significantly decreased from 27.74 (200 bar and 45 °C) and 17.80 (250 bar and 60 °C) to 23.66 and 9.05 for hexane, respectively. This low L* value for hexane extracted oils which even appeared to be darker than the SC-CO_2 extracted oils might be attributed to the fact that hexane might extracted more pigments than SC-CO_2. Even though the color of vegetable oils is often associated with the pigments in raw materials, there could be a possibility that hexane could extract even some other matters (like gums) along with the oil which could make hexane extracted oil darker than that of SC-CO_2.

2.3.5. Oil quality and stability

Table 2.4 shows some quality characteristics and the oxidative stability of the extracted oils obtained by hexane and SC-CO_2. The average acid and peroxide values ranged between 0.70-1.63 mg KOH/g and 0.52-0.89 meq/kg, respectively. Extracted oils showed significantly higher ($P < 0.05$) acid and peroxide values in hexane than SC-CO_2 extraction. Considering those quality characteristics, these differences could be mainly originated from the operating temperatures of the two extraction processes (for example 45 °C for SC-CO_2 versus 70 °C). The high temperature for longtime involved in hexane extraction might contribute to triglycerides break down into fatty acids leading to an increase in the free fatty acids. Therefore the amount of free fatty acids in the hexane extracted oils was increased which could consequently increase the acid

value in the hexane extracted oils. Likely the operating conditions for SC-CO_2 (at 250 bar and 60 °C) seemed to affect the acid value. Moreover, there was a significance difference ($P < 0.05$) in terms of peroxide value between those extracted by hexane and SC-CO_2. Like for acid value, this might be due to process temperature, since high temperature was used for hexane extraction this could be contribute to the formation of peroxides. These results converge on those reported by other authors for seed oils who found that the oils obtained by hexane extraction presented higher acid and peroxide values than oils obtained by SC-CO_2 [32].

The results of OS of the extracted oils are presented in Table 2.4. Unlike acid and peroxide value, SC-CO_2 extracted oils were less stable than hexane extracted oils. The induction time was 2.15 h and 3.45 h for hexane, 1.28 h and 2.93 h for SC-CO_2 (at 200 bar and 45 °C) and 1.13 h and 3.27 h for SC-CO_2 (at 250 bar and 60 °C) for CS and MX oils, respectively. The OS of oil depends on many factors; the number of unsaturated fatty acids present in the oil is one of those factors [33]. As the double bonds number increases, the rate of oxidation increases. By matching this factor with our results it can be seen in Table 2.1 that the percentage of unsaturated fatty acids is higher for SC-CO_2 extracted oils than hexane extracted oils. Hence this high proportion of unsaturated fatty acids might have contributed to the instability of the SC-CO_2 extracted oils. More importantly, this difference in stability might be also related to the phytosterols profile. The phytosterols including sitosterol, stigmasterol, campesterol and Δ-Avenasterol were found to be higher in hexane extracted oils than in SC-CO_2 (Table 2).This might have had an impact on the OS since the effect of these phytosterols on stabilizing oils has been pronounced by many authors [34, 35]. They reported that the phytosterols, particularly Δ-Avenasterol may act as antioxidants and antipolymerization agents in oils. Therefore the high OS of hexane extracted oils might be related with their high phytosterols content. In addition, another possible reason for

this difference in stability could be the phospholipids content. The role of phospholipids contents in the stability of oils has been explained by many previous authors [26].They reported that phospholipids may act as an oxygen barrier at the oil/air interface, and thus reduce the rate of oxygen uptake by the sample during the oxidation. Even though, the phospholipids content was not analyzed in this study, it is known that hexane can extract the phospholipids, while phospholipids are insoluble in SC-CO_2.Thus this might be among the reason why hexane extracted oils showed higher OS.

However, as shown in Table 2.4, the induction time increased dramatically from 1.28 h and 1.13 h for CS oil to 2.93 h and 3.27 h for MX oil for SC-CO_2 extraction (200 bar, 45 ℃ and 250 bar, 60 °C) respectively. Also the induction time was increased up to 3.95 h (data not shown) when the proportion of CP in MX was increased (from 60 % to 75 %). This increase in stability might be attributed to the fact that CP contain oxygenated monoterpenes hydrocarbons and other volatile compounds which can either act as antioxidants alone or by their synergistic effect, thus the stability was improved. These results concur with the findings of earlier workers for the role of CP extract on the stabilization of oils and fats [36].

Table 2.17. Color and viscosity of extracted oils

Parameters	Hexane			SC-CO_2, 200 bar, 45 °C			SC-CO_2, 250 bar, 60 °C		
	CS	CP	MX	CS	CP	MX	CS	CP	MX
Color									
*L** value	23.66	3.09	9.05	27.74	6.67	19.48	26.12	4.96	17.80
*a** value	-2.39	+24.23	+15.24	-3.58	+14.23	+8.95	-3.26	+16.30	+10.03
*b** value	+31.61	+2.98	+12.49	+10.95	+7.15	+29.93	+14.52	+9.11	+22.67
Viscosity*(cP)*	80±0.88[a]	na	78±1.06[a]	79±0.17[a]	na	78±0.00[a]	78±0.5[a]	na	79±1.00[a]

Values with same letter in the same row are not significantly different ($P < 0.05$)

Table 2.18. Acid value (AV), peroxide value (POV) and oxidative stability index (OSI) of extracted oils

Sample	Hexane			SC-CO_2, 200 bar, 45 °C			SC-CO_2, 250 bar, 60 °C		
	AV(mg KOH/g)	POV(meq/kg)	OSI(h)	AV(mg KOH/g)	POV(meq/kg)	OSI(h)	AV(mg KOH/g)	POV(meq/kg)	OSI(h)
CS	1.63±1.63[e]	0.87±0.03[d]	2.15±0.03[c]	1.06±1.06[c]	0.65±0.01[b]	1.28±0.02[b]	1.15±0.8[d]	0.89±1[d]	1.13±0.3[a]
CP	0.92±0.92[b]	0.81±0.02[c]	na	0.70±0.7[a]	0.52±0.04[a]	na	0.94±0.1[b]	0.66±0.5[b]	na
MX	1.53±1.53[e]	0.85±0.04[d]	3.45±0.00[f]	1.06±1.06[c]	0.64±0.03[b]	2.93±0.01[d]	1.03±0.09[b]	0.60±0.78[b]	3.27±0.4[e]

Values are presented as mean ± standard deviation (n=3).

Values with different letter in the same column are significantly different ($P < 0.05$)

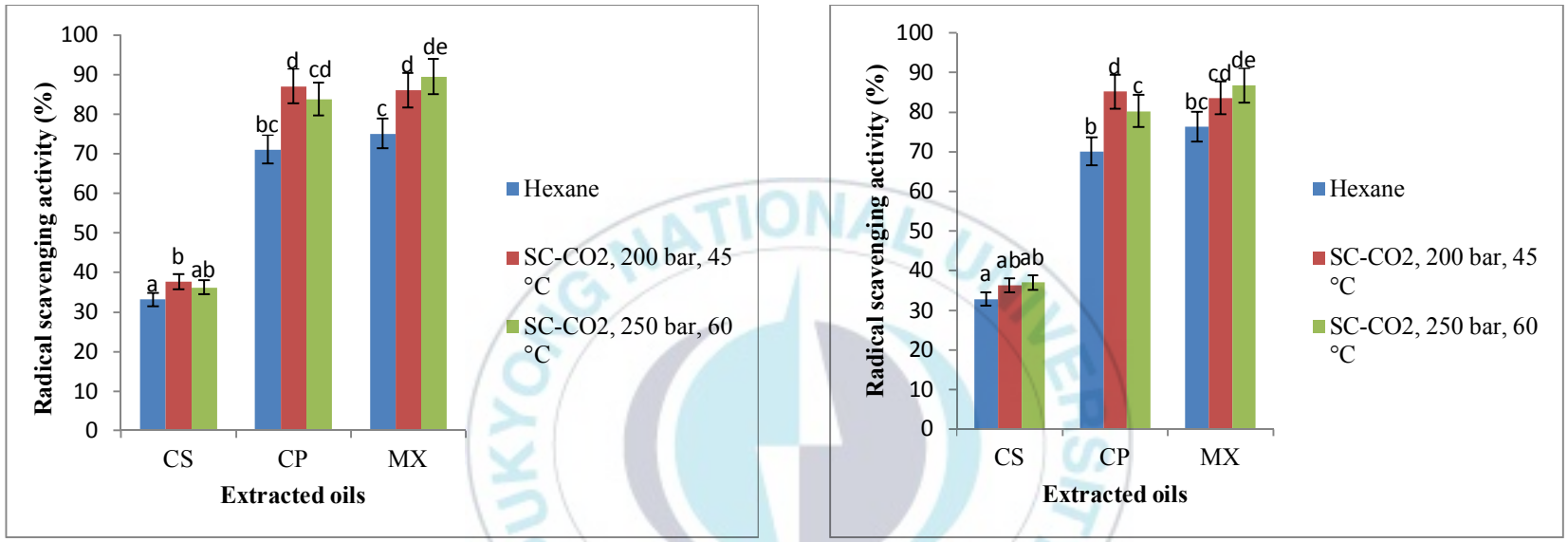
na: not analyzed

2.3.6. Antioxidant activity

The results in Figure 2.3 show the antioxidant activity (as percentage) of extracted oils. In general, the antioxidant activity changed significantly ($P<0.05$) depending on the extraction conditions and the type of sample used for extraction. It is clear from these results that the SC-CO_2 extracted oils showed higher antioxidant activity than hexane extracted oils. The antioxidant activity varied between 32.82 % to 76.33 % for hexane extracted oils, 36.28 % to 87.12 % for oils extracted by SC-CO_2 (at 200 bar and 45 °C) and 36.25 % to 89.51 % for SC-CO_2 (at 250 bar and 60 °C) extracted oils, irrespective of type of method used for analysis (ABTS or DPPH). For hexane extracted oils, the antioxidant activity was in the following order: MX oil > CP oil >CS oil. For SC-CO_2 extracted oils, the antioxidant values varied in the following order: CP oil > MX oil > CS oil.

This difference between hexane and SC-CO_2 extracted oils might be explained by the fact that the SC-CO_2 can extract more oxygenated hydrocarbons monoterpenes than hexane which contribute greatly to the antioxidant activity. For instance the oxygenated monoterpenes (e.g., linalool) is higher in the oils extracted by SC-CO_2 (at 200 bar and 45 °C) than in hexane extracted oils. However, the antioxidant activity might not be attributed only to the oxygenated compounds because some furanocoumarins and some phytosterols can contribute also to the scavenging capacity for free radicals. Not only to act alone but also some studies revealed the synergistic effect between those compounds when they act together. Moreover, higher temperature for longtime might also provoke the degradation of some compounds for hexane extraction. Cacace and Mazza [37] reported that an extraction temperature of > 50 °C can cause the denaturation and affect the stability of some active compounds, so it can make sense to say that the temperature which was used during hexane extraction could have a negative influence on

the antioxidant activity. In addition, the removal of residual solvent by drying after evaporation could affect the composition of the hexane extracted oils since some important minor volatile compounds might be lost, which could adversely affect the antioxidant activity of hexane extracted oils [38].



(a) (b)

Figure 2.15. Antioxidant activity of extracted oils expressed as percentage of inhibition measured by a) ABTS and b) DPPH assay.

Values are presented as mean ± standard deviation (n=3).

Different letters on the histograms imply the significant difference ($P < 0.05$).

2.4. Conclusion

The processing of citrus fruits generates the by-products that are rich sources of bioactive substances. In this study the characteristics of oils extracted from a mixture of citrus by-products have been studied. In general, the SC-CO_2 and hexane extracted oils were significantly different ($p < 0.05$). It appeared that the yield in hexane extraction was higher than SC-CO_2 extraction which in turn might be ascribed to the non selectivity of hexane. Since the evaporation and refining process which is necessary in hexane extraction is not needed for SC-CO_2, it is clear that this could substantially save energy for SC-CO_2 which could make SC-CO_2 more economical than hexane extraction. More importantly, shorter extraction time (2 h for SC-CO_2 versus 20 h for hexane) and its undisputed environmental friendliness make SC-CO_2 a better extraction technique than solvent extraction.

Moreover, the results indicated that the oils extracted by SC-CO_2 are rich in unsaturated fatty acids compared to hexane extracted oils; hence their promising alternative to be applied in food industries. One drawback of these SC-CO_2 oils is their low oxidative stability compared to hexane extracted oils. However, it was interestingly revealed that this problem can be overcome by combining CS and CP, which reflects how MX oil can be a tailor-made in many applications.

Overall, this study showed that the combination of CS and CP which are considered as wastes yielded the oils with high quality, hence a promising application of these oils in many areas.

