Process integration of supercritical fluid with novel techniques in extraction and microencapsulation of bioactive compounds

CHHOUK Kimthet

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CHHOUK Kimthet

Graduate School of Engineering

Nagoya University

Contents

Chapter 1

Introduction

1.1 Introduction

Bioactive compounds are secondary metabolites of plants such as antibiotic, food grade pigments, plant growth factors, mycotoxinsm, alkaloids, and phenolic compounds. The main phenolic compounds are phenolic acids, flavonoids, and tanins. Phenolic acids are a principal class of phenolic compounds, which show wide range of bioactive functions. Phenolic acids are divided into two subgroups based on their structure: the hydroxybenzoic and the hydroxycinnamic acid. Hydroxybenzoic acid group comprises garlic, protocatechui, vanllic, p-hydroxybenzoic, ellagic, syringic, gentistic and salicylic acid. Feulic, caffeic, p-cumaric, sinapic, cinnamic, quinic, and chlorogenic acid are found in hydroxycinnamic acid group. The most common phenolic compounds found in plants are gallic, p-hydrobenzoic, vallic, caffeic, feulic, p-coumaric, sinapic, protocatchuic, hydroxycinnamic and syringic acids [1]. Recent studies indicated that bioactive compounds show wide range of physiological properties such as antioxidant activity, anti-mutagenic, anti-allergenic, anti-inflammatory, and antimicrobial, cardioprotective and vasodilatory effect. Therefore, they are extensively used in food, pharmaceutical, and cosmetic industries [2, 3].

In recent years, bioactive compounds have received increasing attention from many research groups due to their ability to provide or promote benefits for human health. The amount of bioactive compounds of plant depends on many factors such as type of plants, cultivation area, climatic conditions, vegetation phase and genetic modifications [4]. Therefore, researchers have been increased interest to find fruits, vegetable, and plants, agriculture and agro-industrial residues as sources of bioactive phenolic compounds in order to respond to the great demand of bioactive compounds for food and pharmaceutical industries [1].

The quality and quantity of bioactive compounds from plant materials depend on the extraction methods. Extraction is identified as a separation process consisting in separation of substance from matrix. There are several important factors affecting on extraction process including matrix properties of the plant part, solvent, temperature, pressure, and extraction time. The extraction of bioactive compounds from plant can be carried out by various extraction procedures. Due to development of technology, the new extraction method is invented which is more the environment friendly because of less and no consumption of organic solvent. Moreover, the new extraction technology also can reduce operational time, increase the yield, and enhance the selectivity of bioactive compounds from plant materials. Supercritical fluid extraction (SFE) is known as a fast, efficient, and clean method used to recover the drawbacks of conventional methods. At the present time, conventional extraction, such as Soxhlet, is still regard as one of the reference method compare to success of SFE method [5].

1.2 Conventional extraction techniques

Several conventional techniques is used to extraction bioactive compounds from plants. Most of conventional extraction techniques use solvent with the application of heat and/or agitation. There are some well-known classical methods such as Soxhlet, maceration and hydrodistillation. Among these methods, Soxhlet method is widely used to extract bioactive compounds from different natural sources. It is consider as a standard method for comparison with new alternative extraction method [5, 6].

The extraction efficiency of conventional method is based on the choice of solvent. The choice of solvent is the main effect on the polarity of targeted compound. Most widely used solvents are hexane, ether, chloroform, acetonitrile, benzene, methanol and ethanol. These solvents were employed to extract both polar and nonpolar compounds from natural resources such as alkaloid, organochlorine pesticides,

phenol, aromatic hydrocarbon, fatty acid, oil, and other compounds [7]. The conventional methods have some advantages such as easy operation and low processing cost. However, these methods use large amount of solvent, so it can give bad effect to human health and environment due to the large amount of solvent residue and toxicity in final product. Moreover, these methods required extraction, evaporation, and concentration step for recovery, so they take long time for extraction. The degradation of bioactive compounds might occur due to high temperature of the solvent with long times of extraction. As the result, new extraction methods have been developed to overcome the drawback of the conventional methods [5-7].

1.3 Supercritical fluid technology

In order to reduce the use of organic solvent and improve the yield and selectivity of bioactive compounds from plants materials, the novel extraction methods, such as ultrasound, pulsed electric field, enzyme digestion, extrusion, microwave heating, ohmic heating, supercritical and accelerated solvent, has been developed [8]. Nowadays, supercritical fluid extraction is still considered as a novel technology due to fast, efficient, and clean method. It has been used to recover the bioactive compounds from plants material in food, pharmaceutical and cosmetic industries because it is able to generate the product with high purity and without the degradation of active compounds and toxic residues.

In compare to conventional method, SFE has some advantages. SFE can decrease the consumption time because supercritical fluid has higher diffusion coefficient and lower viscosity and surface tension than the liquid solvent, this makes it more penetration to sample matrix and increases the mass transfer. Moreover, the supercritical fluid flows continuously into sample, thus it allows to provide the complete extraction. The selectivity of supercritidcal fluid is better than that of conventional solvent because its solvation power can be adjusted by changing temperature and pressure. The separation of solute from solvent is done by expansion of solvent after extraction, so it can save time. Only small amount of organic solvent was used in extraction, thus it is consider as environmentally friendly method. Supercritical fluid can be recycled, so it can reduce the waste generation. Recently, many studies on SFE of phenolic compounds, essential oil, cartotenoids, tocopherols, tocotrienols, alkaloids, and other classes of chemical compounds have been distributed. Several plant materials were used to this

extraction method such as seeds, fruits, leaves, flowers, rhizomes, roots, peel of fruits, and branches of trees [5-9].

Supercritical state is occurred when substance is subjected to the temperature and pressure above its critical point. Critical point is point over the critical temperature (T_c) and pressure (P_c) where no liquid or gas will take place. At this point, supercritical fluid has diffusion, viscosity, and surface tension like gas and density and solvation power like liquid. These properties make it suitable for extraction of bioactive compounds from plant materials. The selectivity of compounds can be obtained by changing temperature and pressure [5]. The most used solvent in SFE is carbon dioxide. Carbon dioxide is known as a safe, nontoxic and cheap solvent. Moreover, it has low critical temperature (31 ^{o}C) and pressure (7.4 MPa) (Figure 1.1). Carbon dioxide has low polarity, which makes it easily to extract lipids, fats, and non-polar substances. However, some bioactive compounds from plant materials are difficult to extract by using carbon dioxide due to the high polarity of bioactive compounds. Therefore, the small amount of polar solvents (modifiers) is frequently added during extraction in order to increase the dissolving power [5, 10].

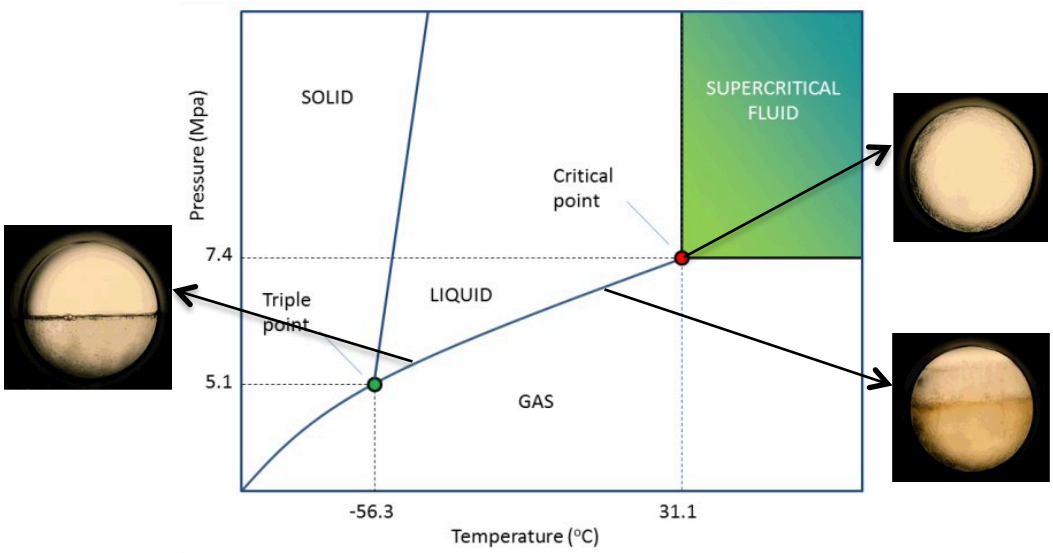

Figure 1.1 Phase diagram of CO_2

The diagram of SFE apparatus is shown in Figure 1.2. The basic of SFE extraction with cosolvent consists of a gas tank, usually CO_2, a chiller, a pump for CO_2, a cosolvent vessel, a pump for cosolvent, a extractor, an oven, a pressure gauge, a back pressure regulator, a trapping vessel and a CO_2 flow meter. Generally, the

parameters that may affect the extraction efficiencies in SFE include temperature, pressure, solvent flow rate, extraction time, and among others. The extraction efficiency is also influenced by the morphology of the solid subtract particle because the solvent must go through the diffusive paths inside the solid particle in order to extract the specific compounds. The efficiency of SFE process is able to improve by using combined extraction technique such as supercritical fluid expended liquid extraction, ultrasound assisted SFE, and SFE integrated hydrothermal process [10].

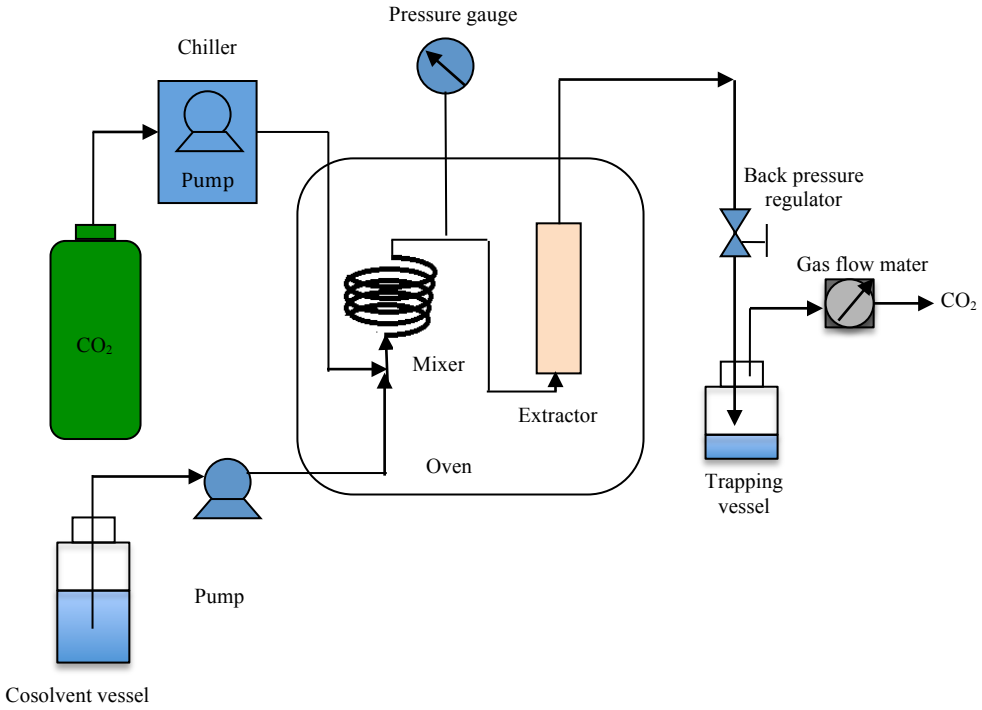

Figure 1.2 The diagram of SFE apparatus

Supercritical fluid expended liquid extraction has been employed for extraction, reactions and separations. The most commonly used gas in supercritical fluid expended liquid is CO_2. CO_2 expended ethanol (CXE) has been employed to substitute of the conventional methods for extraction because it can reduce the viscosity, decrease the interval interfacial tension, and enhance the diffusivity of solvent. Under this condition, it can enhance the solubility of bioactive compounds in solvent. Recently, this method was used to extract γ-linolenic acid from *Arthrospira platensis* (Spirulina) [11], astaxanthin content and antioxidant activity