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Chapter 3

Bioactive Compounds, Antioxidant and Antimicrobial Activity of Oils obtained from a Mixture of Citrus Peels and Seeds using a Modified Supercritical Carbon Dioxide

3.1. Introduction

The consumption of citrus fruit either as fresh produce or as the juice is common due to its dietary benefits and particular flavor. It is broadly grown around the world with the yearly production of about 102 million tons [1]. Because of the production of juice and other products from citrus fruits, the big amounts of citrus by-products are generated every year. As a consequence, this not only wastes useful materials, but also may pose some pollution, disposal, and other related environmental problems due to microbial spoilage [2]. These citrus by-products can be valorized since they contain a wide range of healthy bioactive compounds [3, 4].

Citrus peels (CP) have a higher proportion of natural flavonoids and are among the rich sources of phenolic compounds [5]. In addition, several compounds including flavanone glycosides, polymethoxylated flavones, and flavanones that are unique to citrus have been found to be comparatively rare in other plants [6, 7]. It has been reported that the citrus peels extracts present higher antioxidant activity [5] and exert antimicrobial effects against food borne pathogens [7, 8] due to the presence of quinones, terpenoids, polyphenols, phenolic acids, and tannins [9-11].

Citrus seeds (CS) is another by-product of citrus fruits processing, even though many researchers have drawn much attention to the CP, the importance of CS has been also studied due to the presence of diverse compounds including polyphenols, tocopherols, phytosterols, and high amount of unsaturated fatty acids that can be useful for adding value to many products [4, 12, 13].

Among the different techniques that have been used to obtain the extracts from plant matrices, solvent extraction and distillation are among of them [14]. However, those techniques have some drawbacks including long extraction time, volatile compounds loss, residues of toxic substances, and unsaturated compounds degradation due to high temperature [15, 16]. The supercritical carbon dioxide (SC-CO_2) extraction of natural products has recently drawn many researchers. The SC-CO_2 extraction is not only the eco-friendly extraction but also the minimum degradation of bioactive compounds (since CO_2 has a close-room critical temperature: 31°C), and the prospect of getting solvent-free products [17] have made the SC-CO_2 a promising technique. Again, in SC-CO_2 extraction, the solvating power of SC-CO_2 fluid can be increased or decreased by manipulating pressure and/or temperature, giving a high selectivity. Moreover, the separation of dissolved solutes and SC-CO_2 could be simply done by depressurization [18]. However, the limitation of CO_2 for extraction of polar compounds due to its non-polar characteristic has been a challenge for the extraction of polyphenols and other polar compounds. Nonetheless, the SC-CO_2 polarity can be improved by incorporating modifiers such as ethanol, methanol, water, etc. [14, 19]. Therefore the use of SC-CO_2 extraction and/or with ethanol as a modifier can not only result in the extract with a high bio-potentiality but also it might help for eliminating or notably decrease the necessity for eco- unfriendly organic solvents [20].

Even though many studies have been dedicated to the study of citrus by-products [21, 22], to the best of our knowledge no such study on the bioactive compounds, antioxidant and antimicrobial activity of oils resulting from a combination of CP and CS either by using neat SC-CO_2 or modified SC-CO_2 extraction has been emerged. The combination of CP and CS may not only lead to the increase of the bioactivity of resulting oils due to synergistic effect of the compounds they contain but may also enhance the bioavailability of some active compounds [23, 24]. So,

this work was designed to study the effect of combining the CP and CS on the bioactive compounds, antioxidant and antimicrobial activity of the resulting oils in order to assess if their bioactivity can make them applicable in many fields.

The purpose of the present work was threefold: First, to extract the oils from CS, CP and mixture (MX) of CS and CP by neat SC-CO_2 and/or SC-CO_2 with ethanol as a modifier. Second, to determine the total phenolic, total flavonoid, tocopherol and phytosterol content of extracted oils. Third, to study the antioxidant and antimicrobial activity of the extracted oils and to assess whether the MX oils have potential bioactivity so that they can be used for different applications.

3.2. Materials and methods

3.2.1. Chemicals

Folin–Ciocalteu's reagent (FCR), gallic acid, quercetin, tocopherol standards (α-, β-, γ- and δ-tocopherol), sterol standards (brassicasterol, campesterol, stigmasterol, sitosterol and Δ-avenasterol), 1,1 diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Tryptone Soy Broth (TSB), and Mueller-Hinton agar(MHA) were purchased from Sigma Aldrich (Busan, South Korea). Ethanol, methanol, and 2, 3, 5-triphenyltetrazolium chloride (TTC) were purchased from Samchun Company (Busan, South Korea). Carbon dioxide (99.99 % purity) was obtained from KOSEM Company (Busan, South Korea). All the chemicals and reagents used were of HPLC and analytical grade.

3.2.2. Sample collection and preparation

The citrus fruits (*Citrus junos*), Common name: Yuza, Season: Nov–Jan (2015), Provenance: Nam He (Busan, South Korea) were given by Y.G, Co. Citrus fruits were cleaned, peeled off and the peels and seeds were collected. The CP were freeze dried (-50 °C for four days) while the CS were oven dried at 103 °C and then crushed, sieved (using a 710 μm metal sieve) to get powder for extraction.

3.2.3. Extraction procedure

The SC-CO_2 extraction diagram used in the study is shown in Figure 3.1. For extraction by neat SC-CO_2 (without ethanol), the extraction setup and procedures were the same as those reported in our previous work [25]. For extraction by a modified SC-CO_2 (with ethanol), a second pump was connected to the extraction line which supplied the ethanol (ca. 1 ml/min: flow rate) and then mixed with CO_2. The CO_2+ethanol passed through a heat exchanger and then flowed through the sample in the extraction vessel. The ethanol-oil mixture was received in a vial whereas ethanol-saturated CO_2 left through the flow meter. The residual ethanol was removed out by a rotary evaporator (Model N-1100, Eyela, Japan).

The extraction (either by neat SC-CO_2 or modified) was done at the temperature of 45 °C, pressures of 200 and 300 bar, and the extraction time of 2 h and CO_2 flow rate of 27 g/min.

The yield of extracted oil was calculated with the following formula:

$$\text{Yield (\%)} = \frac{\text{weight of extracted oil}}{\text{weight of sample}} \times 100$$

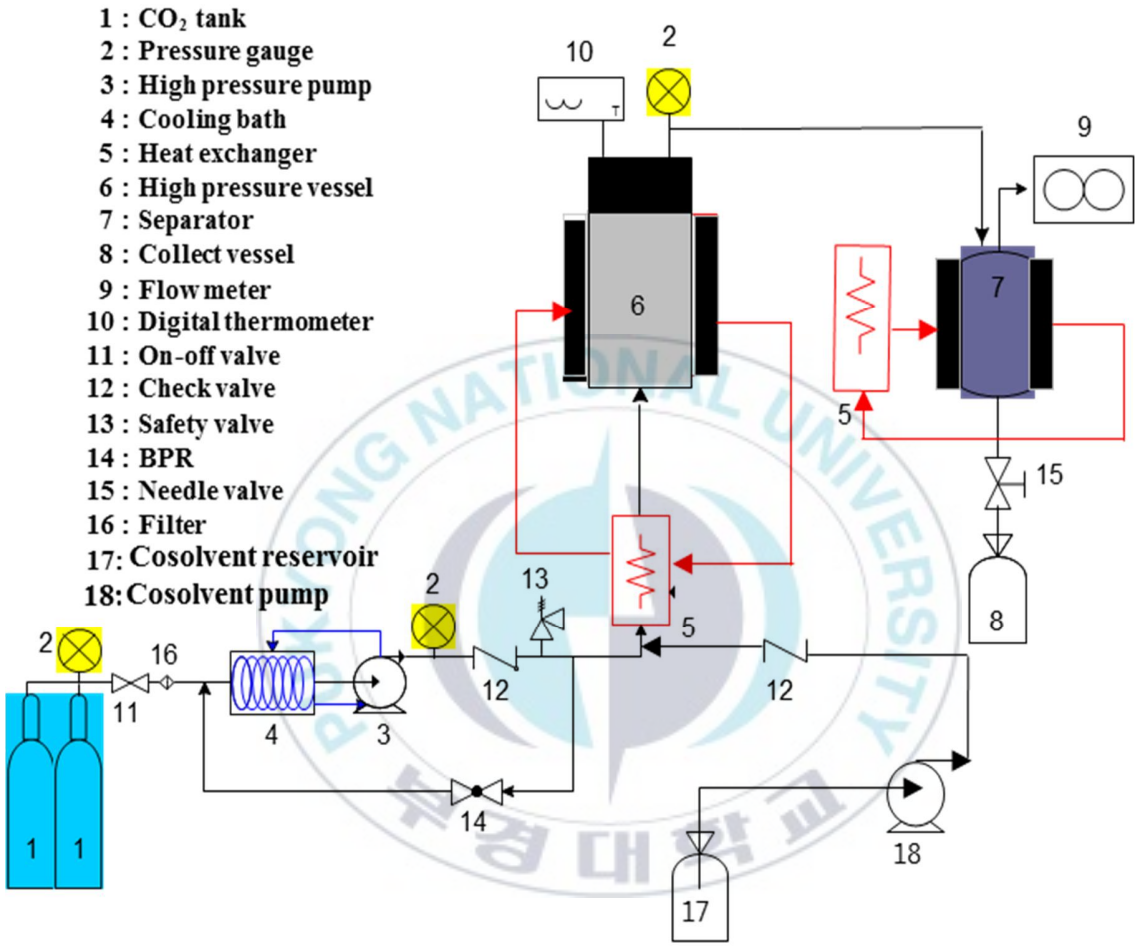


Figure 3.16. Schematic diagram of SC-CO_2 and co-solvent extraction process.

3.2.4. Determination of total phenolic content

The total phenolic content (TPC) was carried out according to the Folin-Ciocalteu method. The reaction mixture was made of 0.5 ml of the diluted extracted oil (5 mg/ml in ethanol), 0.5 ml of 1N Folin-Ciocalteu's reagent and 5 ml of distilled water. 1 ml of sodium carbonate solution (20%) was added and vigorously shaken and stayed for 1 h for reaction. The absorbance was read at 725 nm using UV-Vis spectrophotometer (UVmini-1240, SHIMADZU, JAPAN).The gallic acid standard curve was used for quantification of TPC and was expressed as mg gallic acid equivalent (GAE)/g of extracted oil.

3.2.5. Determination of total flavonoid content

The method developed previously by Meda et al. [26] was used for determination of total flavonoid content (TFC) with some modifications. In brief, a mixture consisted of 0.5 ml of diluted extracted oil (2.5 mg/ml in ethanol), 0.5 ml methanol, 50 µl AlCl3 (10 %), 50 µl 1 M potassium acetate and 1.4 ml distilled water was allowed to stand for 30 min at ambient temperature. Subsequently, the absorbance was read at 415 nm using the same spetrophotometer. The TFC quantification was based on quercetin standard curve and expressed as mg quercetin equivalent (QE)/ g of extracted oil.

3.2.6. Tocopherol content

The tocopherol content of extracted oils was performed by normal-phase HPLC using a Hitachi chromatography system. Approximately 20 mg of extracted oil were dissolved in 1ml HPLC grade hexane, filtered through 0.45 µm filters and 20 µl were injected directly into a column (5 µm, 4.6×150 mm) (Agilent Technologies, Hewlett-Packard, CA, USA). The mobile phase was hexane/isopropanol (99.5:0.5 v/v), the flow rate was 1.0 ml/min and the detection wavelength was 294 nm by a Hitachi L-2420 UV-Vis detector. The individual tocopherol standards were

diluted with hexane (HPLC grade) at different concentrations to construct an external standard curve which was used to identify and quantify the tocopherols in the extracted oils.

3.2.7. Phytosterol content

The extracted oils were first saponified and then analyzed for phytosterol content. Approximately 1 g of extracted oil and 100 ml 1 M ethanolic KOH were put in an Erlenmeyer flask and heated at 60 °C for 45 min. The mixture was put in a 250-ml separatory funnel with 100 ml of hexane, shaken and washed with 50 ml of distilled water to take out the hydro-soluble components. The hexane layer was dried over anhydrous sodium sulfate and evaporated completely in a rotary evaporator at 40 °C. Prior to analysis, the residue was dissolved in 3ml of hexane, filtered through 0.45 μm and then 20 μl was injected into the same HPLC column. The mobile phase was hexane: ethanol (70:30 v/v) at the flow rate of 1.0 ml/min and the detection wavelength was 205 nm by the same UV-Vis detector. The individual sterols standards were diluted at different concentrations to construct an external standard curve which was used to identify and quantify the phytosterols in the extracted oils.

3.2.8. Antioxidant activity determination

3.2.8.1. DPPH Radical Scavenging Assay

The DPPH assay of extracted oils was carried out in accordance with a previously reported method [27] with some modifications. 1.5 mL of oil with different concentrations of ethanol was added to 1.5 mL of 0.1 mM ethanol DPPH radical solution. The absorbance was read 517 nm using UV-Vis spectrophotometer (UVmini-1240, SHIMADZU, JAPAN) after 30 min of incubation at ambient temperature (in the dark). Ethanol was used as a control. A percent inhibition against concentration curve was plotted, and the concentration of oil required for inhibiting 50 % was determined and expressed as IC_{50} values.