from *Haematococus pluvialis* [12], DHA-containing lipid from *Schizochytrium sp.* [13], bioactive compounds from *Moringa oleifera* leaves [14].
Ultrasound assisted supercritical fluid is known as an effective method to extract bioactive compounds from plant materials. When ultrasonic introduced into SFE, it can produce the cavitation; this leads to break the cell wall of plant materials. Under this condition, the solvent is easy to penetrate into the material and mass transfer of solvent also improves. As the result, the extraction rate and yield are significantly enhanced. Lately, ultrasound assisted supercritical fluid was used extraction of valuable compounds from plant material such as oleanolic, ursolic acid and oridonin from *Rabdosia rubescens* [15], α-humulene oil from clove [16], and triterpenic acid from *Hedyotis corymbosa* [17].
Supercritical fluid integrated hydrothermal process is also considered as a potential alternative technique to conventional supercritical carbon dioxide. Adding supercritical CO_2 to water under subcritical condition can reduce the pH of solvent due to the formation of carbonic acid especially at high pressure. In this case, the solvent is more suitable for extraction bioactive compounds from plants materials, especially the compounds that have an affinity to acidic solvent. Recently studies showed that this method could be employed to extract β-glucane from barley grain [18], and xanthone from *Garcinia Mangostana* pericarp [19].
Beside extraction, supercritical fluid has also applied in micronization of bioactive compounds form plant materials, which is known as the supercritical anti-solvent micronization technique. In this technique, the bioactive compounds dissolve organic solvent. When carbon dioxide was added to the organic solvent under desirable condition, the decreasing the density of the solvent is occurred, resulting the micronization of bioactive compounds. This method has been found to successfully apply in micronization of bioactive compounds with and without polymer such as cilostazol [20], celecoxib [21], β-carotene [22], curcumin [23], and quercetin [24].

1.4 Purpose of this study

The purpose of this study is application of process integration of supercritical fluid with novel techniques in extraction and microencapsulation of bioactive compounds from plant materials.
The specific objectives of this study are following:

1. To investigate the effect of operational parameters in extraction of bioactive compounds from various plant materials by using process integration of supercritical carbon dioxide with novel techniques
2. To compare the efficiency of process integration of supercritical carbon dioxide with novel techniques with conventional method
3. To identify the bioactive compounds in extract of each sample
4. To produce the microencapsulation of the bioactive compounds using supercritical anti-solvent method.

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Chapter 2

Extraction of bioactive compounds from plant materials using process integration of supercritical carbon dioxide with novel techniques

2.1 Extraction of phenolic compounds and antioxidant activity from garlic husk using carbon dioxide expanded ethanol

2.1.1 Introduction

Allum sativum L., a wild aromatic plant commonly called garlic, has been used as food ingredient, functional food and traditional medicine for many centuries due to its distinctive flavor, therapeutic and medical properties [1, 2]. Garlic has many

therapeutic effects, such as hypolipidaemic, antitherosclerotic, hypoglycaemic, anticoagulant, antihypertennsive, antimicrobial, antioxidant, antidote and hetoprotective. Furthermore, it can be used to prevent some type of diseases such as cardiovascular disease, cancer, diabetes, flu and cold. Garlic was widely consumed as fresh in food processing industry [3-5]. However, the garlic husk, a by-product from food processing industry, contributes 25% of garlic bulk regards as agricultural waste [6, 7]. Generally, agriculture wastes are lignocellulosic material. The main compositions of these wastes are cellulose, hemicellulose and lignin. It has been reported that lignin and carbonhydrates (Cellulose and hemicellulose) are a potential source for insoluble phenolic compounds such as caffeic, p-coumaric, ferulic acid and other phenolic compounds [8, 9]. Garlic husk contains 6.3% lignin, 41% cellulose, 18% hemicellulose [7]. Recent studies have found that the extract from garlic husk contains phenolic compounds with antioxidant and anti-bacterial activities. The major phenolic compounds in garlic husk are caffeic, p-coumaric, ferulic and di-ferulic acid [10, 11].

Nowadays, the demand of these natural phenolic compounds is increased for their antioxidant activity. Solid-liquid conventional methods can be used to extract these compounds from natural plants. However, these methods provide low extraction efficiency and harmful effect to human health and environment such as the massive use of organic solvent and production of toxic residue in final product. Therefore, new extraction method has to be developed for a better and safer extraction [12, 13].

Supercritical carbon dioxide and carbon dioxide expanded organic solvent have been focused as an alternative solvent to conventional organic solvent for extraction process. Carbon dioxide expanded ethanol (CXE) has been recognized to substitute the conventional method. By adding carbon dioxide (CO_2) into pressurized ethanol, it can reduce viscosity, decrease the interval interfacial tension, and enhance diffusivity of solvent. This condition can improve the solubility of bioactive compounds in solvent [14-16]. Moreover, CXE can be operated at low pressure compare to supercritical carbon dioxide and use less amount of organic solvent compared to pressurized liquid extraction [17]. In recent year, CXE has been widely studied for extraction of valuable compounds from natural product [14-18]. Reyes et al. obtained higher extraction yield, astaxanthin content and antioxidant activity from *Haematococus pluvialis* than the conventional solvent extraction. However, there is no data reported regarding extraction of phenolic compounds from garlic husk using CXE.

The aim of the present work is to investigate the extraction of phenolic compounds and antioxidant activity from garlic husk using CXE in comparison with the

conventional solvent method of extraction. Moreover, the effects of different extraction parameters such as temperature and CO_2 flow rate on total phenolic compounds and antioxidant activity were investigated. The major phenolic compounds in CXE extraction were also identified.

2.1.2 Materials and methods

2.1.2.1 Materials and chemicals

Galrlic sample were purchased from a local market in Japan. The dried garlic husk was separated from garlic bulb. The dried garlic husk was collected and dried via freeze drying (EYELA FDU-1200, Japan). Then, the dried sample was ground into fine particle and passed through 1 mm sieve screen. After that, the ground samples were packed in alluminum plastic bag and stored at 4 ^{o}C prior to extraction.
Carbon dioxide (purity 99%) was supplied by Sogo Kariya Sanso, Inc. Japan. Ethanol (99.5%) was purchased from Nacalai Tesque (Japan). Acetonitrille plus from obtained by Kanto Chemical Co. Inc. (Japan). Methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, sodium nitrite, aluminum chloride, sodium hydroxide, acetic acid, folic acid were supplied from Wako Chemical (Japan), Folin-Ciocalteu agent solution was purchased from Sigmaldrich. Pure distilled water was used in all experiments.

2.1.2.2 Carbon dioxide expanded ethanol extraction

The schematic of supercritical carbon dioxide expanded ethanol extraction system is illustrated in Figure 2.1.1. The experiment apparatus consists of chiller, two high-pressure pumps (PU-2080, JASCO, Japan), CO_2-ethanol mixer (Swagelok, USA), extractor (Thar Tech, Inc., USA), electric heater (ESPEC ST-110, Japan), back-pressure regulator (BPRs; AKICO, Japan). First, one gram of ground sample was load in 10 mL stainless-steel extractor. The top and bottom of the extractor were filled with glass beads. The extractor was placed in an electric oven. Ethanol was introduced to extractor by means of HPLC pump until 10 MPa. The pressure was kept constant by mean of back-pressure regulator. Ethanol was then heated to desired operating temperature in range of 50-200 ^{o}C. When the extractor reached desired temperature and pressure, CO_2 was introduced in the extractor at flow rate of 0.5-2 mL/min at the pump condition. At the same time, ethanol was also applied to extractor at flow rate of 3 mL/min and extraction time commenced. The extraction time for each experiment was 2 h. After extraction, solvent was evaporated and

extract was dissolved in ethanol and kept in refrigerator at temperature of 4 ^{o}C until analysis was carried out. Each experiment was conducted in duplicates

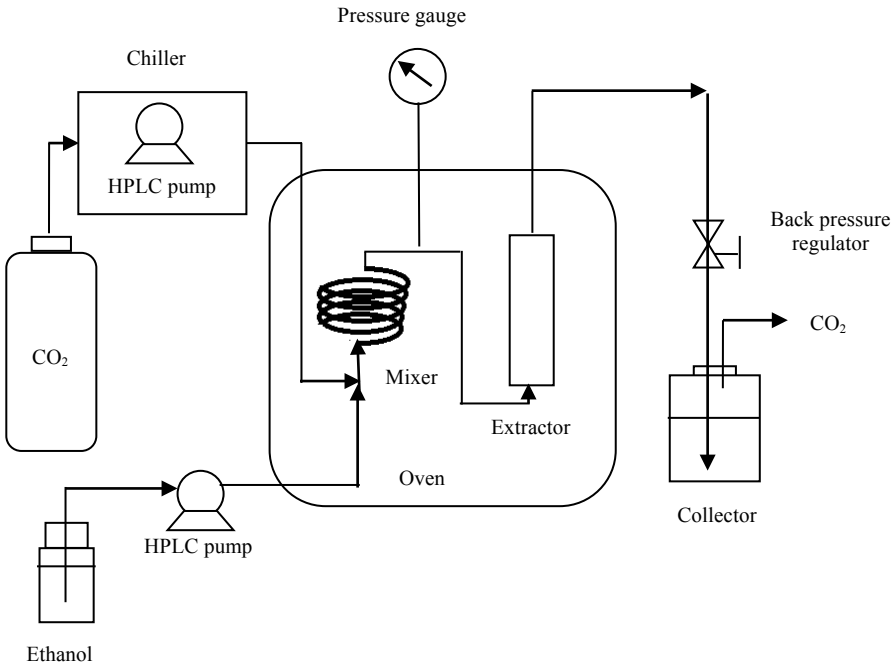


Figure 2.1.1 Schematic diagram of carbon dioxide expanded ethanol

2.1.2.3 Pressurized ethanol extraction

Pressurized ethanol extraction was done in order to compare the performance with method of CXE. Briefly, 1 g of garlic husk was placed in a 10 mL stainless-steel extractor. The same extraction system of CXE with the same setup was applied to this system except that there was no CO_2 was applied into system. The pressurized ethanol extraction was conducted under following condition: temperature of 150 ^{o}C, pressure of 10 MPa, flow rate of ethanol 3 mL/min and extraction time of 2 h. The extract was collected and stored at the same manner as mentioned in the CXE section.

2.1.2.4 Soxhlet extraction

3 g of ground garlic husk was placed in the thimble and extracted with 300 mL of each solvent (methanol or ethanol) for 6 h in Soxhlet apparatus. Thereafter, the solvent was evaporated using rotary evaporator (SB-1200, Eyela Co., Ltd, Japan). Soxhlet was performed as conventional method technique in order to compare the yield and composition of the extracts with those of CXE.

2.1.2.5 Total phenolic compounds content assay

Total phenolic compounds content (TPC) of the extracts were determined using Folin-Ciocalteu method, in accordance to method of Kallel et al. [11]. 1 mL of sample were mixed with 1 mL of Folin-Cioacateu reagent. After 3 min, 1 mL of 7.5 % (w/w) sodium carbonate and 7 mL of de-ionized water were added to mixture. After 2 h of incubation in dark place at room temperature, the total phenolic contents were estimated by measuring at 750 nm absorbance using UV-VIS spectrophotometer (V-550, Jasco, Japan).