3.2.8.2. ABTS·+ Radical Scavenging Assay

The ABTS·+ Radical Scavenging Assay of extracted oils was determined by a method reported previously by Re et al. [28] with some modifications. In brief, the pre-formed ABTS·+ radical was obtained by reacting 2.45 mM potassium persulfate with ABTS solution (7 mM) for fourteen hours (in the dark at room temperature). Thereafter, the solution was diluted (with ethanol) to get the absorbance of 0.7 ± 0.2 at 734 nm. The extracted oils were dissolved in ethanol at different concentrations, and an aliquot of 1ml of each concentration was added to 3 ml of already prepared ABTS·+ radical solution and shaken vigorously. After one hour in the dark, the absorbance was measured at 734 nm using the same UV-Vis spectrophotometer, and IC_{50} values were calculated.

3.2.9. Antimicrobial activity

3.2.9.1. Test microorganisms

The four pathogenic bacteria were used to test the antimicrobial activity of extracted oils. These bacteria include two Gram-negative bacteria: *Salmonella typhimurium* KCCM 11862 and *Escherichia coli* ATCC 25922; two Gram-positive bacteria: *Staphylococcus aureus* KCCM 11335 and *Bacillus cereus* ATCC 13061.

3.2.9.2. Disk diffusion assay

The Preliminary screening of the antimicrobial activity was performed with the method of disc diffusion. MHA media was prepared and then poured into plates. The plates were inoculated and spread with 100 µl of bacterial suspensions (1×10^7 CFU/ml). Next, filter paper discs (6 mm Ø) were put on the surface of plates and impregnated with 15 µl of extracted oils. After staying for 2 h (4 °C), all plates were incubated at 37 °C (overnight), and the diameter of the zone of inhibition (in mm) was measured.

3.2.9.3. Determination of the minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined by 96-flat well microtiter broth dilution method in 0.15 % agar amended TSB as recommended by Mann and Markham [29]. Stock solutions and serial dilutions of the extracted oils were prepared in TSB + 0.15 % agar. 100 µl of each dilution was placed into rows of wells in microtiter plates (96×250 µl wells). An equal volume of inoculum was dispensed into the proper wells and mixed with the growth medium, and then the plates were incubated at 37 °C with the covers on. After 24 h of incubation, 40 µl of 2, 3, 5-triphenyltetrazolium chloride (TTC) (3 mg/ml) prepared in distilled water was added to each well and incubated again for 30 min. After that, the change to red color was the indication of biological activity of bacteria. The MICs were recorded to the wells which showed no change of color of TTC.

3.2.10. Statistical analysis

The results were reported as the mean ± standard deviation of three replicates. The analysis of variance (ANOVA) was performed to compare the results using SPSS for Windows (version 20.0.0, SPSS Inc.).

3.1. Results and discussion

3.3.1. Yield of extracted oils

The results of yield are demonstrated in Figure 3.2. The yield of extracted oils showed to be significantly different ($p < 0.05$) depending on extraction conditions and raw materials (namely CS, CP, and MX). The yield of extracted oils from CS, CP, and MX using neat SC-CO_2 at 200 bar was 15.45 %, 1.57 %, and 8.22 %, respectively. However, the yield of extracted oils from CS, CP, and MX using neat SC-CO_2 at 300 bar was 22.12 %, 1.87 %, and 11.97 %, respectively. On the other hand, SC-CO_2+ethanol at 200 bar yielded 15.67 %, 1.66 %, and 8.39 % for CS, CP, and

MX, respectively. SC-CO_2+ethanol at 300 bar showed 23.04 %, 1.91 %, and 12.73 % for CS, CP, and MX, respectively. According to these results, as pressure increased, the extraction yield increased significantly ($p < 0.05$) for all samples. This increase of yield, resulting due to the increase of pressure, might be justified by the fact that pressure is among one of the main driving parameters for SC-CO_2 extraction [18]. This occurs as a result of the variation in pressure, which causes variation in CO_2 density, which thereby affects the solubility of analytes in CO_2. As the density of CO_2 increases (from 18.46 mol/L at 200 bar to 20.23 mol/L at 300 bar at the constant temperature of 45 °C), there is a diminution of distance between the molecules. Hence, the analytes and CO_2 interact, leading to the higher solubility of analytes in CO_2, which thereupon increases the yield [30]. Furthermore, this increase in yield as result of an increase in the pressure might result because, at lower pressure, the selectivity of CO_2 is higher due to lower density. When the density increases (with increasing pressure at constant temperature), it owes even the solubility of more dense compounds which consequently increases the yield. Similarly, previous works [15] reported that during the extraction of coriander seeds by SC-CO_2, the fraction of the non-volatile part was markedly increased when the pressure increased from 100 to 350 bar, with a clear reduction in the volatile fraction, which therefore increased the total yield.

Furthermore, it has been reported that the addition of ethanol (as a modifier) enhances the solvent power of SC-CO_2, and promotes the sample matrix swelling, thus increasing the surface area and the inner volume for contact with SC-CO_2 [31], which might increase the yield. Even though the addition of ethanol showed an influence on the yield ($P < 0.05$) at 300 bar, the yield did not appear to increase significantly ($P > 0.05$) by addition of ethanol at 200 bar. This might be attributed to the fact that although ethanol was added, it would not have helped in increasing

the density of CO_2 to the level at which it could extract much denser compounds compared with that possible at 300 bar.

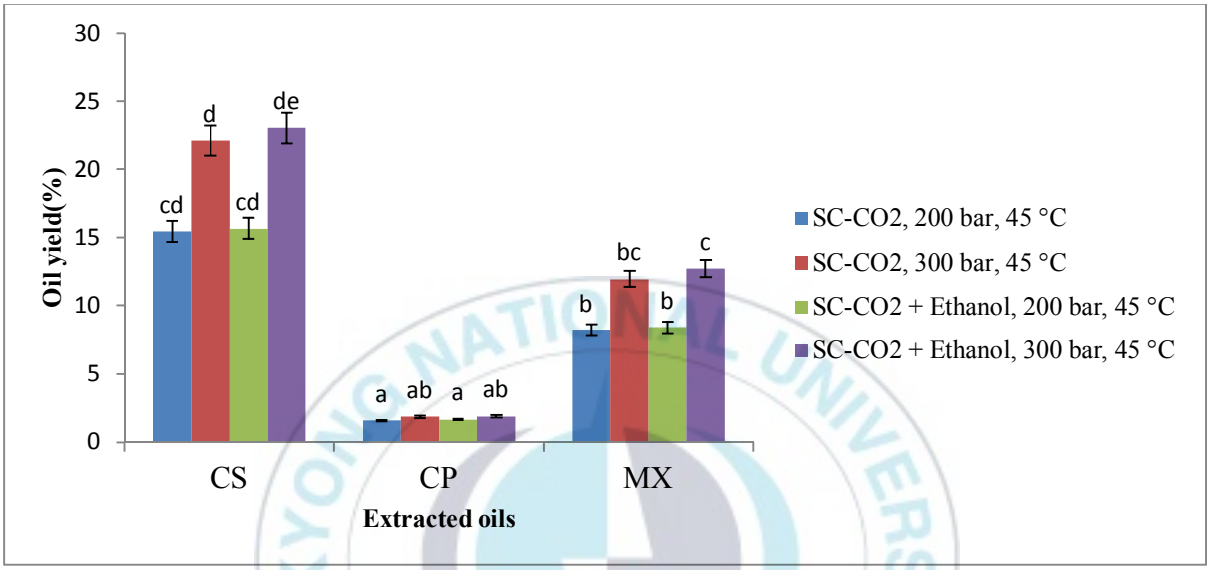


Figure 3.17. The yield of extracted oils

Values are presented as mean ± standard deviation (n = 3).

Different letters on the histogram imply the significant difference ($P < 0.05$).

3.3.2. Total phenolic and total flavonoid content

The TPC of the extracted oils is presented in Table 3.1. The CP oils showed significantly ($P < 0.05$) higher TPC among others. The TPC was in the following order: CP oil > MX oil > CS oil. The highest TPC (43.64 mg GA/g) was found in CP oil extracted by SC-CO_2+ethanol at 200 bar, while CS oil at the same conditions showed TPC of 29.18 mg GA/g only. The lowest TPC (12.17 mg GA/g) was shown in CS oil extracted by neat SC-CO_2 at 300 bar, whereas the CP oil at the same conditions showed TPC of 22.40 mg GA/g. Generally, phenolic compounds might work like protective agents against UV lights, predators, and pathogens in fruits and vegetables [32]. So, it is plausible that the CP oils showed a higher proportion of TPC because the peels might have a higher proportion of these compounds because they are the external covering of the fruit, which is a more likely site of synthesis of phenolic compounds [33]. These results are in convergence with those reported by previous researchers [34] who found higher levels of phenolic compounds in citrus peel than in the other parts.

Regarding the addition of ethanol as a modifier, the oils extracted by SC-CO_2+ethanol showed significantly ($P < 0.05$) higher TPC than neat SC-CO_2 extracted oils. The TPC ranged between 12.17 to 27.94 mg GA/g for the oils extracted by neat SC-CO_2, whereas the TPC range was between 27.31 to 43.64 mg GA/g when SC-CO_2+ethanol was used. Therefore, by adding ethanol, the TPC increased regardless of the sample type. This increase in TPC, as a result of ethanol addition, might be explained by considering the polarity. In fact, SC-CO_2 is a non-polar solvent; even at high density, the SC-CO_2 capability of dissolving polar compounds is limited. The addition of a modifier (ethanol in this case) to SC-CO_2 could ameliorate the extraction efficiency of polar compounds by increasing their solubility [35]. Since the phenolics are polar in nature, it can be deduced that the addition of ethanol might have an enormous impact on the extraction of

phenolic compounds by accelerating desorption process. It may exert its effect by competing with the phenolics for their active binding site which may cause the release of those compounds and thus an increased TPC [7, 36].

Additionally, there was a significant difference ($P < 0.05$) among oils extracted at different conditions. The TPC showed a range of 15.32 to 43.64 mg GA/g for the extracted oils (either neat SC-CO_2 or modified) at 200 bar while the TPC fell in the range of 12.17 to 40.71 mg GA/g for the extracted oils (either neat or modified) at 300 bar. This decrease of TPC by increasing pressure might be understood by considering their density and solubility. Usually the solubility of phenolics increases with increasing pressure [37, 38], resulting in the increase in phenolics release from the plant matrix [39]. However, even though the release of phenolic compounds increases with pressure during the extraction, the overall TPC of the extracted oils is decreased because the extraction yield also increases at the same time (Figure 3.2). Therefore, our results indicate that pressure can have a larger effect on the solubility of other heavier molecules than on the solubility of phenolic compounds, which might increase the overall extraction yield but decrease the phenolics concentration in the extracted oils. As discussed above, the increase of pressure increases the solubility of a wide range of molecules, so this affects the concentration of TPC in the extracted oil adversely because it is like diluting effect, and hence the decrease of TPC concentration in extracted oils. This behavior was also reported by previous researchers who demonstrated that rate of phospholipids and paraffinic compounds (i.e., waxes) increased by increasing pressure during SC-CO_2 extraction [40, 41].

Our results are similar to those published by Lou et al. [42] (5.71 to 30.00 mg GA/g) for kumquat extracts and Zhang et al. [43] (29.38–51.14 mg GA/g) for Chinese wild mandarin peel extract. Indeed, range of variation in our results is higher than those reported by Karimi et al. [44]

(3.93–4.83 mg GAE/G) for the phenolics of *Citrus aurantium* peels and Chen et al. [45] (15.595 to 18.950 mg GA/g) for total phenolic content of ethanol extracts of *C. reticulata* Blanco cv. Ougan fruits peels. But these results show lower values than those reported by Ghasemi et al. [46] (104.2 to 172.1 mg GAE/g DW) for the extracts of *C. reticulate* Blanco fruits peels and Goulas and Manganaris [47] (112.2–196.2 mg GAE/G) for citrus fruits pulps and peels grown in Cyprus. The results of TFC of the extracted oils are presented in Table 3.1. The TFC ranged between 2.20 and 6.75 mg QE/g for all extracted oils. In general, unlike TPC, the CS oils showed significantly ($P < 0.05$) higher TFC than others. The TFC was in the following order: CS oil > MX oil > CP oil. The highest TFC was shown by CS oil extracted by neat SC-CO_2 at 300 bar with 6.75 mg QE/g while the lowest was found at the same conditions for CP oil with 2.2 mg QE/g. The TFC of MX oils varied from 4.01 to 5.14 mg QE/g for all conditions. Once more, the extraction conditions appeared to influence the TFC. The pressure increase seemed to increase the TFC for CS and MX while decreasing the TFC for CP when neat SC-CO_2 was used. However, the TFC showed an increase for CP while decreasing for CS and MX when SC-CO_2+ethanol was used. For instance, the TFC was increased from 5.17 and 4.95 mg QE/g at 200 bar up to 6.75 and 5.14 mg QE/g at 300 bar, respectively, for CS and MX when neat SC-CO_2 was used while the TFC was slightly reduced from 2.71 to 2.20 mg QE/g for CP at the same conditions. Conversely, the TFC was decreased from 5.65 and 5.06 mg QE/g at 200 bar to 4.46 and 4.01 mg QE/g at 300 bar for CS and MX, respectively, when SC-CO_2+ethanol was used, while CP showed an increase at the same conditions.