2.1.2.6 DPPH free radical scavenging activity assay

DPPH radical scavenging activity was quantified by the method described in Fernández-Agulló et al. [19]. DPPH was measured by UV/Vis spectrophotometer. The extract was diluted in methanol in the ranged of 0.01 to 2 mg/mL. The sample was mixed with 2.7 mL of DPPH solution (6×10^{-5} M in methanol). After being thoroughly stirred, the mixture was kept in dark place at room temperature for 60 min. Thereafter, the absorbance of the mixture at 517 nm wavelength was measured. The percentage of DPPH radical scavenging activity was calculated by the following equation (1):

$$\%\text{DPPH Scavenging activity} = \left[\frac{(A_{517\ \text{of control}} - A_{517\ \text{of sample}}}{A_{517\ \text{of control}}}\right] \times 100 \quad (1)$$

where

$A_{517\ \text{of control}}$: the absorbance of control reaction (containing the entire reagent except the sample).

$A_{517\ \text{of sample}}$: the absorbance of the reaction of sample and reagents

IC_{50} is the concentration of the sample that is necessary for 50% scavenging of DPPH free radical. Its value was determined by the plot of %DPPH scavenging activity against the concentration.

2.1.2.7 HPLC analysis

The phenolic compounds were measured by modifier procedure described in Kallel et al. [11]. Analysis was performed using high performance liquid chromatography (HPLC) with diode array detection (SPD-M10AVP, Shimadzu, Japan). The sample was filtered with 0.45 μm filter paper and 20 μL of injected and separated in Intersil ODS-3 column (5 μm; 4.6 mm × 250 mm, GL Science, Japan) at 40 ^{o}C. The mobile phase was a mixture of 0.1 % formic acid in water and 0.1 % of formic acid in acetonitrile (85:15 v/v). The flow rate was at 0.5 mL/min, and separation was determined at detection wavelength of 280 nm.

2.1.3 Results and discussion

2.1.3.1 Preliminary study

Table 2.1.1 The effect of pressurized ethanol and CXE method on extraction yield total phenolic compounds and antioxidant activity.

Extraction method	Extraction Yield (%)	Total Phenolic compounds (mg GAE/ g of dried garlic husk)	Antioxidant activity (IC_{50}) (mg/mL)
Pressurized ethanol	6.48±0.14	28.35±0.07	0.91±0.01
CXE	8.51±0.01	29.83±1.14	0.89±0.00

The purpose of preliminary experiment is to determine which method is efficiency for extraction of phenolic compounds from garlic husk. The pressurized ethanol and CXE method were used to extract phenolic compounds from garlic husk. For pressurized ethanol, extraction was carried out at temperature of 150 ^{o}C, pressure of 10 MPa, ethanol flow rate of 3 mL/min [20, 21]. For CXE method, the same process parameters that applied in pressurized ethanol were used in this process. The flow rate of CO_2 was 0.5 mL/min at pump condition. It was found that both methods could extract phenolic compounds from garlic husk. Moreover, the addition of CO_2 in pressurized ethanol increased not only the extraction yield, but also total phenolic compounds content and antioxidant activity in the extract as shown in Table 2.1.1. The addition of CO_2 in pressurized ethanol can reduce the surface tension and viscosity. The polarity of mixed solvent is lower than ethanol. Also, it increases

diffusivity of solvent into material. Thus, it may increase the solubility of phenolic compounds in solvent [16, 17]. The following Sections 2.1.3.2 and 2.1.3.3 summarize the effect of operating parameters such as temperature and CO_2 flow rate of CXE extraction on the amount of the phenolic compounds extracted from garlic husk and their radical scavenging activity.

2.1.3.2 Effect of extraction temperature

Temperature is one of critical parameter, which significantly influences on the amount of total phenolic compounds in CXE extraction. The effect of temperature in range of 50-200 ^{o}C on amount of total phenolic compounds was investigated at a fix pressure of 10 MPa, CO_2 flow rate of 0.5 mL/min and ethanol flow rate of 3 mL/min. As shown in Figure 2.1.2, the total phenolic compounds content was gradually increased from 4.69 to 56.02 mg GAE/g of dried garlic husk with increasing temperature from 50 to 200 ^{o}C. At high temperature, the solubility of compounds could be increased due to decrease the polarity of solvent, which facilitate the solvent penetrate into material [22]. Also, the cell tissues of material may be disrupted during extraction; this makes the mass transfer rate increase [23, 24]. Furthermore, the lignin, which is the component of plant cell wall, might be decomposed into phenolic compounds at high temperature [21, 25]. Brebu and Vasile reported that the functional group of lignin was cleaved at high temperature. As the result, the low molecular weight product was obtained. When rearrangement of backbone is complete, it leads to release of volatile product. Also, the cleavage of the aryl-ether linkages results in formation of highly reactive and unstable free radicals. Then these products have further reaction through rearrangement, electron abstraction or radical-radical interaction to form the stable products. Therefore, the decomposition of the polymer structure in lignin results in formation of aromatic hydrocarbons, phenolics, hydroxyphenolics and guaiacyl-syringyl-type compounds [21]. The antioxidant activity of phenolic extraction was determined by the 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activity. The value of IC_{50} was dramatically decreased with increasing the temperature from 50 to 200 ^{o}C (Figure 2.1.2). It is possible that thermal degradation of lignin may occur in extraction and release new formation compounds, which have antioxidant properties. The lowest value of IC_{50} of 0.41 mg/mL was achieved at temperature of 200 ^{o}C. The highest value of IC_{50} of extract (4.69 mg/mL) was obtained at temperature of 50 ^{o}C. The lowest value of IC_{50} means the highest antioxidant activity in extract. Several studies indicated that heating improves the antioxidant activity in fruits and vegetables due to the enhancement antioxidant properties of naturally occurring compounds or the

formation of compounds such as result in Maillard reaction that have antioxidant activity [26-28]. It is reported by Sharma et al. that the antioxidant activity of red onions was increased after heating at temperature of 120 ^{o}C for 30 min [24].

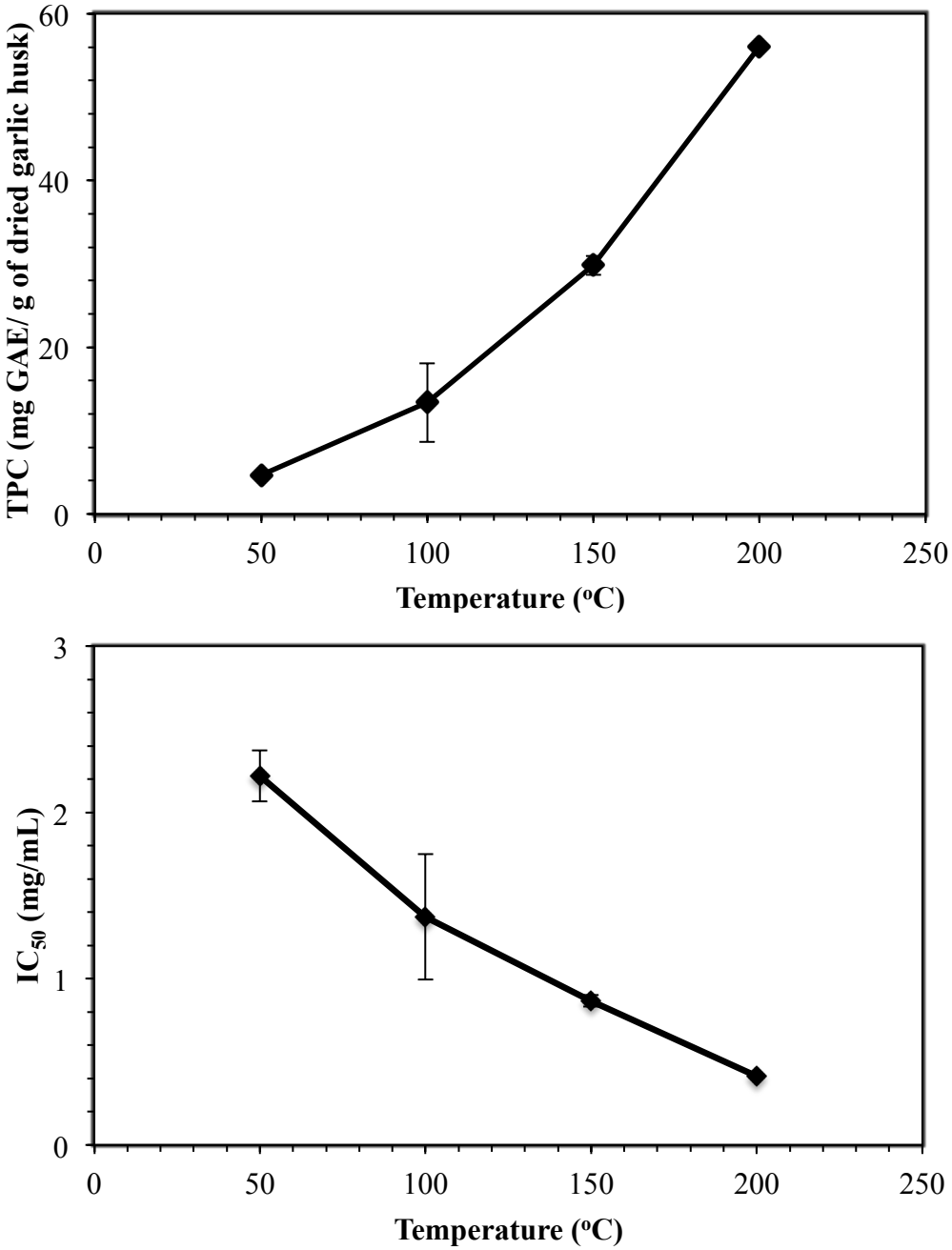


Figure 2.1.2 Effect of temperature on total phenolic compounds content and antioxidant activity (IC_{50})