Moreover, similar to TPC the addition of ethanol showed a significant increase of TFC ($P < 0.05$). For example, the TFC was increased from 5.17, 2.71, and 4.95 mg QE/g for oils extracted by neat SC-CO_2 at 200 bar to 5.65, 3.32, and 5.06 mg QE/g for those extracted by SC-

CO_2+ethanol for CS, CP, and MX, respectively, at the same pressure. This might again be attributed to the polar nature of ethanol, which possibly helps in extracting more polar flavonoids which might increase the TFC for SC-CO_2+ethanol extracted oils. These data are partially in accordance with those reported by Chen et al. [45] (0.30–31.1 mg QE/g DW) and Ghasemi et al. [46] (4.67–5.79 mg rutin equivalents/g DW). However, these results report lower values than those of Zhang et al. [43] (29.38–51.14 mg GA/g) for Chinese wild mandarin peel extract but higher than those reported by Goulas and Manganaris [47] (1.27–2.28 mg rutin/g) for citrus fruits pulps and peels grown in Cyprus. These discrepancies (either for TPC or TFC) between our results and those reported earlier can be attributed to many factors, including raw materials, extraction methods, pedoclimatic conditions, genotype and variety and sample matrix preparation, which might alter the TPC and TFC of resulting extracts.

Table 3.19. Total phenolic content (mg GAE /g of oil) and total flavonoid content (mg QE /g of oil) of extracted oils

	SC-CO_2, 200 bar, 45 °C			SC-CO_2, 300 bar, 45 °C			SC-CO_2 + ethanol, 200 bar, 45 °C			SC-CO_2 + ethanol, 300 bar, 45 °C		
	CS	CP	MX	CS	CP	MX	CS	CP	MX	CS	CP	MX
TPC	15.32 ± 0.02^{ab}	27.94 ± 0.1^{cd}	24.1 ± 0.1^{c}	12.17 ± 0.3^{a}	22.4 ± 0.04^{bc}	20.16 ± 0.02^{b}	29.18 ± 0.17^{d}	43.64 ± 0.5^{ef}	40.94 ± 0.3^{e}	27.31 ± 0.2^{cd}	40.71 ± 1^{e}	37.28 ± 0.8^{de}
TFC	5.17 ± 0.01^{de}	2.71 ± 0.01^{ab}	4.95 ± 0.2^{d}	6.75 ± 0.05^{f}	2.2 ± 0.02^{a}	5.14 ± 0.1^{de}	5.65 ± 0.02^{e}	3.32 ± 0.01^{b}	5.06 ± 0.4^{de}	4.46 ± 0.06^{cd}	3.67 ± 0.1^{bc}	4.01 ± 0.01^{c}

Values are presented as mean ± standard deviation (n = 3).

Values with different letter in the same row are significantly different ($P < 0.05$).

3.3.3. Tocopherol and phytosterol content

Table 3.2 shows the tocopherol content of extracted oils. The increase of pressure resulted in significant decrease ($P < 0.05$) of tocopherol content while ethanol addition did not lead to a remarkable difference ($P > 0.05$). The CS oils showed the highest tocopherol content, followed by MX oils, and CP oils showed the lowest (CS oil > MX oil > CP oil). Among CS oils, the highest total tocopherol content was 33.8 mg/100 g recorded at 200 bar (neat SC-CO_2), while the lowest was 22.5 mg/100g recorded at 300 bar (SC-CO_2+ethanol). Similarly, the highest total tocopherol content (16.76 mg/100 g) for MX oil was recorded at 200 bar (neat SC-CO_2), while the lowest (8.49 mg/100g) was recorded at 300 bar (SC-CO_2+ethanol). However, CP oils showed almost no significant difference among the total tocopherol content at different conditions ($P > 0.05$). This decrease of tocopherol content by increasing pressure could be the result of an increase in SC-CO_2 density, which might increase the solubility of a wide range of heavier molecules, which causes dilution of the tocopherol content of the extracted oil, and hence the decrease of tocopherol concentration. Similar to our results, Illés et al. [15] reported that the amount of tocopherols in coriander seed oil extracted under mild conditions (100–200 bar and 25 °C) was about two times higher than the ones extracted at 250–350 bar and 35 °C . Among the individual tocopherols, α-tocopherol was predominant in all the extracted oils ranging from 16.7 to 24.93 mg/100 g, 5.88 to 13.50 mg/100 g, and 0.56 to 1.01 mg/100 g for CS, MX, and CP respectively. It was followed by γ-tocopherol, β-tocopherol, and δ-tocopherol, which was lowest in amount. Our results are slightly higher than those of Matthaus and Özcan [4], who reported a range of 1.70–20.5 mg/100g for oils of citrus seeds from Vietnam and Turkey.

The phytosterol content of extracted oils is presented in Table 3.3. The total phytosterol content ranged from 245.91 to 367.76 mg/100 g and 90.96 to 140.44 mg/100 g for CS and MX,

respectively. The phytosterols viz. sitosterol, campesterol, avenasterol, stigmasterol, and brassicasterol were identified, and sitosterol was found to be, by far, the most predominant phytosterol in all oils with the range of 66.52–276.08 mg/100 g followed by campesterol (ranging from 14.75 to 49.16 mg/100 g). The third one was avenasterol, except of oils extracted by SC-CO_2+ethanol at 300 bar, where stigmasterol turned out to be the third one. Brassicasterol was not detected in almost all extracted oils. According to our results, the phytosterols content appeared to be affected significantly ($P < 0.05$) by both pressure and the addition of ethanol. For extracted oils, the total phytosterols content increased significantly ($P < 0.05$) by raising pressure. This increase of phytosterols content resulting in an increase of pressure might be ascribed to the fact that a considerable amount of phytosterols is expected to be attached deep inside the seed tissues and when the pressure is increased, the SC-CO_2 reaches inside, thus successfully extracting more phytosterols. This was proven by previous works [48], who studied the optimization of SC-CO_2 extraction of phytosterol-enriched oil from Kalahari melon seeds, and concluded that the phytosterols were increased by increasing pressure. This pattern of SC-CO_2 extraction for increasing phytosterols was also reported by other researchers [49], who found SC-CO_2 to extract better the phytosterols than petroleum ether, which can be ascribed to its ability to penetrate the inner part of material being extracted. Correlating this with our results, it is plausible that the increase of pressure might cause the increase in penetration of SC-CO_2 (due to density increase) which in turn might result in high release rate of phytosterols.

Regarding the influence of ethanol on the extraction of phytosterols, it was shown that the phytosterol content was increased when ethanol was added. Because when ethanol (possessing OH group) is added, it increases the polarity of the CO_2, hence facilitating the extraction of phytosterols. Within the same line of explanations, Nyam et al. [48] found that the addition of

ethanol (about 2 mL/min: flow rate) increased the phytosterols concentration. As far as the individual sterols are concerned, our results concur with previous studies: Lazos and Servos [50]; Matthaus and Özcan [4], who reported that the composition of phytosterols in Citrus seed oil is dominated by sitosterol, which accounted for about 70 % or even more of the total phytosterols.

Table 3.20. Tocopherol content (mg/100 g of oil) of extracted oils determined by HPLC

Tocopherols	RT	SC-CO_2, 200 bar, 45 °C			SC-CO_2, 300 bar, 45 °C			SC-CO_2 + ethanol, 200 bar, 45 °C			SC-CO_2 + ethanol, 300 bar, 45 °C		
	(min)	CS	CP	MX	CS	CP	MX	CS	CP	MX	CS	CP	MX
α-tocopherol	3.33	24.93	1.01	13.5	19.4	0.73	7.16	23.1	0.56	10.67	16.7	1.01	5.88
β-tocopherol	3.56	2.94	0.19	1.05	1.77	nd	0.82	2.52	0.39	1.25	1.29	0.27	0.65
γ-tocopherol	5.12	5.83	0.11	2.08	3.31	0.2	1.53	4.95	0.42	2.03	4.02	nd	1.96
δ-tocopherol	5.70	0.1	nd	0.13	0.1	nd	0.15	0.47	nd	0.25	0.49	nd	nd
Total		33.8 ± 0.2^{cd}	1.31 ± 0.1^{ab}	16.76 ± 0.6^{bc}	24.58 ± 0.3^{c}	0.93 ± 0.3^{a}	9.66 ± 0.2^{b}	31.04 ± 0.6^{cd}	1.37 ± 0.2^{ab}	14.2 ± 0.7^{bc}	22.5 ± 1.1^{c}	1.28 ± 0.1^{ab}	8.49 ± 0.08^{b}

Table 3.21. Phytosterols content (mg/100 g of oil) of extracted oils determined by HPLC

Phytosterols	RT	SC-CO_2, 200 bar, 45 °C			SC-CO_2, 300 bar, 45 °C			SC-CO_2+ethanol, 200 bar, 45 °C			SC-CO_2 + ethanol, 300 bar, 45 °C		
	(min)	CS	CP	MX	CS	CP	MX	CS	CP	MX	CS	CP	MX
Brassicasterol	18.27	nd	na	nd	nd	na	nd	0.21	na	nd	0.68	na	nd
Campesterol	22.49	39.36	na	15.74	40.79	na	16.00	44.15	na	14.75	49.16	na	22.12
Stigmasterol	23.13	7.33	na	2.85	11.48	na	4.01	8.79	na	3.52	23.78	na	14.26
Sitosterol	26.38	183.8	na	66.52	219.54	na	90.01	255.04	na	102.5	276.08	na	91.1
Δ-Avenasterol	27.64	15.42	na	5.85	16.17	na	6.07	19.5	na	9.34	18.06	na	12.96
Total		245.91 ± 1^{e}	na	90.96 ± 0.02^{a}	287.98 ± 0.8^{f}	na	116.09 ± 0.9^{b}	327.69 ± 0.3^{g}	na	130.11 ± 0.1^{c}	367.76 ± 1.7^{h}	na	140.44 ± 0.4^{d}

Values are presented as mean ± standard deviation (n = 3).

Values with different letter in the same row are significantly different ($P < 0.05$).

nd: not detected

na: not analyzed

RT: retention time

3.3.4. Antioxidant activity

The antioxidant activity of extracted oils expressed as IC_{50} values is presented in Figure 3.3. Low IC_{50} value corresponds to a strong antioxidant activity. The IC_{50} values differed significantly ($P < 0.05$) depending on the extraction conditions and/or raw materials (CS, CS, and MX). The DPPH values of extracted oils are presented in Figure 3.3(a) where the values ranged from 2.73 to 0.52 mg/ml for all extracted oils. The lowest IC_{50} value (strong antioxidant activity) was recorded for CP and MX oil extracted by SC-CO_2+ethanol at 200 bar with 0.52 and 0.53 mg/ml, respectively, with no significance difference ($P > 0.05$) among them, whereas the same oils (CP and MX oil) showed IC_{50} value of 0.71 and 0.68 mg/ml, respectively, for SC-CO_2+ethanol at 300 bar. The IC_{50} values of CS oils were significantly ($P < 0.05$) high compared with those of CP and MX with 0.78 and 1.31 mg/ml, respectively, for SC-CO_2+ethanol at 200 bar and 300 bar. On the other hand, the IC_{50} values showed a significant increase ($P < 0.05$) (decrease of antioxidant activity) for all the oils (compared with SC-CO_2+ethanol) when neat SC-CO_2 was used, regardless of the pressure used. In this category, the lowest IC_{50} value was recorded for MX and CP oil extracted at 200 bar with 0.89 and 0.9 mg/ml, respectively, whereas the same oils (MX and CP) showed an IC_{50} value of 1.12 and 1.31 mg/ml, respectively, at 300 bar. Again, CS oils showed higher IC_{50} value in this category. These results suggest that the IC_{50} value of extracted oils relied significantly on two parameters: extraction conditions and sample material. Regarding the extraction conditions, as it was discussed above, it was evident throughout this work, that the increase of pressure increased the yield significantly ($P < 0.05$), but at the same time, some quality characteristics (like TPC, TFC, and tocopherols) decreased significantly. In fact, the phenolic and flavonoid compounds substantially contribute to the antioxidant activity due to the redox properties of their hydroxyl groups [51] which intervene in scavenging free radicals or

donating hydrogen atoms or electrons [52]. Likewise, tocopherols are important natural antioxidants that have been repeatedly reported for their antioxidant activity in foods and biological systems [53, 54]. Given the fact that these compounds contribute a lot to the antioxidant activity and relating this to our results, it is logical to assume that the decrease in antioxidant activity (higher IC_{50} value) might be linked to a reduction in TPC, TFC, and tocopherol content.

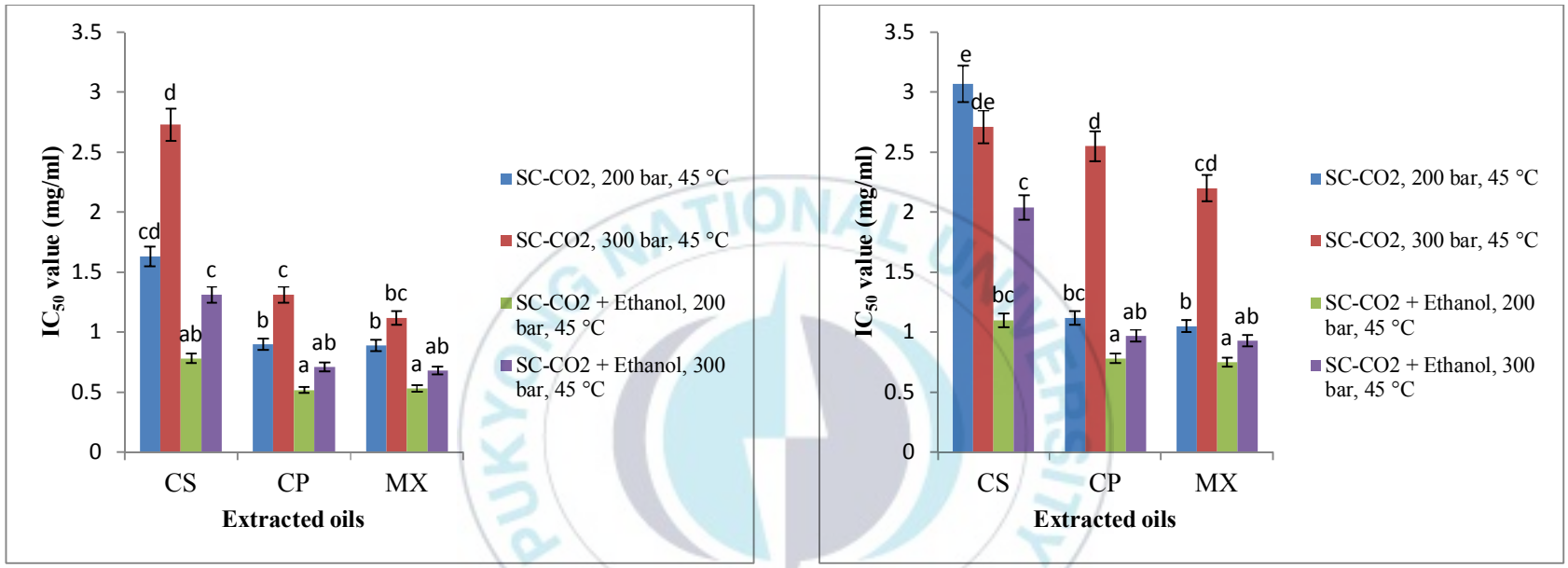
Furthermore, the IC_{50} value was shown to decrease (an increase of antioxidant activity) significantly ($P < 0.05$) by the addition of ethanol as a modifier. For instance, it dropped from 0.90 to 0.52 mg/ml for CP at 200 bar when ethanol was added. This might be due to ethanol helped extract more polar compounds (particularly phenolics, and flavonoids) which might contribute a lot to the antioxidant activity. Here it should be noted that even though some less polar and lipophilic phenolic compounds can exert antioxidant activity, the more polar and hydrophilic phenolics can exhibit higher antioxidant activity [55]. Even though the individual phenolic and flavonoid compounds were not determined in this work, it can be suggested that the addition of ethanol might extract even more polar and complex polyphenols, which might increase the antioxidant activity.

Concerning the antioxidant activity of oils (CS, CP, and MX oil), the IC_{50} values for CP and MX were significantly lower ($P < 0.05$) than CS. As observed in Table 3.1, it is quite remarkable that the TPC are higher for CP and MX than CS oil; however, the same Table also shows high TFC for CS oil compared with that for CP and MX oil. Similarly, Table 3.2 markedly indicates that total tocopherol content is greater for CS than CP and MX oil. Nevertheless, the IC_{50} value was lower (high antioxidant activity) for CP and MX than CS oil despite the high TFC and TTC in CS. This demonstrates that the TPC might exert more activity than TFC and tocopherols.

Interestingly, there was no significant difference ($P < 0.05$) between CP and MX oils in terms of antioxidant activity, even though the CP oils showed higher TPC than MX oils. This might presumably be ascribed to the synergistic effect which might greatly contribute to MX oils. In order to elucidate this effect, their composition can be taken into account. If their composition is compared, it is pretty obvious that MX oils have additional compounds (like phytosterols and tocopherols) compared with CP oils because they resulted from a combination of CP and CS. So these extra compounds (particularly tocopherols) might not only directly contribute to the antioxidant activity, but they might act synergistically with other compounds also, which may subsequently increase the overall antioxidant activity. This coincides with previous studies [55], who demonstrated the synergistic effects of polyphenols and tocopherols as a consequence of the transfer of electrons from the polyphenols to the tocopherolxyl radical to regenerate tocopherol. Besides, the phytosterols (present in MX oil but not in CP oil) might also contribute to the scavenging capacity for free radicals. Moreover, it has been shown in earlier studies that phytosterols with an ethylidene group in the side chain (particularly avenasterol) can act effectively as antioxidants and as anti-polymerization agents in oils [7, 36] and suggested that a synergistic effect of those sterols with other oils compounds may occur. The carotenoids, which are lipophilic antioxidants have been reported to be extracted more by SC-CO_2 when the vegetable oil is used as co-solvent (due to the presence of triglyceride species in vegetable oil) [24]. Although the carotenoids were not determined in this work, it might be expected that when CS and CP were combined, the triglycerides in citrus seeds might help to extract more carotenoids from peels which might consequently contribute to the MX oil antioxidant activity.

As far as the methods used are concerned, the DPPH assay showed significantly ($P < 0.05$) low IC_{50} values compared with ABTS assay (Figure 3.3(a) and (b)) for all extracted oils and even the

correlation among those assays was not high ($r^2 = 0.685$). This difference could be explained by analyzing their behaviors. ABTS assay is usually used to measure the antioxidant capacity of hydrophilic compounds[56]. However, DDPH assay has been regularly applied in both aqueous-organic extracts of plant foods[57, 58] and vegetable oils [59]. Based on the nature of our extracted oils, it seems like DPPH presented an advantage over ABTS because it might be able to react with both hydrophilic and lipophilic antioxidants compound present in a sample, which could have contributed to the augmentation of overall antioxidant activity for DPPH. Contrarily, ABTS is hydrophilic in nature; its ability to react might sometimes be limited only to the hydrophilic compounds in the extract and it may not react with lipophilic compounds, which in turn might contribute to the diminution of overall antioxidant activity compared with that of DPPH.



(a) (b)

Figure 3.18. Antioxidant activity of extracted oils expressed as IC_{50} values (mg/ml) determined by a) DPPH and b) ABTS assay.

Values are presented as mean ± standard deviation (n = 3).

Different letters on the histograms imply the significant difference ($P < 0.05$)

3.3.5. Antimicrobial activity

The disk diffusion assay results of the oils are depicted in Table 3.4. Generally, the oils showed significantly ($P < 0.05$) lower inhibition for gram-negative bacteria than gram-positive bacteria. Also, the increase of pressure showed a significant reduction ($P < 0.05$) in the inhibition capacity of the extracted oils. The highest antimicrobial activity was obtained with the oil from MX extracted by SC-CO_2 + ethanol at 200 bar against *Staphylococcus aureus* and *Bacillus cereus*, with an inhibition zone diameter of 19 mm; followed by CP oil extracted at the same conditions, with an inhibition zone diameter of 16 and 18 mm, respectively, against *Staphylococcus aureus* and *Bacillus cereus*. As far as gram-negative bacteria were concerned, the inhibition zone diameter showed a significant decrease ($P < 0.05$) compared with that with gram-positive with MX oil (SC-CO_2 + ethanol at 200 bar), showing the highest inhibition zones of 15 and 16 mm against *E. coli* and *Salmonella typhimurium*, because CP oil extracted at the same conditions showed inhibition zones of 15 and 14 mm against the same bacteria. Indeed, unlike for gram-positive bacteria, the CS oils showed no inhibition against gram-negative bacteria regardless of extraction conditions.

The MIC results for tested bacteria are presented in Table 3.5. Similarly to the results of the disk diffusion assay, Gram-positive bacteria were more sensitive than Gram-negative bacteria, and the MIC values for them ranged from 0.20 to 1.35 mg/ml depending on the extracted oil. In this category, the lowest MIC value (highest antimicrobial activity) was shown by MX oil (extracted by SC-CO_2+ethanol at 200 bar) with 0.20 and 0.25 mg/ml against *Bacillus cereus* and *Staphylococcus aureus,* respectively, whereas CP oil extracted at the same conditions showed the MIC value of 0.25 mg/ml for both bacteria. On the other hand, the MIC values for Gram-negative bacteria varied between 0.27 and 1.75 mg/ml with the MX and CP oils extracted by SC-

CO_2 + ethanol at 200 bar showing the lowest MIC value amongst. According to both results (either disk diffusion assay or MIC), it is quite obvious that gram-negative bacteria were less susceptible than gram-positive bacteria to the oils. This less susceptibility of gram-negative bacteria to the action of extracted oils was perhaps due to the reason that they have an external membrane enclosing the cell wall, which limits the lipophilic compounds of the oil to diffuse through its lipopolysaccharide covering, thus reducing the antimicrobial activity. This has also been shown by other studies which reported that gram-negative bacteria are less sensitive to the plant extracts than gram-positive bacteria [12]. Regarding the type of extracted oils, it was evident that the oils obtained at 200 bar (either by neat or modified SC-CO_2) exhibited higher antimicrobial activity for both disk diffusion assay (high diameter inhibition zone) and MIC (low MIC values) than the corresponding ones at 300 bar. This reduction of antimicrobial activity resulting in augmentation of pressure can be explained in the following way. According to our data, it was clear that increasing pressure affected the yield positively, but at the same time, affected the quality. It not only affects the unsaponifiable matters (like tocopherols) but also reduces the volatile fraction of extracted oils [15]. The volatile fraction in citrus oils is mainly composed of monoterpenes hydrocarbons, sesquiterpenes hydrocarbons, and oxygenated monoterpenes. These compounds play an important role in bacterial inhibition due to their hydrophobic property, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, distracting the structures and rendering them more permeable, which leads to an outflow of proteins and other cell contents, and therefore the bacteria dies [60]. Moreover, it was revealed in our study that the composition of sesquiterpene hydrocarbons and oxygenated monoterpenes decreased significantly when the pressure increased. For example, the composition of γ-terpinene (sesquiterpene hydrocarbon) showed a decrease from 13.35 % and 9.85 % to 7.65 %

and 5.47 %, respectively, for CP and MX oil (data not shown) when the pressure was increased from 200 bar to 300 bar (for neat SC-CO_2). Similarly, the composition of linalool (oxygenated monoterpene) was decreased from 3.74 % and 3.66 % to 1.81 % and 1.06 %, respectively, for CP and MX oil extracted at the same conditions (data not shown). By correlating these results with those for antimicrobial activity, it can be stated that the decrease of antimicrobial activity as a result of increased pressure could be linked with the reduction of volatile compounds in extracted oils, which consequently might provoke a diminution of antimicrobial activity. Moreover, the addition of ethanol as a modifier caused a slight increase in the antimicrobial activity of the oils. This may be due to the augmentation of polyphenols, as a result of polarity increase, which can contribute to the inhibition [61]. Besides, the MX oil showed slightly high antimicrobial activity compared with the CP oil, although in most cases there was no significant difference ($P > 0.05$). This might be due to the presence of additional compounds (like tocopherol, sterols, and other lipophilic compounds) present in MX oils which could contribute to the synergism with the volatile ones and increase the overall antimicrobial activity [60]. Overall, the mechanism of plant extracts to inhibit the microorganisms is complex because not only major compounds but some minor compounds also can contribute to the inhibition. The mechanism might involve synergism or antagonism effects between those compounds or specificity of some compounds to certain microorganisms [60, 62]; therefore, it is difficult to predict the exact individual compound for inhibition of such bacteria.

Table 3.22. The diameter of the zones of inhibition (in mm) of extracted oils tested against gram positive and gram negative bacteria; disk diameter (6.0 mm).