2.1.3.3 Effect of CO_2 flow rate

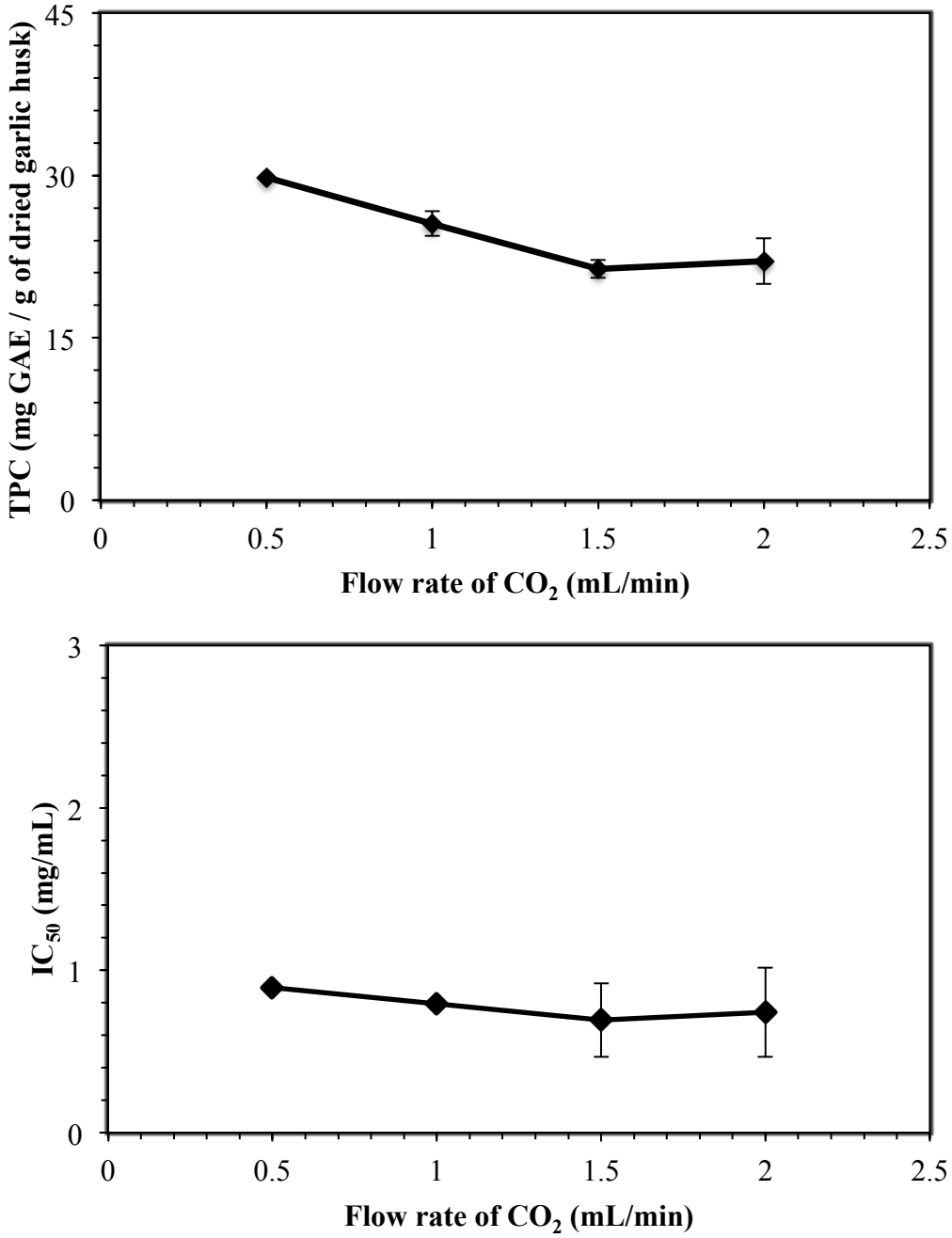


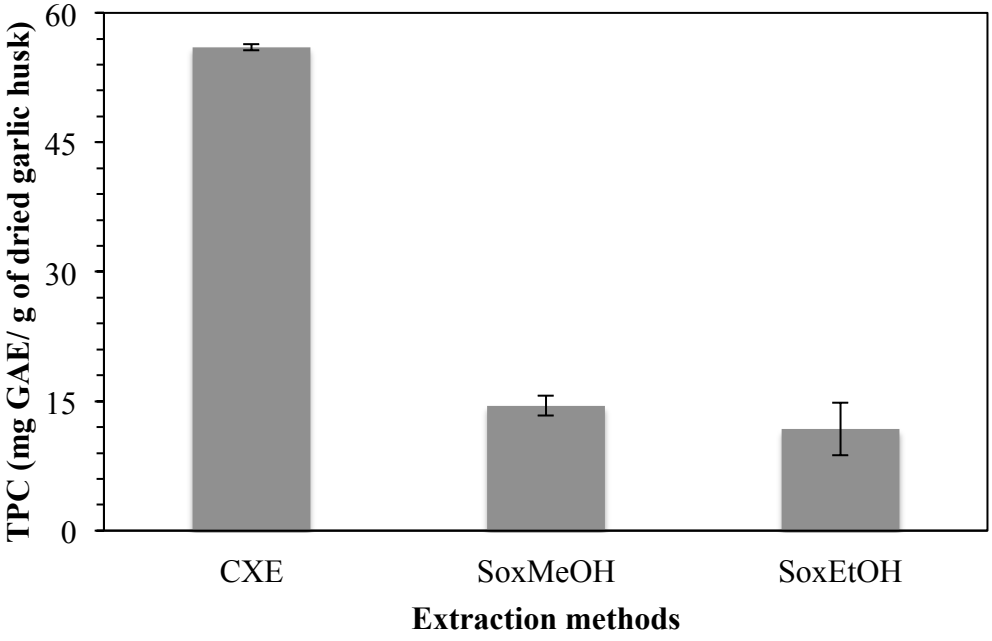
Figure 2.1.3 Effect of CO_2 flow rate on total phenolic compounds content and antioxidant activity (IC_{50})

The effect of CO_2 flow rate on extraction of phenolic compounds was carried out at a fix temperature of 150 ^oC and pressure of 10 MPa as indicated in Figure 2.1.3. It was found that increased flow rate of CO_2 result in the decreased total phenolic compounds content. The total phenolic compounds content was declined slightly from 29.83 to 21.36 mg GAE/g of dried garlic husk with increasing flow rate of CO_2 from 0.5 to 1.5 mL/min at the constant ethanol flow rate of 3 mL/min. Then, the total phenolic compounds content was almost constant when flow rate of CO_2 was augmented beyond 1.5 mL/min. One of the reasons for the negative effect of CO_2 flow rate is decreased polarity of the mixed solvent of ethanol and CO_2 due to lower ratio of ethanol to CO_2 [29]. This may result in the decreased solubility of phenolic compounds in the mixer of solvent. Another reason maybe mass transfer effect. It ispossible that increasing flow rate of CO_2 in CXE can increase the diffusivity of solvent [16, 17]. However, due to flow rate is too high, the solvent may pass around the sample matrix and it doesn't diffuse into the pore to extract the compounds from material. Thus, it reduces the contact time between the material and solvent and the solubility of compounds into solvent also could be decreased in this condition [30-32].

The effect of CO_2 flow rate on the antioxidant activity also is illustrated in Figure 2.1.3. The result showed that the flow rate of CO_2 has only a small effect on antioxidant activity (IC_{50}). The value of IC_{50} of extract was slightly decreased from 0.91 to 0.69 mg/mL with increasing the flow rate of CO_2 from 0.5 mL/min to 1.5 mL/min. However, there is no significant difference was found for the value of IC_{50} when CO_2 flow rate was increased from 1.5 mL/min to 2 mL/min.

2.1.3.4 Comparison with traditional Soxhlet extraction

The results of phenolic compounds and antioxidant activity of Soxhlet extraction using methanol or ethanol as solvent were compared to those obtained from the CXE extraction method at temperature of 200 ^oC and CO_2 flow rate of 0.5 mL/min as shown in Figure 2.1.4. The result indicated that CXE produced highest amount of total phenolic compounds (56.26 mg GAE/g of dried garlic husk) and antioxidant activity (0.41 mg/mL) compared to Soxhlet extraction using methanol or ethanol. This result supported by the fact that CXE have ability to enhance the extraction efficiency compared to organic solvent by reducing viscosity, decreasing the interval interfacial tension, and increasing diffusivity of solvent. Generally, in Soxhlet extraction the extraction processes consisting of desorption from solid surface and diffusion of solid matrix to extraction phase. Thus, extraction rate is slow. In CXE extraction, coupling CO_2 and ethanol can increase the compressibility



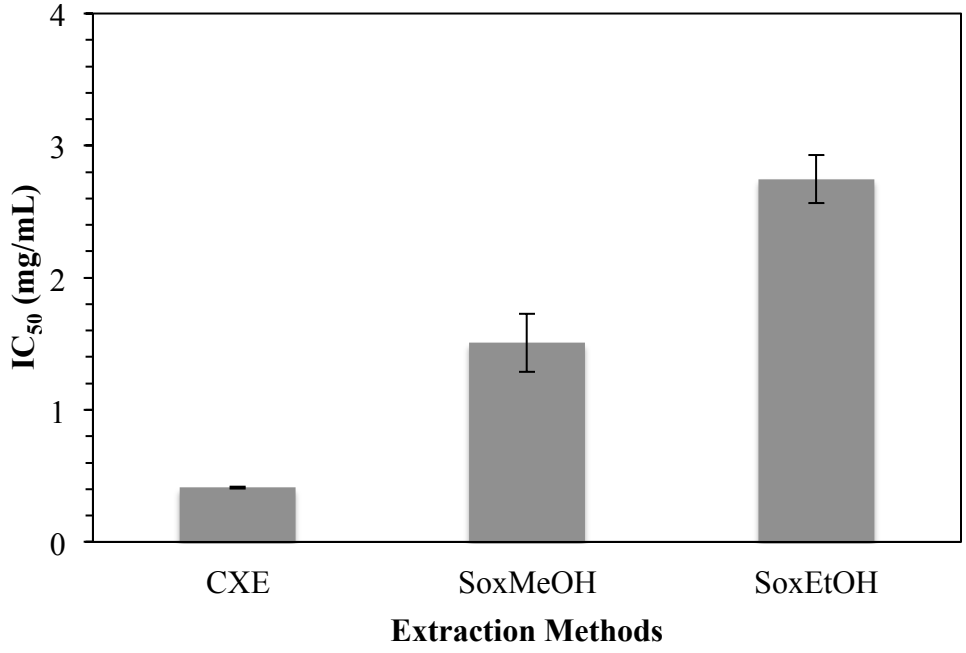


Figure 2.1.4 Comparison of total phenolic compounds content and antioxidant activity (IC_{50}) of CXE with those of Soxhlet extraction using methanol or ethanol

of solvent, this improve the solubility of phenolic compounds in extraction. Moreover, adding CO_2 compressed in ethanol improves the intermolecular interactions with the phenolic compounds in term of polar interaction and hydrogen bond interaction. It also decreases its static relative permittivity, surface tension and viscosity, which enhances the mass transfer rate in extraction [29]. On the other hand, CXE is continuous flow process. The continuous addition fresh solvent might help to maintain the high desorption and diffusion rate during extraction. Therefore, it could be conclude that the extraction efficiency with CXE is better than Soxhlet extraction. Al-Hamimi et al. [29] reported CXE extraction of cis-vebenol give faster extraction rate compared to solid liquid extraction. In addition, total phenolic compounds content (14.47 mg GAE/g of dried garlic husk) and antioxidant activity (1.13 mg/mL) was attained by using methanol as solvent in Soxhlet extraction were higher than those was obtained by using Soxhlet extraction using ethanol as solvent. This result can be explained that the methanol is more polar solvent than ethanol, thus the methanol could extract more phenolic compounds which are polar compounds compared to ethanol [33]. Similar result was obtained by Lapornik et al. [33]. The authors reported that methanol have better polar characteristic as solvent for extraction of phenolic compounds than ethanol. Compared to other research's work, CXE extraction method exhibited higher total phenol phenolic compounds content and similar antioxidant activity compared to the work of Kallel et al. [11]. However, methanol-water was used as a solvent in their study and it might be not suitable to apply for pharmaceutical or food industry.

2.1.3.5 HPLC analysis of phenolic compounds content

HPLC analysis was used to identification of majority phenolic compounds in the extract from garlic husk. Identification was accomplished by comparing their retention times with standards. Sample extracted from CXE extraction performed at temperature of 200 ^{o}C, pressure of 10 MPa, ethanol flow rate of 3 mL/min, CO_2 flow rate of 0.5 mL/min and extraction time of 2 h was analyzed for phenolic compound through HPLC-PDA (Figure 2.1.5). Peaks 3, 5, 6, 8, and 10 were identified as (3) Garlic acid, (5) Hydrobenzoic acid, (6) Caffeic acid, (8) P-Coumaric, and (10) Trans-ferulic acid. However, peak 1, 2, 4, 7, 9 were unknown. The chromatographic profile showed similar behavior to other researcher, although these authors have used other extraction methods. Ichikawa et al. found six phenolic compounds in garlic husk such as N-tran-Coumaroyloctopamine, N-trans-feruloyloctopamine, guaiacylglycerol-β-ferulic acid ether, guaiacylglycerol-β-

caffeic acid, trans-coumaric acid ether, and trans-ferulic acid. It was observed that those phenolic compounds showed major antioxidant activity in garlic husk [10]. Also, eleven phenolic compounds was identified by the work of Kallel et al. such as ferulic acid, garlic acid, hydrobenzoic acid, caffeic acid, p-coumaric acid, di-ferulic acid, chlorogenic acid, caffeic acid-O-glucoside, coumaroylquinic acid, coumaric acid-O-glucoside and caffeoylputrescine. The main phenolic compounds in the garlic husk were caffeic, p-coumaric, ferulic and di-ferulic acid [11].