Bacteria	SC-CO_2, 200 bar, 45 °C			SC-CO_2, 300 bar, 45 °C			SC-CO_2+ethanol, 200 bar, 45 °C			SC-CO_2 + ethanol, 300 bar, 45 °C		
	CS	CP	MX	CS	CP	MX	CS	CP	MX	CS	CP	MX
Bacterial strains G (+)												
Staphylococcus aureus	8±1.00^{a}	14±1.00^{bc}	15±1.00^{bc}	ni	9±1.00^{a}	9±1.00^{a}	8±1.00^{a}	16±0.00^{c}	19±0.00^{cd}	ni	9±1.00^{a}	10±1.00^{ab}
Bacillus cereus	8±0.00^{a}	16±1.00^{c}	17±1.00^{c}	ni	10±1.00^{ab}	11±1.00^{ab}	8±1.00^{a}	18±1.00^{cd}	19±0.00^{cd}	ni	14±2.00^{bc}	14±2.00^{bc}
Bacterial strains G (-)												
Escherichia coli	ni	11±1.00^{ab}	13±1.00^{b}	ni	8±0.00^{a}	8±0.00^{a}	ni	15±1^{bc}	15±1.00^{bc}	ni	8±1.00^{a}	12±2.00^{b}
Salmonella typhimurium	ni	12±1.00^{b}	13±1.00^{b}	ni	8±1.00^{a}	ni	ni	14±1^{bc}	16±1.00^{c}	ni	11±0.00^{ab}	10±0.00^{ab}

ni: no inhibition

Values are presented as mean ± standard deviation (n = 3).

Values with different letters in the same row are significantly different ($P < 0.05$).

Values with different letters in the same column are significantly different ($P < 0.05$).

Table 3.23. Minimal inhibitory concentration (MIC) (mg/ml) of extracted oils

Bacteria	SC-CO_2, 200 bar, 45 °C			SC-CO_2, 300 bar, 45 °C			SC-CO_2+ethanol, 200 bar, 45 °C			SC-CO_2 + ethanol, 300 bar, 45 °C		
	CS	CP	MX	CS	CP	MX	CS	CP	MX	CS	CP	MX
Bacterial strains G (+)												
Staphylococcus aureus	> 2.00	0.375	0.50	nt	1.35	1.35	> 2.00	0.25	0.25	nt	1.00	1.00
Bacillus cereus	> 2.00	0.40	0.35	nt	1.25	1.00	> 2.00	0.25	0.20	nt	0.70	0.75
Bacterial strains G (-)												
Escherichia coli	nt	0.675	0.575	nt	> 2.00	> 2.00	nt	0.275	0.325	nt	> 2.00	1.35
Salmonella typhimurium	nt	0.675	0.625	nt	1.75	nt	nt	0.325	0.30	nt	1.50	> 2.00

nt: not tested

3.4. Conclusion

The processing of citrus fruits generates the by-products that are rich sources of bioactive substances. In this study, the bioactive compounds, antioxidant and antimicrobial activity of oils extracted from a mixture of citrus peels and seeds were studied. The results demonstrated that the antioxidant and antimicrobial activity might be attributed but not merely limited to the phenolic, flavonoid, tocopherol and phytosterol content of extracted oils since there might be other minor components which might play a role through synergistic or mutual effects, which consequently might contribute to the overall activity.

In addition, the extraction conditions (pressure) showed to qualitatively and quantitatively affect the extracted oils, which might be a concomitant of SC-CO_2 density and solubility. Moreover, the addition of ethanol as a modifier showed to boost the antioxidant and antimicrobial activity of extracted oils. More importantly, it appeared that the MX oil had the same or even higher potentiality than CP oil, which in turn might reflect how MX oil can be a tailor-made in many applications. Overall, this study showed that the extraction of a mixture of CS and CP, which are considered as wastes, using SC-CO_2 and/or modified SC-CO_2, might not only result in the oils with high bioactivity but might also be a promising work in many areas since the SC-CO_2 extraction is an environmentally-friendly extraction technique.

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Chapter 4

Formation, Characterization and Release Behaviors of Citrus Oil-Polymer Micro-particles using PGSS Process

4.1. Introduction

The citrus oils have been used in many applications including food, cosmetics, perfumery and pharmaceuticals industries, and in agriculture for the preparation of insecticides, painting, and adhesives, as well as textiles and plastics industries [1]. Recently, the use of essential oils from citrus has been increased due to their biological activity since they contain a wide range of bioactive compounds. Especially, the application of those citrus oils as a natural antioxidant and antimicrobial agents has been demonstrated [2-4]. The advances in new applications of citrus oils are bolstered by the fact that the public is increasingly getting concerned about the use of industrial manufactured chemicals as additives in foods and other applications.

Citrus oils can be easily degraded when they are subjected to moderate or high temperatures, the action of oxygen and light due to their volatile compounds and other non-stable components; hence if they are just physically added to food and/or in other formulation, they can be easily degraded and/or oxidized. In addition, to achieve an efficient bioactivity of the oil or another plant extract, a strict and exact dosing is required [5]. The dosing of essential oils is difficult due to their insolubility in water, which as a result limits their bioactivity and bioavailability [6]. Hence, an accurate and adequate formulation of the citrus oil is required, which takes all these aspects into consideration for effective application.

Solid forms (micro-composites or microcapsules), semi-liquid forms (liposomes, gels, etc.) and liquid forms (liquid solutions, micelles emulsions, etc.) are among the possible formulations that have been used [7]. Various conventional methods for the producing such formulations have

been studied, which include coacervation, emulsion techniques, spray-drying, and the use of foam mediums [8]. However, those methods have presented many disadvantages.

The polymer processing, the formation of polymer composites, and encapsulation of active ingredients using the supercritical carbon dioxide (SC-CO_2) has shown to be an alternative to those conventional techniques due to its eco-friendliness', run at mild operational conditions and the ability to get solvent-free and homogenous products. Particularly, the use of the particle from gas saturated solutions (PGSS) process has effectively been used for encapsulating different liquid ingredients and other active materials using polymers as wall material. The PGSS process involves two major steps:

1) The SC-CO_2 saturation of a mixture (polymer + active material to be encapsulated).

2) The gas-saturated solution expands into a spray chamber at room pressure via a nozzle. After the expansion of the mixture into a spray chamber, the temperature of the mixture diminishes drastically due to the Joule-Thomson effect, thus causing the solidification of the polymer [9].

In addition, the PGSS has been successfully used to obtain composites or encapsulates, in order to ameliorate the preservation of product and to control the rate of dissolution of some active compounds and their delivery system [10]. Therefore, due to all of those advantages of PGSS, we chose this technique to encapsulate the citrus oils.

The objective of this work was to encapsulate the citrus oil in PEG by the PGSS technique. To study the influence of process parameters including the pre-expansion conditions (temperature and pressure) and the mixing ratio on the characteristics of formed micro-particles. Moreover, the oxidative stability and *In vitro* release of encapsulated citrus oil were performed.

4.2. Materials and methods

4.2.1. Materials

The citrus fruits (*Citrus junos*), Common name: Yuza, Origin: Nam He (Busan, South Korea), Season: Nov–Jan (2017) were used. The preparation of the sample material (namely CP and CS) for extraction was the same as reported in our previous work [11]. All chemicals used were of either analytical or HPLC grades.

4.2.2. Oil extraction

The SC-CO_2 extraction diagram used in this study is depicted in Figure 2.1. The extraction setup and procedures were the same as those reported in our previous work [11]. The SC-CO_2 extraction conditions were the pressure of 200 bar, the temperature of 45 °C and flow rate of 35 g/min. The extraction time was two hours, and the sample was the mixture of CS and CP (mixing ratio was 6:4, for peels and seeds, respectively).

4.2.3. Microencapsulation process

The encapsulation process was performed using the PGSS apparatus shown in Figure 4.1. Experiments were carried out at pressures of 200-400 bar, temperatures of 40 and 50 °C and mixing ratio (citrus oil-to-PEG ratio) of 0.2-0.4 g oil g^{-1} PEG. The MX oil was used in this process due to its higher bioactive compounds and bioactivities as was demonstrated by our preliminary experiments. The PGSS process started by pumping the CO_2 from the cylinder into the reactor (carrying the mixture of PEG-8000 +citrus oil) until the desired pressure was reached. The nozzle size was 300 μm and the mixture was stirred at 400 rpm. The time of reaction was 1 h. After 1 h of reaction, the depressurization was done by suddenly opening the needle valve and the micro-particles were collected in the precipitation chamber which was kept at room pressure.

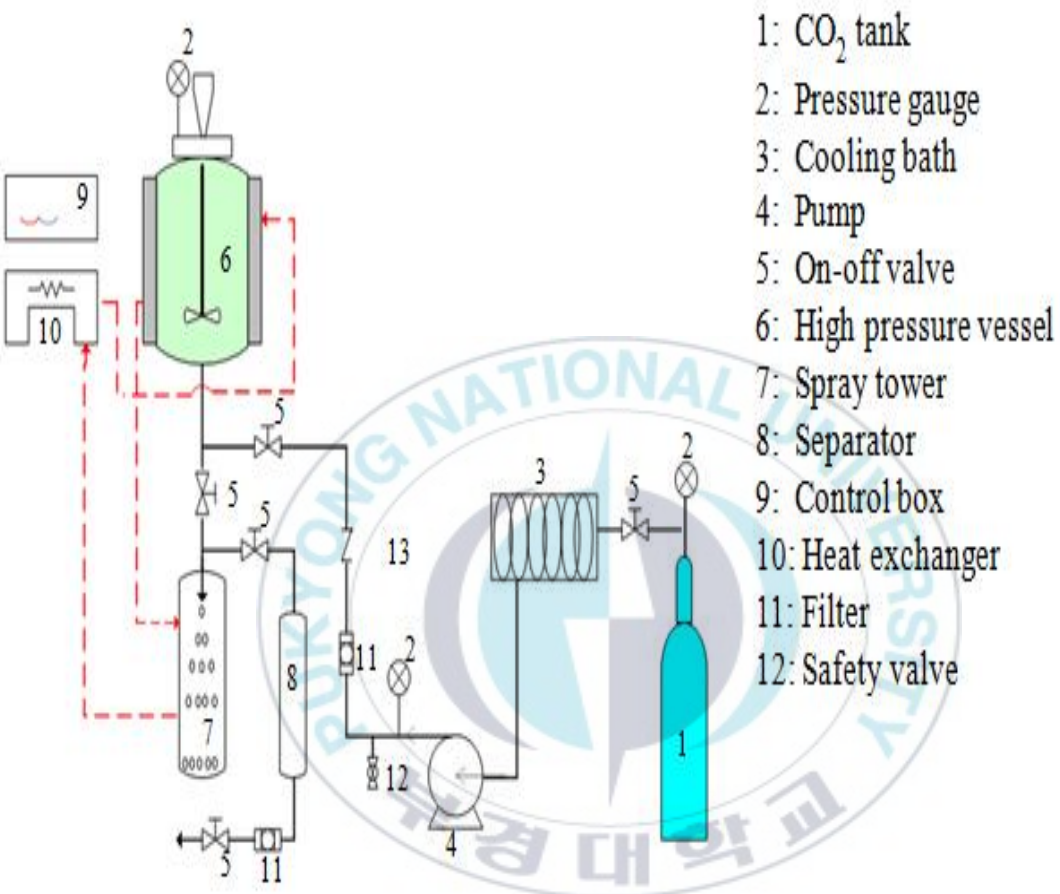


Figure 4.1. Schematic diagram of PGSS process.

4.2.4. Characterization of micro-particles

4.2.4.1. Powder wettability

The method described by Fuchs et al. [12] was used to determine the micro-particles wettability. The particles (1 g) were sprayed on the surface of distilled water (100 ml, 20 °C) and the time taken for particles to submerse below and disappear from water surface was measured.

4.2.4.2. Density Measurements

The bulk and tap densities were measured with the method described by Chinta et al. [13] with some modifications. For bulk density, the sample (3 g) was placed into the graduated cylinder to achieve uniform horizontal level. The same sample was tapped 100 times prior to the tap density measurement and then expressed as g cm^{-3}.

4.2.4.3. Particle size analysis

The particle size analyzer (PSA, LS 13320, Beckman Coulter, Brea, CA) was used to determine the size distribution of particles. A plot of the relative distribution of volume of particles vs. particle size was created and the frequency curve peak provides the modal diameter.

4.2.4.4. Encapsulation efficiency (EE)

A gravimetric method described by Bradley [14] was used to measure the total oil of particles by hexane extraction after a total solubilization of wall material using distilled water. The amount of non-encapsulated oil (surface oil) was determined according to a method shown by Tan, Chan, and Heng [15]. A mixture of 2 g of particles and 16 mL of hexane was shaken for 3 minutes at ambient temperature. Then, the mixture was settled and filtered using a Whatman No. 1 filter paper. The collected particles on the filter paper were washed 2 times with 10 mL of hexane (at each time) to wash off completely any remaining surface oil. The obtained particles were dried to a constant weight in order to remove off any residual solvent.