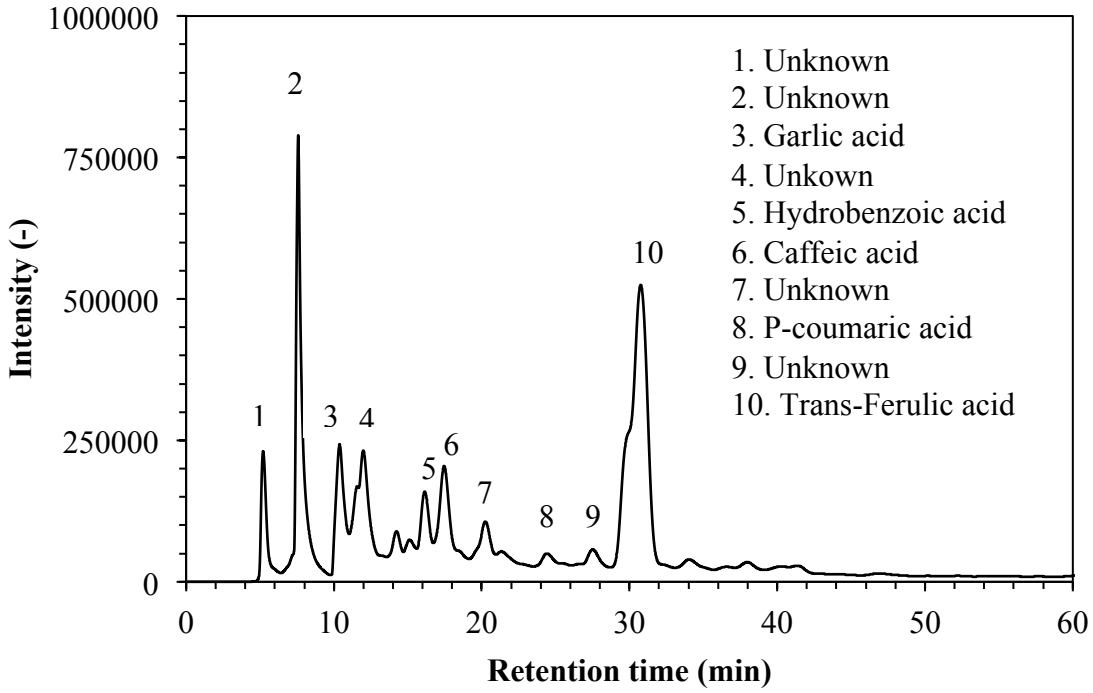


Figure 2.1.5 High performance liquid chromatography with diode array detection (HPLC-DAD) of CXE extract at temperature of 200 ^{o}C and CO_2 flow rate of 0.5 mL/min at 280 nm

2.1.3.6 Characterization of solid residues

In general, agriculture wastes are lignocelluosic materials, which compose of lignin, cellulose and hemicellulose. The structure and chemical composition of these wastes vary from species to species. Lignin is a complex polymer, which consists of different functional groups such as aliphatic hydroxyl group, phenolic group, and carboxylic group. It was noted that the degradation of cell-wall constituent might occur by decomposition reaction during CXE extraction. This may result in the

transformation of chemical structure of lignin. Therefore, the thermal degradation and the chemical structure of solid residue of garlic husk after CXE extraction was investigated by thermogravimetry (TG) and Fourier transform infrared (FTIR) spectroscopy spectrophotometer, respectively. Figure 2.1.6 shows the thermal degradation of the garlic husk and its residue also was investigated by TG.

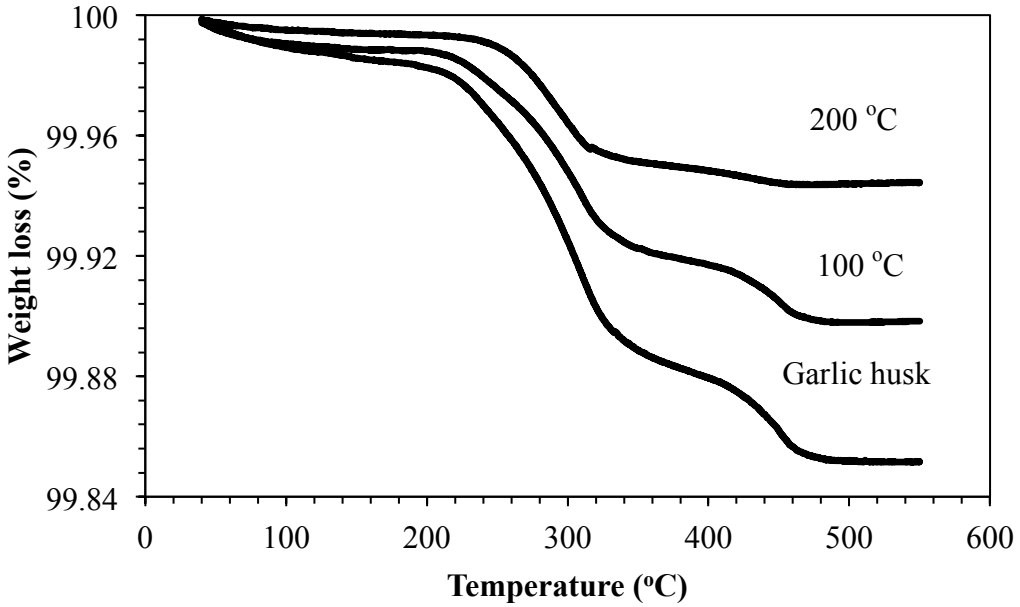


Figure 2.1.6 TG/DTA of garlic husk before and after CXE extraction

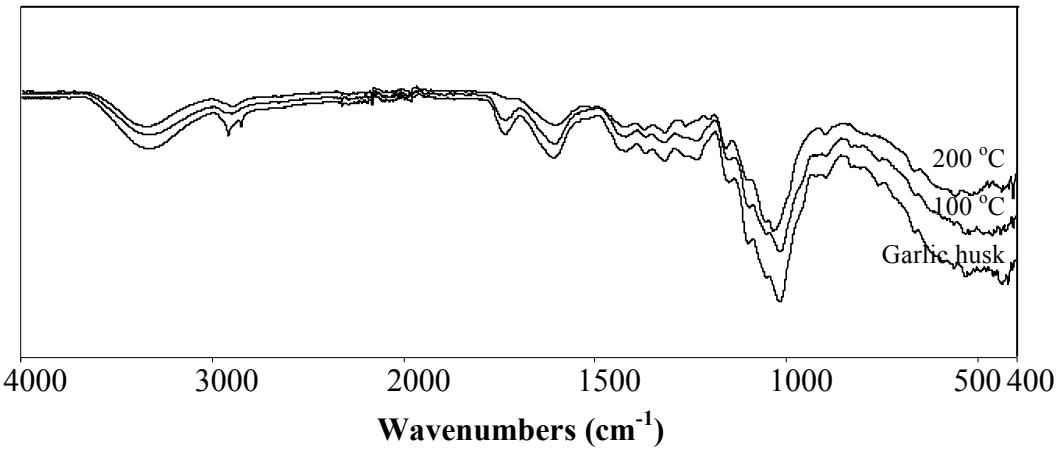


Figure 2.1.7 FTIR of garlic husk and its solid residue after CXE extraction

As seen in Figure 2.1.6, the degradation behavior of sample before and after CXE extraction was different depending on temperature and its composition (such as cellulose, hemicellulose, and lignin content) in garlic husk resulting from the extraction process. Figure 2.1.6 also illustrates that the thermal degradation behavior of garlic husk and its solid residue at 100 ^{o}C have similar trend. However, the thermal degradation of its solid residue at temperature of 200 ^{o}C behaved quite differently. This phenomenon maybe due to the large amount of hemicellulose, cellulose as well as lignin extracted by CXE and only amount of those compounds leaved in garlic husk with thermal behavior at high temperature. Figure 2.1.7 illustrates the FTIR spectra of garlic husk and garlic husk solid residues after CXE extraction at temperature of 100 and 200 ^{o}C. The typical functional group and the infrared signal with the possible compound were briefly described in Table 2.1.2. After CXE extraction, the peak spectrum in the region 3335.23-3316.38 cm^{-1} corresponding to O-H stretching was found in all sample solid residues. This intensity of this peak decreased with increasing temperature. It is possible that the alcoholic group was lost due to decomposition occurred in high temperature. The sharp peak spectra in the region 2860-2970 cm^{-1} indicated to stretching of C-H group. In this region, the peak of garlic husk was shaper than those of solid residue after CXE extraction. It is showed that the C-O bond in garlic husk was consumed during extraction. Moreover, the absorbance intensity due to C=O stretching (1700-1730 cm^{-1}) was found in garlic husk and its solid reside at temperature of 100 ^{o}C, but it was disappeared from solid residue at temperature of 200 ^{o}C. It can be concluded that ketone and carbonyl group were hydrolyzed when high temperature was applied in CXE system. The peak spectra in solid residue at the region of 1608.83 cm^{-1}, 1316.98 cm^{-1}, 1016.34 cm^{-1} corresponding to C=C stretching, C-H bending, C-O stretching and C-O deformation, respectively, was obtained in all solid residues. The intensity of absorbance in these regions declined when the temperature was augmented from 100 to 200 ^{o}C. It is denoted that aromatic skeletal mode, C-H and C-OH compounds were also hydrolyzed during CXE extraction. As observed in Figure 2.1.7, the FTIR spectra of garlic husk and its solid residues were similar and the intensity of some peaks was significantly different. It can be conclude that the functional group compositions of garlic husk have changed after CXE extraction at temperature of 100 and 200 ^{o}C.

Table 2.1.2. Main functional groups of major constituents of garlic husk

Wave number (cm^{-1})	Functional groups	Compounds
3600-3000	O-H stretching	Acid, methanol
2860-2970	C-H_n stretching	Alkyl, aliphatic, aromatic
1700-1730, 1510-1560	C=O stretching	Ketone and carbonyl
1632	C=C	Benzene stretching ring
1613, 1450	C=C stretching	Aromatic skeletal mode
1470-1430	O-CH_3	Methoxyl-O-CH_3
1440-1400	O-H bending	Acid
1402	C-H bending	-
1232	C-O-C stretching	Aryl-alkyl ether linkage
1215	C-O stretching	Phenol
1170,1082	C-O-C stretching vibration	Pyranose ring skeletal
1108	O-H association	C-OH
1060	C-O stretching and C-O deformation	C-OH (ethanol)
700-900	C-H	Aromatic hydrogen
700-650	C-C stretching	-

2.1.4 Conclusion

Phenolic compounds and antioxidant activity were successfully extracted from garlic husk using CXE method. For the effect of operational parameters in CXE process, CO_2 flow rate had smaller effect on the amount of total phenolic compounds and antioxidant activity than temperature. The high amount of total phenolic compounds of 56.26 mg GAE/g of dried garlic husk and antioxidant activity (IC_{50}) of 0.41 mg/mL was achieved at temperature of 200 ^{o}C and CO_2 flow rate of 0.5 mL/min. Compared to the pressurized ethanol, the result shows that CXE not only improves the extraction yield, but also increases the total phenolic compounds and antioxidant activity because CXE have ability to enhance the extraction efficiency compared to organic solvent by reducing viscosity, decreasing the interval interfacial tension, and increasing diffusivity of solvent. Moreover, the amount of total phenolic compounds and antioxidant activity extracted with CXE surpassed those obtained with Soxhlet extraction. It can be concluded that CXE is an efficient method to extract phenolic compounds and antioxidant activity from garlic husk.