The encapsulation efficiency (EE) was calculated using the following formula:

$$EE = \frac{TO - NSO}{TO} \times 100$$

Where TO is the total oil and NSO is the non-encapsulated surface oil

4.2.4.5. Oxidative stability

The samples (CP oil, CS oil or particles) were sealed in a glass vial, kept at 25 and 50 °C and the stability was measured. To measure the peroxide value, the extraction of the oil from micro-particles was carried out by the method reported by Partanen et al. [16]. The oxidation degree was measured by peroxide value determination at time zero (immediately after particle formation) and after 3 months of storage. The IDF standard method was used to determine the peroxide value using UV/VIS spectrophotometer (UVmini 1240, Shimadzu Co., Japan) and the peroxide concentration was calculated using a Fe^{+3} standard curve, as reported by Shantha and Decker [17]. In all cases, the bulk citrus oil (i.e. unencapsulated oil) was also stored and analyzed under the same conditions.

4. 2.5. *In Vitro* Release of micro-particles

Particles containing encapsulated oil, 300 mg, were put into a glass bottle containing 50 mL of 96 % phosphate buffer saline (PBS) + 3 % Tween with different pH (2, 4, 6, 7.4) and then incubate at 37 °C. At specific times, samples (250 µL) were taken and the absorbance was read at the wavelength of 315 nm using a microplate reader (Synergy HTX BioTek Instruments, Winooski, VT, USA), and 250 µL of the medium (PBS+Tween) was replaced.

4. 2.6. Statistical analysis

The results were reported as the mean ± standard deviation of three replicates. The analysis of variance was performed to compare the results using SPSS for Windows (version 20.0.0, SPSS Inc.).

4. 3. Results and discussion

Table 4.1 presents a detail of experimental conditions tested, together with the main results regarding the encapsulation efficiency, density measurements, wettability and mean particle size of microparticles.

4. 3.1. Encapsulation efficiency

As presented in Table 4.1, the EE of citrus oil in PEG varies in the ranges of 43.95 to 83.87 %. From these results, it can be seen that the EE increased when the pressure was increased up to 35 MPa then further increase caused a decrease of EE. For instance, the EE was increased from 48.06 % to 75.75 % when the reaction pressure was increased from 20 MPa to 25 MPa. Also, the EE was increased up to 83.87 % when the pressure was further augmented to 35 MPa. This may be due to a stronger cooling effect (Joule-Thomson effect) during depressurization due to the increase in the amount of CO_2 dissolved in the polymer melt at higher pressures [18]. This effect can cause a faster solidification of the polymer, facilitating the encapsulation with lower losses of oil. However, the EE declined from 83.87 % to 43.95 % when the pressure was raised from 35 MPa to 400 MPa, respectively. This can be attributed to a partial extraction of oil by supercritical CO_2. This can also be explained considering that the solubility of citrus oil or another oil in CO_2 increases when pressure is increased, becoming completely miscible with CO_2 at pressures above the mixture critical point which makes it difficult to be separated during depressurization hence the EE reduced [18].

The pattern was the same with the mixing ratio. At low mixing ratio, the EE was lower, but by increasing the mixing ratio the EE seemed to increase, then after decreased. According to our results, the EE was 54.16 % when a mixing ratio of 0.2 g oil g^{-1} PEG was used, which was increased to 75.75 % by increasing mixing ratio up 0.3 g oil g^{-1} PEG with a further increase of

ratio causing a declination of EE. This can be explained considering that as more oil is added, more is encapsulated since there is a portion of oil lost by CO_2 extraction or evaporation during the process since the oil is soluble in CO_2. So, at lower oil/PEG ratio, there might be not enough oil to be encapsulated. On the other hand, the oil/PEG ratio above 0.3 g oil g^{-1} PEG reduced the EE. For example, the EE was significantly diminished from 81.08 % to 69.23 % when the ratio increased from 0.3 g oil g^{-1} PEG to 0.4 g oil g^{-1} PEG. This might be ascribed to the reason that at higher ratio, there might be the over-loading of citrus oil to wall material (PEG) hence there might be the lower amount of polymeric wall material (compared to oil) available to provide a structural matrix that keeps the citrus oil encapsulated which consequently might cause a decrease of encapsulation efficiency [19, 20]. With respect to the pre-expansion temperature, a clear trend of variation of the encapsulation efficiency with this parameter was not observed.

4. 3.2. Particle size

Table 4.1 presents the variation of particle size with the main process parameters. It can be seen that the mean particle diameter decreased as pressure increased. This trend can be related to the variation of the solubility of CO_2 in the polymer, which increases as pressure increased [21]. With a higher amount of CO_2 dissolved in the polymer at a higher pressure, the cooling effect produced by the release of CO_2 from the polymer during the expansion is stronger. An increase of the amount of dissolved CO_2 also improves the atomization of the melt during the expansion, through a reduction of melt viscosity [22], and an increased flash-boiling atomization effect caused by the release of more gaseous CO_2 from the polymer. Both effects contribute to the formation of smaller particles. Results indicate that particle size also depended on the oil/PEG ratio. As shown in the table, smaller particle diameters were obtained with lower oil/ PEG ratios. Moreover, particles produced with higher oil/PEG ratios appeared to be more agglomerated,

indicating that some fraction of superficial, non-encapsulated oil made particles sticky and promoted the agglomeration of particles and the increase of particle size. The temperature was a less determining factor for particle size than pressure and the mixing ratio.

4.3.3. Bulk density, tap density, Carr index and wettability

The bulk density of powders is affected by chemical composition and particle size as well as by processing conditions [23]. In principle, density increases as volume decreases at a given constant mass. In the present study, the bulk densities of powders were between 0.15–0.42 g cm^{-3}. The higher densities observed in the two samples (E_9 and E_7) might be related to their high degree of EE which could result in a retention of oil inside the powder particles hence the increase of mass. A comparison of the bulk density of the particles revealed that it was dependent on the mixing ratio. The low mixing ratio showed the highest bulk density (0.15 g cm^{-3}) compared with the higher mixing ratio (0.20 g cm^{-3}).

Similar observations were also recorded during the comparison of the tap densities. Both bulk density and tap density are important to measure the flow properties of powders. The flow property is generally referred to as the Carr index.

The values of Carr index of the samples were calculated by using the following equation:

$$\text{Carr Index (\%)} = \left(1 - \frac{\rho B}{\rho T}\right) \times 100$$

Where ρ_B: bulk density

ρ_T: tap density

The Carr index is an indication of the flowability of a powder. A Carr index greater than 25 % is considered to be an indication of poor flowability and a Carr index below 15 % shows excellent flowability [23]. The particles prepared with 0.3 g oil g^{-1} PEG showed the minimum Carr index (6.66 %) indicating the best flow properties among others. An increase in the amount of citrus oil

in the mixture (from 0.3 g oil g^{-1} PEG to 0.4 g oil g^{-1} PEG) reduced the flow properties from excellent (Carr index 6.66 and 7.89 %) to 14.28 %. Desobry et al. [24] reported that higher bulk density of microparticle products suggests that the particles could fit more compactly and this has less effect on oxidation rate.

The ability of powders to mix with water or other powders is one of the important physical properties related to reconstitution with water or dry blend formulation. Wettability of powders is characterized as the ability to rehydrate in water, which is the ability of a bulk powder to absorb water [25]. In this study, the time taken for powders to disappear from the surface of the water was used as a measure of the degree of wettability (Table 4.1). The wetting times taken for the powders E_9 and E_3 were 3.3 and 4 min, respectively and this might be correlated with their densities, which would have increased their sinkablility in water. These results are in agreement with several previous reports [26, 27].

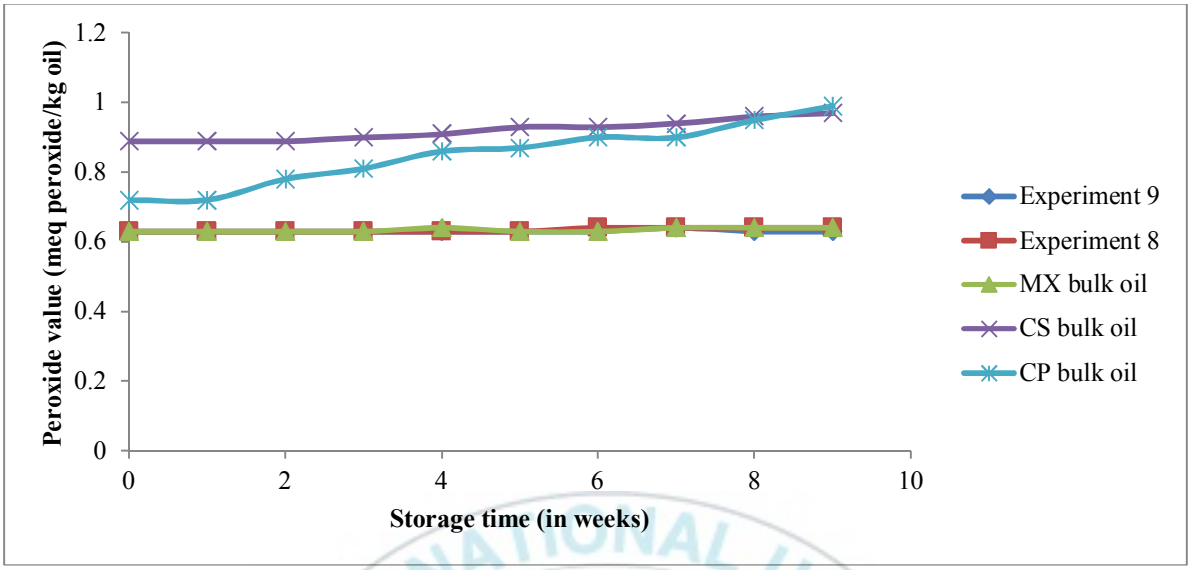
Table 4.24. Experimental conditions and results of wettability, bulk density, tap density, encapsulation efficiency (EE) and mean diameter of encapsulated powders

Experiment	Pressure (MPa)	Temperature (°C)	Mixing ratio (g g^{-1})	Wettability (min)	Bulk density (ρ_B) (g cm^{-3})	Tap density (ρ_T) (g cm^{-3})	Carr Index (%)	EE (%)	Mean diameter (μm)
E_1	20	50	0.4	5.30	0.20	0.26	23.07	51.06	373.32
E_2	25	50	0.2	8	0.21	0.26	19.23	54.16	339.91
E_3	25	50	0.3	4	0.31	0.35	11.42	75.75	213.77
E_4	20	40	0.2	10	0.15	0.20	25	48.06	282.068
E_5	40	40	0.3	7	0.17	0.20	15	60.11	246.51
E_6	35	50	0.4	6	0.24	0.28	14.28	69.23	220.10
E_7	35	50	0.3	5	0.35	0.38	7.89	81.08	199.88
E_8	40	40	0.2	10	0.16	0.20	20	43.95	250.22
E_9	35	40	0.3	3.3	0.42	0.45	6.66	83.87	190.56

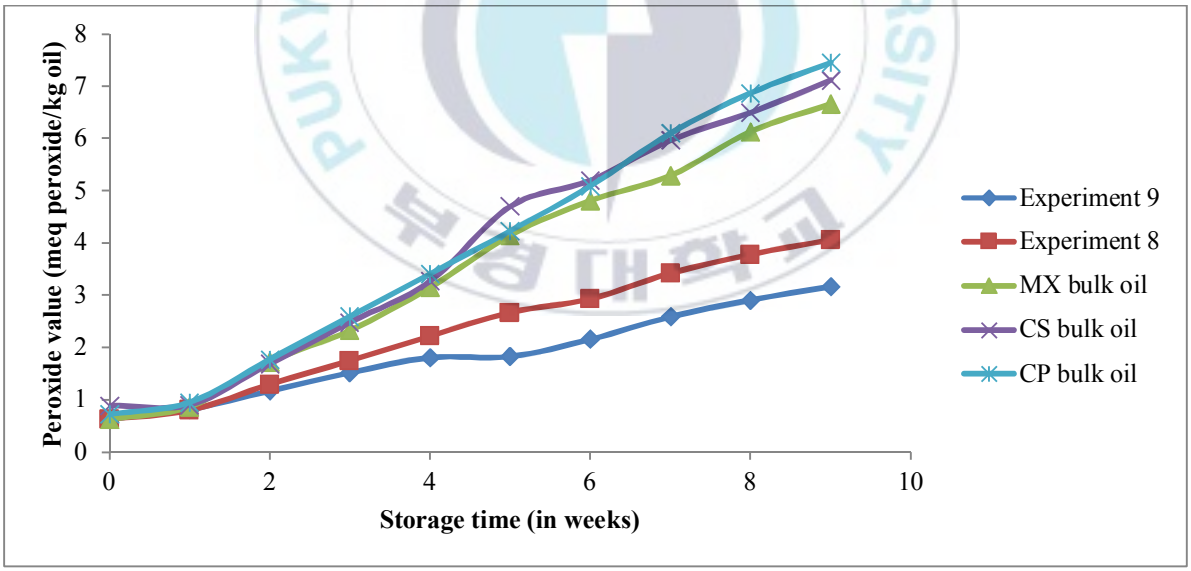
4.3.4. Oxidative stability

The oxidative stability of encapsulated mico-particles was tested by measuring their peroxide values during storage of the powders at 25 °C for 9 weeks. It was also analyzed under the accelerated condition of 50 °C for 9 weeks. The un-encapsulated citrus bulk oil (CS, CP, and MX oil) was also tested under the same conditions. The results are presented in Figure 4.2(a) & (b). At day 0 before storage, the encapsulated oils (E_8 and E_9) and bulk MX oil (which was used for encapsulation) had the same initial peroxide value of 0.63 meq kg^{-1} oil which implies that lipid oxidation did not occur during the encapsulation process. On the other hand, at day 0 the CS and CP oils showed a slightly higher peroxide value of 0.89 and 0.72 meq kg^{-1} oil, respectively, which shows how the MX oil was more stable than CS and CP oils during extraction (one of the reason it was chosen for encapsulation). The figure 4.2(a) shows the oxidative stability of encapsulated oil and non-encapsulated bulk oils during the storage time of 9 weeks at 25 °C. As we can see, the peroxide values of encapsulated oil (E_8 and E_9) and non-encapsulated MX oil (used for encapsulation) did not change during storage of 9 weeks, suggesting that, irrespective of microencapsulation, the MX oil used in this study was very stable against oxidation at ambient temperature. However, the same non-encapsulated oils of CS and CP showed a significant increase of peroxide values (from 0.89 and 0.72 at day 0 to 0.97 and 0.99 at week 9, respectively). This indicates that the MX oil might show a higher stability due to the combination of carotenoids, tocopherols, phytosterols and phenolic compounds since it is a combination of both peels and seeds. Unlike the storage condition of 25 °C, under the storage condition of 50 °C (Figure 4.2(b)), the peroxide values were observed to start rising in all cases after 1 week. The increases continued until week 9, resulting in 3.17, 4.06, 6.66, 7.12 and 7.45 meq kg^{-1} oil for micropaticles (E_9), microparticles (E_8), bulk MX oil, bulk CS oil and bulk CP oil,