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2.2 Extraction of curcumin from turmeric using ultrasound assisted supercritical carbon dioxide

2.2.1 Introduction

Turmeric, belongs to the Zingiberaceae family, is widely cultivated in tropical part of South and Southeast Asia [1-3]. Turmeric was used as a condiment, food preservative and herbal medicine in Asia countries for many centuries [4, 5]. The yellow color of turmeric is attributed to a group of phenolic compounds known as curcuminoids. The components of curcuminoids are curcumin, demethoxycurcumin and bisdemethoxycurcumin. Curcumin is the major compounds in curcuminoids, insoluble in water and contributes 2-5% of total weight of turmeric [1, 3]. Previous investigations of turmeric indicated that curcumin exhibited a wide variety of biological activities, including anti-inflammatory, anti-bacterial, anti-carcinogenic, antifungal, and antimicrobial activities. Those studies have also shown that curcumin can be used in diversity application such as food product and medical industries for treatment of various diseases like Alzheimer, AIDS and cancer [2, 3, 5].

In conventional method, curcumin was extracted from turmeric by solid-liquid extraction, sonication, and Soxhlet extraction. However, these procedures required long extraction time and large amount of solvent. Moreover, these conventional methods also can provide poor efficiency and toxicity in final product [4, 6, 7].

Supercritical carbon dioxide (SC-CO_2) extraction was found to be a good alternative to conventional extraction process because carbon dioxide (CO_2) is non-flammable, non-explosive, inexpensive, odorless, colorless, non-toxic and clean solvent. Furthermore, CO_2 has low critical temperature and pressure (T_c=31 ^oC, P_c=7.38 MPa). The optimal condition can be achieved by changing the temperature and pressure. On the other hand, CO_2 has low dielectric constant, which lead to low yield and recovery of the target solute from the material. Thus, cosolvent (water, methanol and ethanol) is added in order to improve the polarity of solvent in extraction process [8, 9].

Ultrasonic assisted supercritical carbon dioxide (USC-CO_2) extraction has been reported in extraction of functional compounds from natural plant. This technique is a potential alternative to the conventional extraction methods as well as enhancing the efficacy of SC-CO_2 extraction. The combine extraction process triggers micro-stirring and solvent cavitation in extraction process, which result in some physical change in extraction system, including cracked or damaged the cell

wall and an increased in diffusion and interfacial turbulence of solvent. This causes faster rate of the mass transfer of solvent into material, thus it enhances the solubility of solute in the extraction [9-12]. Recently, USC-CO_2 has been widely studied for the extraction of valuable compounds from natural products. Yang and Wei [9] obtained higher yield of oleanolic acid and ursolic acid from *Hedyotis corymbosa* than those obtained by using conventional SC-CO_2 extraction and heat-reflux extraction. However, almost no report conducted to examine the use of USC-CO_2 for the determination of curcumin from turmeric.

The aim of the present work is to investigate the extraction of curcumin from turmeric using USC-CO_2 compared to SC-CO_2. The influence of extraction time on extraction yield and curcumin content was also analyzed. In addition, the characterization of solid residue after extraction was evaluated using thermogravimetry (TG), Fourier transform infrared spectroscopy (FTIR) and scanning electron microscope (SEM) analysis.

2.2.2 Materials and methods

2.2.2.1 Materials and chemicals

Turmeric was purchase from Tochimoto Tenkaido Co., LTD. (Japan). The dried sample was ground into fine particle size and passed through the analytical sieve screen to obtain the particle size of 0.5 mm. After that, the ground sample was packed and stored in refrigerator at 4^{o}C.

Carbon dioxide (purity 99%) was supplied by Sogo Kariya Sanso, Inc. Japan. Ethanol (99.5 %) and hexane (99.5 %) were supplied by Wako Chemical (Japan). The Acetonitrile was obtained from Kanto Chemical Co. Inc. (Japan). Pure distilled water was used in all experiments.

2.2.2.2 Ultrasound assisted carbon dioxide extraction

A schematic of the USC-CO_2 extraction system is given in Figure 2.2.1. The apparatus consists of a chiller, two high-pressure pumps (PU-2080, JASCO, Japan), a heater, an extractor (15 ml, SUS 316, GL Sciences, Japan), water bath, ultrasound supplier (W-118, HONDA, Japan), and back-pressure regulator (BPR; AKICO, Japan). First, 1 g of the ground sample was loaded into extractor where the top and the bottom were filled with glass beads. Water bath was used, pre-heated at the desired temperature prior to placement of extractor inside the water bath. When the water bath reached the set temperature, the extractor was immediately positioned

horizontally inside the water bath. After that, the CO_2 was introduced in extractor until the desired pressure was obtained. Then, CO_2 valve was closed. After 10 min, the carbon dioxide with 10% ethanol was introduced into extractor at flow rate of 0.3 mL/min. At the same time, ultrasound (45 kHz) was also applied to the extractor and extraction time commenced. After extraction, the solvent was evaporated and the extract was dissolved in ethanol and kept in a refrigerator at 4 ^{o}C until analysis. Each experiment was performed in duplicated or triplicated.

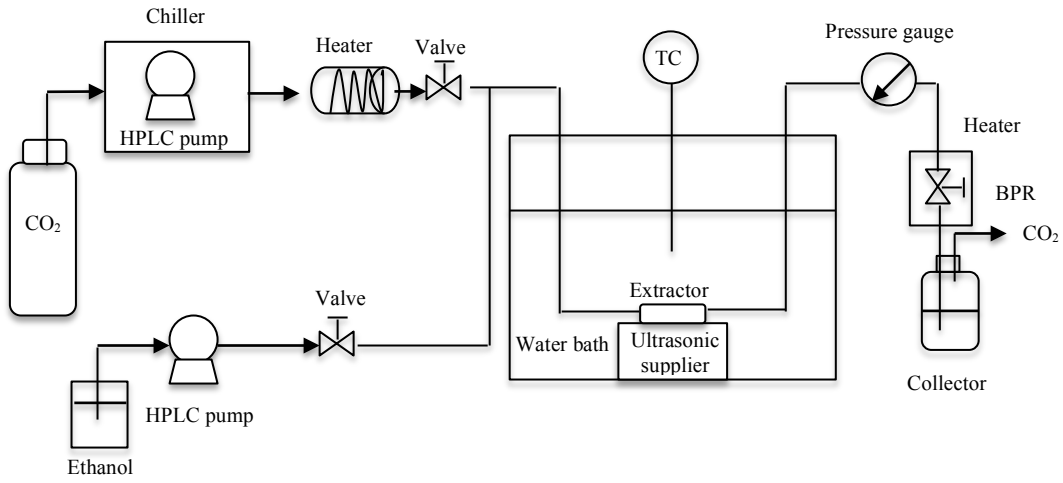


Figure 2.2.1 Schematic diagram of the USC-CO_2 extraction system

The extraction yield was measured by taking 1 mL of extract for the calculation. The extract was dried in an electric oven at 50 ^{o}C until the constant weight obtained. After that, it was cooled in a desiccator at room temperature before weighing to constant weight. The extraction yield was calculate by following Equation (1):

$$\%\text{Extraction Yield} = \frac{W_{extract} \times V_{total\ of\ extract}}{W_{raw\ material}} \times 100 \qquad (1)$$

Where,

$W_{extract}$: Weight of extract in 1 mL of sample after oven drying, g/mL.

$V_{total\ of\ extract}$: Total volume of extract, mL.

$W_{raw\ material}$: Weight of raw material used in extraction, g.

2.2.2.3 HPLC analysis

The curcurmin was identified and determined using the method described by Kiamahalleh et al. [3]. The analysis was conducted using high performance liquid chromatography (HPLC) with diode array detector (SPD-M10AVP, Shimadzu, Japan). The sample was filtered with a 0.2 μm disposable membrane filter and 20 μL was injected. The chromatographic was carried out using an Inertsil ODS-3 column (5 μm, 4.6 mm ×250 mm, GL Science, Japan) at 40 ^{o}C. The mobile phase consisted of a mixture of acetonitrile and water (90:10 v/v). The flow rate was 1 mL/min and the separation was performed at a detection wavelength of 420 nm.

2.2.3 Results and discussion

2.2.3.1 Effect of ultrasound on extraction yield and curcumin content

The effect of ultrasound on extraction yield and curcumin content from turmeric was determined at temperature of 50^{o}C, pressure of 25 MPa, CO_2 flow rate of 3 mL/min with 10% of cosolvent at various extraction times. The experimental extraction curves for extraction yield and curcumin content were exhibited in Figure 2.2.2 (a) and (b), respectively. It is shown that the extraction yield obtained with ultrasound significantly increased from 6.28 %w/w to 7.51 %w/w with increasing extraction time from 30 to 60 min. Then, the extraction yield was remained nearly constant when extraction was prolonged more than 60 min, indicating that optimum time for extraction yield with ultrasound is around 60 min. However, the extraction yield without ultrasound gradually increased from 1.89 %w/w to 6.83 %w/w when extraction time increased from 30 to 90 min. After that, the extraction yield was almost stable. Curcumin is hydrophobic phenolic compounds, which have many medical properties such as anti-inflammatory, anti-bacterial, anti-carcinogenic, antifungal, and antimicrobial activities. As shown in Figure 2.2.2 (b), curcumin content obtained with ultrasound augmented dramatically from 0.35 %w/w to 1.69 %w/w with increasing extraction time of 30 to 90 min. When extraction time increased higher than 90 min, the curcumin content was remained constant. In contrast, the curcumin obtained without ultrasound increased gradually from 0.28 %w/w to 1.57 % w/w when the extraction increased from 30 to 120 min. This could be explained that when the extraction time was shortened, it was observed that the extraction yield and curcumin content were low because of a limiting time for

solvation to take place. This merited a low solubility of solutes in solvent, hence reducing the extraction yield as well as curcumin content. In contrast, the appropriated extraction time gave positive effect on extraction yield and curcumin content because it provided sufficient extraction for soluble compounds to dissolve in solvent [13, 14]. Based on those figures, it was also found that the extraction yield and curcumin content with USC-CO_2 was larger and faster extraction than those with SC-CO_2. This was supported by the fact that ultrasound can produce micro-stirring and solvent cavitation which can improve the extraction yield and curcumin from turmeric due to the damage of cell wall of plant and an increase of mass transfer of solvent when ultrasound was applied in SC-CO_2 extraction [9, 10]. Kawamura et al. [10] found that the extraction yield of luteolin and apigenin from the leaves of *Perilla frutescans* were enhanced using ultrasound assisted SC-CO_2 extraction. The augmentation of extraction yield is because of cavitation produced by ultrasound. Ultrasound produces the cavitation bubbles, which drive to release the component content in cell and enhance the mass transfer, resulting improve the extraction yield.

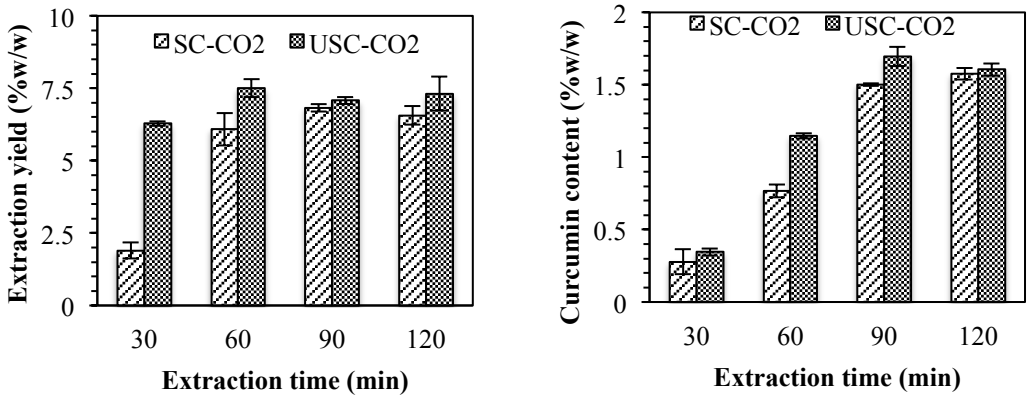


Figure 2.2.2 Effect of ultrasound on (a) extraction yield and (b) curcumin content

2.2.3.2 TG analysis

Figure 2.2.3 illustrates the influence of temperature on turmeric and its residues after SC-CO_2 and USC-CO_2 extraction. The trend of weight losses at all temperatures occurred in turmeric were similar to that observed form its solid residue after SC-CO_2 and USC-CO_2 extraction. However, the lowest change in

mass was observed for solid residue after USC-CO_2 extraction. The difference in amount of cell wall components indicates an explanation for these differences. Figure 2.2.3 also shows that the decomposition of turmeric and solid residues after USC-CO_2 and SC-CO_2 extraction was exhibited in three stages. Stage I, ranging from approximately from 40 to 220 ^oC, released water and volatile compounds presented in turmeric Stage II, the weight losses occurred at 220 to 300 ^oC because of the degradation of carbon hydrate in lignin sample. These degraded composition are converted to volatile compounds. In stage III, within temperature of 300 to 460 ^oC, the weight losses was happened due to degradation of volatile product derived from lignin and the formation gaseous product was taken out from material.

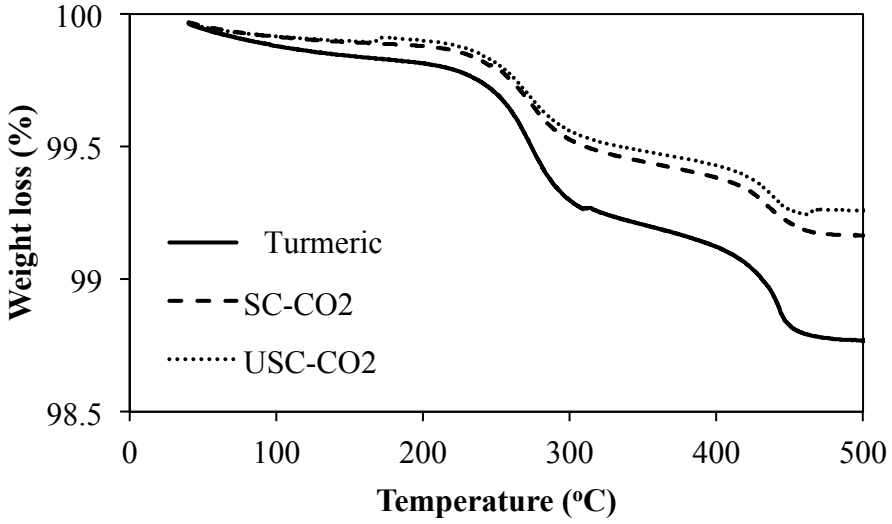


Figure 2.2.3 TG of turmeric and its solid residues after SC-CO_2 and USC-CO_2 extraction

2.2.3.3 FTIR analysis

Fourier transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of absorption or emission of solid in order to study physic-chemical properties of lignocelluloses material. The FTIR spectra of untreated and treated turmeric by SC-CO_2 and USC-CO_2 is shown in Figure 2.2.4. The result shows that the peak spectrum in the region 3600-3000 cm^{-1} relations with O-H stretching was found in each spectrum. The intensity of these peaks decreased in treated turmeric residue. The treated residue with USC-CO_2 have smaller peak than that of SC-CO_2. The peak spectra of solid residue in the region between 2860-2970

cm^{-1} are due to C-H_n stretching was obtained. This suggested that the residue also have aromatic structure. The peak at 1625 cm^{-1} representing C=C. This indicates the benzene stretching ring still found in the solid residue. The peak at region 1601 cm^{-1} reflecting C=C stretching was disappeared in solid residue both in SC-CO_2 and USC-CO_2. This implied that the aromatic skeletal mode was removed from solid residue. The sharp peak in region at 1510-1560 and 1375 cm^{-1} assigned to C=O stretching and C-H bending, respectively, were found in all solid residue after extraction with low intensity. The peak at 1470-1430, 1280, 1206 cm^{-1} are ascribed to O-CH_3, C-O-C stretching, C-O stretching were disappear in the solid residues. The peak at 1152 and 1023 cm^{-1} are due to C-O-C stretching vibration and C-O deformation, respectively. The intensity of this peak declined in all solid residues. The peaks at the region between 700-900 cm^{-1} indicating to C-H (aromatic-hydrogen) was vanished in treated residue with SC-CO_2 and USC-CO_2. These results shows that the addition of ultrasound to SC-CO_2 could breakdown cell wall and removed some functional group from cell wall, thus it improve the efficiently of extraction process.

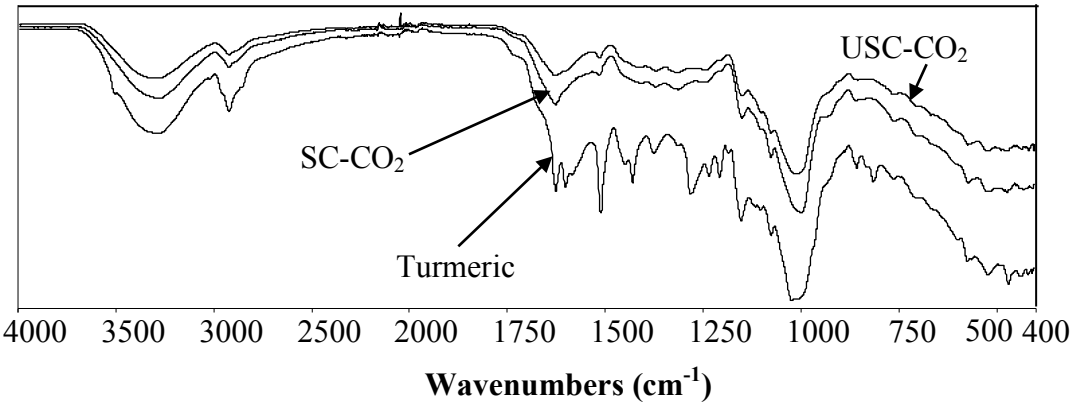
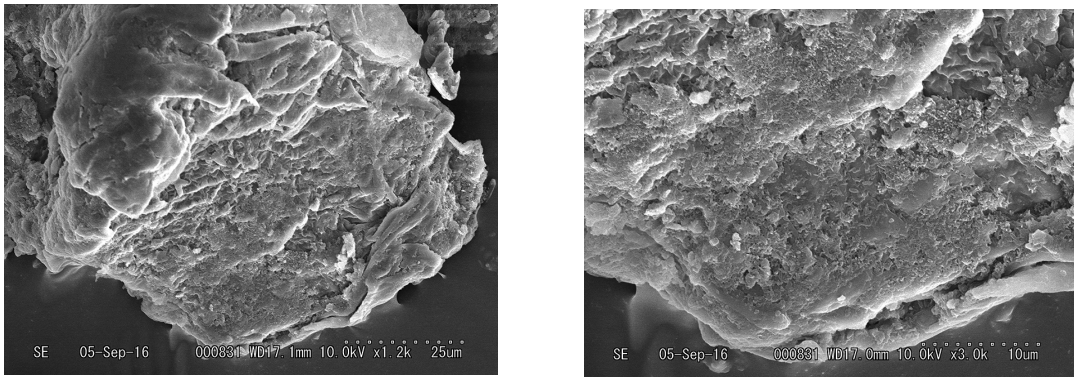


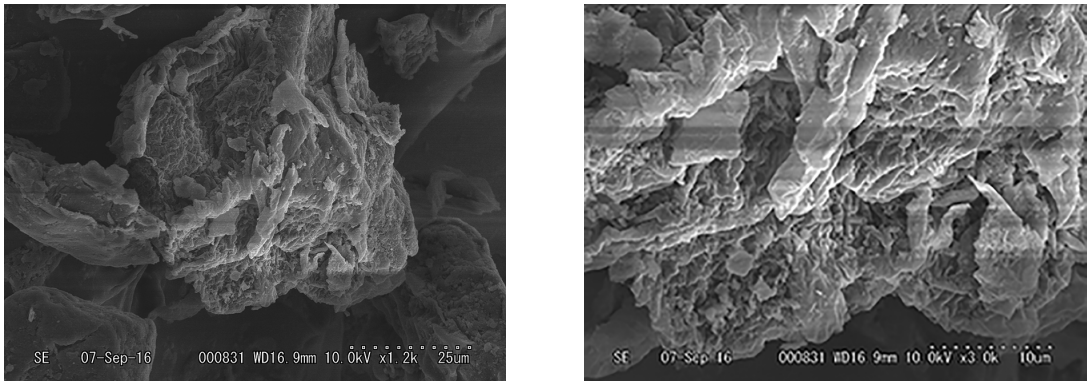
Figure 2.2.4 FTIR of *Curcuma longa* L. and its residues after SC-CO_2 and USC-CO_2 extraction

2.2.3.4 Morphological analysis

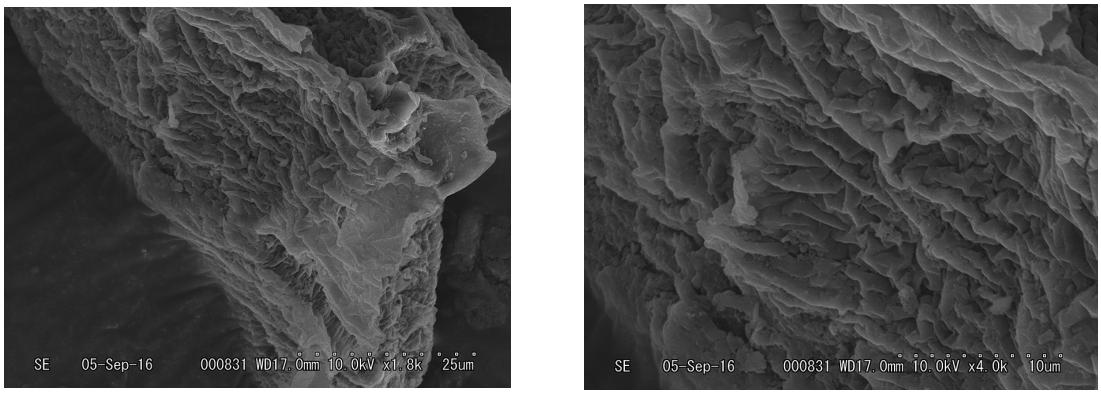
The effect of ultrasound on the physical structure of *Curcuma longa* L. before and after treatment with SC-CO_2 and USC-CO_2 was characterized through the scanning electron microscopy (SEM) as shown in Figure 2.2.5.



(a) Turmeric



(b) Solid residue after SC-CO_2



(c) Solid residue after USC-CO_2

Figure 2.2.5 SEM images of *Curcuma longa* L. and its solid residue after SC-CO_2 and USC-CO_2 extraction

Based on these figures, it could be known clearly that surface morphology of turmeric after USC-CO_2 extraction was different from those of raw material as well as SC-CO_2 extraction. Before extraction, the surface morphology of turmeric seemed hard and had no any pores on surface. After SC-CO_2 extraction, the pores could clearly found in some part of solid residue. In case of USC-CO_2, the pore is even more and appear everywhere on surface of solid residue. Moreover, the size of pore on surface morphology became bigger compared to the pore on solid surface after SC-CO_2 extraction. It is notable that sample extracted by SC-CO_2 alone could only open pore in some part of material's surface. However, ultrasound power assisted SC-CO_2 could cause the surface morphology change and open all pore on surface of plant material significantly. It is possible that cavitation bubble caused by ultrasound could decompose the surface of plant material and helps to remove the target compounds from the internal part of cell wall during extraction process. Furthermore, the ultrasound could enhance diffusivity of solvent into material, which lead to texture shrunken. This condition facilitated the compounds to dissolve in solvent, which caused high extraction yield in USC-CO_2 [10; 15]. Similar to this study, Reátegui et al. [15] reported that ultrasound change the surface of the blackberry bagasse, enhancing the accessibility of extracted compounds to solvent. Thus, high amount of antioxidant compounds from blackberry (*Rubus* sp.) bagasse was obtained in USC-CO_2 extraction.