respectively. According to these results, it is quite obvious that the encapsulation improved the oxidative stability of the oil compared to non-encapsulated oils. The rate of oxidation was doubled (3.17 vs 6.66) when the bulk oil was used. Again, the microparticles (E_9) showed high stability compared to microparticles (E_8). This might be linked with the EE since the free oil in E_8 might have promoted the oxidation hence the higher peroxide value.



(a)



(b)

Figure 4.2. Oxidative stability of micro-particles: A) at 25 °C and B) at 50 °C

4.3.5. *In-Vitro* release

The *in vitro* release profiles of citrus oil from the microparticles (Experiment 9) were investigated for 14 h in buffer solutions with pH of 2, 4, 6 and 7.4. Figure 4.3 illustrates that the release of citrus oil is divided into three stages based on the release rate (the slope of the release profile). The initial burst release was observed for the first 4 h. Citrus oil was released up to 50.6 %, 33 %, 29.04 % and 18.25 % in buffer solutions with pH=2, pH=4, pH=6 and pH=7.4, respectively. The release of citrus oil at this stage might involve the diffusion of citrus oil bound at the surfaces and cavities of particles [28, 29]. The release rate slightly decreased for the second stage, i.e., during 8 h for pH=4 and pH=7.4; but the rate was slightly increased for pH=2 and pH=6. The total concentration of citrus oil released at this stage were 96 %, 80.67 %, 59.24 % and 51.05 %, respectively, for pH=2, pH=4, pH=6 and pH=7.4. The release mechanism at this stage might be explained by the diffusion of citrus oil inside the particles. In other words, the driving force for the release of citrus oil was the concentration gradient. This might be due to the penetration of medium into the particulate system, which caused swelling of the matrix. The conversion of the glassy polymer into rubbery matrix subsequently took place; eventually, the encapsulated oil was diffused or released from the swollen rubbery matrix. For the third stage of release, i.e., from 8 h to 14 h, the liberation of citrus oil was significantly slow with the release rate reaching a plateau. The diminution of the release rate might come from the reduced concentration gradient (i.e., a reduced difference in citrus oil concentration between dense phase and media). The concentration of citrus oil released at 14 h were 100 %, 85.27 %, 63.08 % and 52.6 % for pH=2, pH=4, pH=6, and pH=7.4, respectively.

The amount of citrus oil released and the release rate was affected by the pH of the media. At low pH, i.e., 2 and 4, citrus oil was released from particles very quickly, and the released citrus oil content was relatively high as compared to the release at high pH, i.e., 6 and 7.4. The greater

release of citrus oil in acidic medium might be explained by the swelling and partial dissolution of particles [30]. In contrast, the particles aggregated and precipitated in phosphate buffer solutions with pH of 6 and 7.4, which might cause a reduction of particle surface area exposed to the media; as a result, the release rate and released content of citrus oil were relatively low [29].

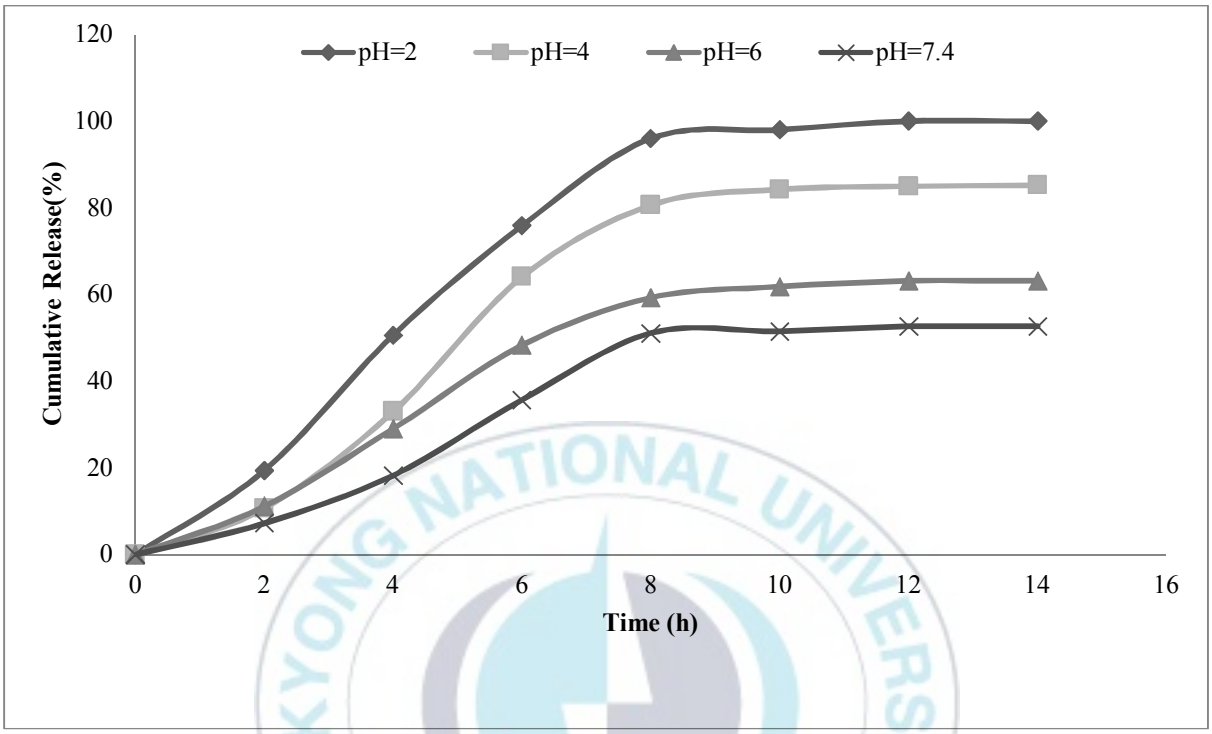


Figure 4.3. In vitro release profiles of citrus oil from particles of Experiment 9 in different pH media. Results were reported as mean of three replicates.

4.4. Conclusion

In this study, the feasibility of the PGSS process for the encapsulation of PEG and citrus oil was investigated. The PGSS experiments involved the examination of the influence of pressure, temperature and citrus oil/PEG ratio on the encapsulation efficiency of citrus oil, particle size, and other characteristics. Also, the oxidative stability and release behaviors of encapsulated citrus oil were assessed. The pressure and citrus oil/PEG ratio were shown to affect significantly the encapsulation efficiency, particle size, and other characteristics, though the influence of the temperature was not very clear. Moreover, the encapsulation showed to improve significantly the oxidative stability of encapsulated citrus oil and the release properties of encapsulated oil was dependent on the pH of the medium.

Overall, it can be concluded that microencapsulation of citrus oil using PGSS process could be applied for production of powders with good properties and higher oxidative stability that could be used in food processing industries or in other formulations.

4.5. References

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Abstract (In Korean)

초임계 이산화탄소를 이용하여 추출한 유자 부산물 오일의 특성 분석 및 캡슐화

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요약

유자를 음료 및 기타 제품으로 가공하는 것은 세계에서 가장 큰 가공 산업 중 하나이다. 이 산업에서 발생되는 부산물(유자 껍질과 씨)은 가공되지 않은 과일의 약 50 %이다. 이는 잠재적 가치의 자원을 낭비할 뿐만 아니라 처분의 문제를 야기한다. 이러한 부산물은 건강에 이로운 생리활성 화합물을 다량 포함하고 있기 때문에 유용한 자원으로 전환될 수 있다. 이러한 목적을 위해 초임계 이산화탄소를 이용하여 유자 부산물로부터 추출 된 오일을 분석하고, 가치를 부가하기 위해 캡슐화하였으며, 이는 식품, 의약품, 향수 및 화장품 산업과 같은 많은 분야에서 오일을 사용할 수 있다.

첫 번째 연구에서는 헥산과 초임계 이산화탄소를 이용하여 유자 씨(Citrus Seed, CS)와 유자 껍질(Citrus Peels, CP)의 혼합물에서 추출한 오일의 특성을 연구하였다. SC-CO2 추출 조건은 온도 45 °C와 60 °C, 압력 200 bar와 250 bar이었으며, 헥산 추출은 70 °C에서 진행되었다. 헥산 추출물은 SC-CO2 추출물보다 유의적으로 높은 오일 수율을 보였다 ($p<0.05$). 화학 성분은 GC-MS로 분석하였으며, phytosterols, monoterpenes, sesquiterpenes 및 oxygenated monoterpenes가 오일의 주요 화합물로 확인되었다. 지방산 조성은 GC에 의해 측정되었고 linoleic acid가 주요 지방산임을 확인하였다. 산화 안정성 분석은 Rancimat법에 의해 수행되었고 헥산으로 추출한 오일은 초임계 이산화탄소로 추출한 오일에 비해 높은 산화 안정성을 보였지만, DPPH 및 ABTS 분석법으로 시험한 항산화 활성은 초임계 이산화탄소 추출 오일이 헥산 추출 오일 보다 높은 소거 활성을 나타내었다.

두 번째 연구의 목적은 초임계 이산화탄소를 약간 변형하여 사용함으로써 얻은 CS와 CP 및 혼합물 오일의 생리활성 화합물, 항산화 및 항균 활성을 조사하는 것이다. 추출 조건은

순수 초임계 이산화탄소 및 에탄올을 보조용매로 한 초임계 이산화탄소를 이용하며, 45℃에서 200와 300 bar의 압력이었다. 수율은 압력이 올라감에 따라 유의적으로 증가함을 보였다 ($p < 0.05$). CP 오일은 더 높은 총 페놀 함량을 보였으나, 총 플라보노이드 함량은 CS 오일이 더 높았다. 토코페롤과 피토스테롤 함량은 HPLC를 사용하여 분석하였고, α-토코페롤과 시토스테롤이 각각 추출 된 오일의 주요 화합물인 것을 확인하였다. 항산화 활성은 DPPH 및 ABTS 분석으로 측정되었고, DPPH 결과 CP 및 혼합물을 200 bar에서 초임계 이산화탄소 + 에탄올로 추출한 오일은 0.52 및 0.53 mg/ml의 IC_{50} 값으로 높은 활성을 보였다. 항균 활성에 대해 혼합물 오일은 다른 오일에 비해 높은 활성을 나타내었고, 그람 음성 균보다 그람 양성 균에 더 민감했다.

세 번째 연구는 PGSS 공정을 이용하여 유자 오일-미립자의 형성, 특성화 및 방출 특성을 다루었다. 유자 오일은 PGSS 공정으로 폴리에틸렌글리콜(PEG)에 캡슐화되었다. 프로세스 조건, 즉 압력, 온도 및 오일/폴리머 혼합비의 영향이 입자의 특성 및 캡슐화 효율에 미치는 영향을 분석하였다. 입자크기 분석 결과, 190.56 내지 373.32 ㎛ 범위의 크기를 갖는 입자가 얻어졌으며, 캡슐화 효율은 43.95 ~ 83.87 % 범위였고 공정 변수에 의존적으로 변하였다. 배양액의 저장 온도와 pH에 따라 산화 안정성과 in vitro 방출이 각각 크게 변화하였으며, 캡슐화에 의해 산화 안정성이 유의적으로 향상되었다.

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