2.2.4 Conclusion

Curcumin was successfully extracted from turmeric using USC-CO_2 extraction. The extraction was carried out at 50 ^{o}C, 25 MPa, CO_2 flow rate of 3 mL/min with 10% cosolvent at various extraction time. The highest curcumin content of 1.69% w/w was obtained at 90 min. The extraction yield and curcumin content obtained with USC-CO_2 were greater and faster extraction time compared than those obtained with SC-CO_2. Based on the result from TG, FT-IR and SEM analysis, USC-CO_2 has greatly power to remove the solute from material compared to SC-CO_2 alone.

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2.3 Comparison of conventional and ultrasound assisted supercritical carbon dioxide extraction of curcumin from turmeric (*Curcuma longa* L.)

2.3.1 Introduction

Turmeric (*Curcuma longa* L.) contains curcuminoids, which are antioxidant substances with many biological properties, including anticancer, antibacterial and antiviral activities [1-3]. Among curcuminoids, curcumin is the most representative. Recent studies show that curcumin is currently used in dietary spice, foods, textile coloring and medicines due to its beneficial properties, such as anti-inflammatory, anti-bacterial, antifungal, antimicrobial, anti-carcinogenic activities, also contributing to the treat multiple myeloma, Alzheimer's, psoriasis, myelodysplastic syndrome and anti-human immunodeficiency virus (Anti-HIV) cycle replication. Because of the curcumin benefits, there is a great interest in developing new technology to extract such compounds with high purity [4-6].

Extraction of functional compounds from raw material is a promising area in the food and pharmaceutical industries. On the other hand, the quality of extracts from raw material strongly depends on the employed extraction method [7]. Various conventional extraction methods were used to extract functional compounds from plant materials such as Soxhlet extraction, maceration, and hydrodistillation. However, these methods can cause the degradation of labile compounds, require long extraction time, and leave the tract of toxic solvent in final product [8].

Supercritical fluid extraction is one of green and clean technology for food and pharmaceutical industries because it can maximize the recovery and quality of the extraction and minimize the energy cost due to faster and more selective compared to conventional methods. Moreover, the solubility can be improved by adding small amount of polar solvent. The use of cosolvent not only enhances the solubility, but also helps to disrupt the interaction of solute and solid material, resulting the increased in the transport of solute from solid pores to its surface [9, 10].

Adding ultrasound might improve the supercritical fluid extraction because ultrasound can generate the cavitation, resulting the breaking cell wall of plant materials. In this case, it facilitates the solvent penetrate in material and improve the mass transfer, thus increasing the extraction rate and yield [11]. Recently, ultrasound assisted supercritical fluid extraction has been applied to extract functional compounds from different raw materials such as blackberry bagasse [10], malagueta paper [11], adlay seed [12], *Scutellaria barbata* D. Don [13], grape marc [14],

cumbaru almond [15], and, clove [16]. However, no reports have been found in the literatures that consider the Ultrasound assisted supercritical carbon dioxide (USC-CO_2) extraction of curcumin from turmeric.
Therefore, the objective of this study was to investigate the extraction of curcumin from turmeric using USC-CO_2. The effect of temperature, pressure, extraction time, CO_2 flow rate and percentage of cosolvent on extraction yield and curcumin content were also evaluated. Moreover, the performance of USC-CO_2 was check by comparison of extraction yield and curcumin content with conventional method.

2.3.2. Materials and methods

2.3.2.1 Materials and chemicals

Turmeric with 12% moisture content was bought from Tochimoto Tenkaido Co., LTD., Osaka, Japan. 99.9% purity of CO_2 was obtained from Sogo Kariya Sanso, Inc., Nagoya, Japan. 99.5% ethanol, 99.5% hexane were supplied by Wako Chemical, Japan. The acetonitrile was purchased at Kanto Chemical Co. Inc., Tokyo, Japan.

2.3.2.2 Methods

The flow chart of extraction and analysis is shown in Figure 2.3.1.

2.3.2.3 Ultrasound assisted supercritical carbon dioxide (USC-CO_2) extraction

The USC-CO_2 unit is composed of a 15 mL stainless steel cell (SUS 316, GL Sciences, Japan), which supports pressure up to 25 MPa. The unit is equipped with a chiller to control temperature of CO_2, two high-pressure pumps, (PU-2080, JASCO, Japan) with capacity up to 10 mL/min, a electric heater to control the temperature of cell, water bath, ultrasound supplier (W-118, Honda, Japan), pressure gauge and back-pressure regulator (BPR; AKICO, Japan) to keep the desired pressure constant. The schematic diagram of USC-CO_2 extraction is illustrated in Figure 2.3.2.
The extractor was filled with 1 g of ground sample and void volume was completed with glass beads on the top and bottom. The extraction process was carried out at various values of the operating condition: temperature (40-60 ^oC), pressure (15-25 MPa), CO_2 flow rate (2-4 mL/min), and colsolvent (10-20%). In order to study the dynamic of extraction process, extract time was performed at 30-120 min.

Ultrasound power of 45 kHz and 600 W (Ultrasonic multi cleaner W-118, Honda, Japan) was applied during extraction. After extraction, the extract was collected and solvent was evaporated using rotary evaporator (SB-1200, Eyela Co. Ltd, Japan). Lastly, the extract was dissolved in ethanol and kept in refrigerator for further analysis and solid residues were dried and used for scanning electron microscope (SEM), thermogravimetry (TG), and Fourier transform infrared spectroscopy (FTIR) analysis. Each experiment was conducted in duplicates or triplicates.

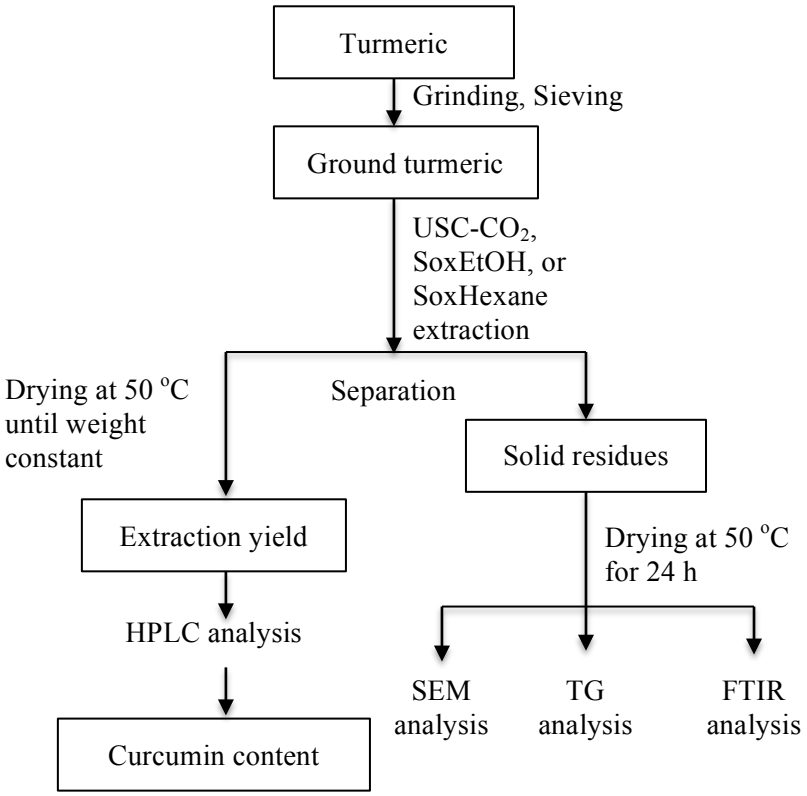


Figure 2.3.1 The flow chart of extraction and analysis

The extraction yield was measured by taking 1 mL of extract for the calculation. The extract was dried in an electric oven at 50 °C until the constant weight obtained. After that, it was cooled in a desiccator at room temperature before weighing to constant weight. The extraction yield was calculate by following Eq. (1):

$$\%\text{Extraction Yield} = \frac{W_{extract} \times W_{total\ of\ extract}}{W_{raw\ material}} \times 100 \qquad (1)$$

Where,
$W_{extract}$: Weight of extract in 1 mL of sample after oven drying, g/mL.
$V_{total\ of\ extract}$: Total volume of extract, mL.
$W_{raw\ material}$: Weight of raw material used in extraction, g.

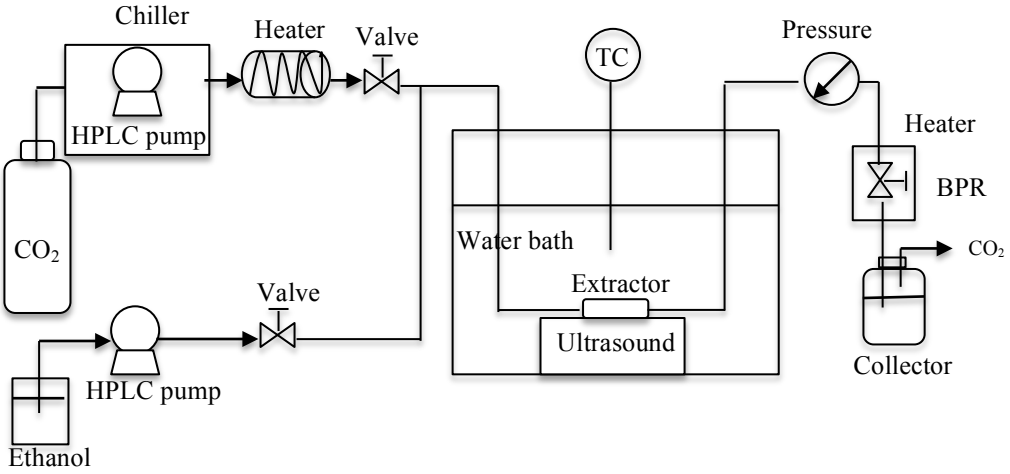


Figure 2.3.2 Schematic diagram of USC-CO_2 extraction system

2.3.2.4 Conventional extraction technique

The Soxhlet extraction was also investigated and the results obtained were compared to the result of the USC-CO_2 exaction process. Briefly, 5 g of sample was placed in a timble and extracted with 250 mL of ethanol or hexane for 6 h. After that, the solvent was separated from extract by using a rotary evaporator.

2.3.2.5 HPLC analysis

The qualitative and quantitative analysis of curcumin in extract was performed by high performance liquid chromatography (HPLC) with diode array detector (SPD-M10AVP, Shimadzu, Japan), according to method described by Kiamahalleh *et al.* 2016 [6]. The sample was filtered with a 0.2 μm disposable membrane filter and 20 μL was injected. HPLC analysis was carried out using an Inertsil ODS-3 column (GL Sciences, Japan), with length of 250 mm, internal diameter of 4.6 mm and thickness of 5 μm. The operating temperature of the chromatographic separation was maintained at 40 ^{o}C. The mixture of acetonitrile and water (90:10 v/v) was used as

mobile phase with a flow rate of 1 mL/min and the separation was performed at a detection wavelength of 420 nm.

2.3.3 Results and discussion

2.3.3.1 Effect of extraction time

The effect of extraction time on extraction yield and curcumin content from turmeric was determined at temperature of 50 ^{o}C, pressure of 25 MPa, CO_2 flow rate of 3 mL/min with 10% of cosolvent. The experimental extraction curved for extraction yield and curcumin content are exhibited in Figure 2.3.3. The results shows in Figure 2.3.3 indicated that the extraction time for the high extraction yield and curcumin content obtained by USC-CO_2 were 60 min and 90 min, respectively. The ultrasound can produce micro-stirring and solvent cavitation which can improve the extraction yield and curcumin content from turmeric due to the damage of cell wall of plant and an increase of mass transfer of solvent when ultrasound was applied in SC-CO_2 extraction [17, 18]. Kawamura et al. 2016 [18] also found that the extraction yield of luteolin and apigenin from the leaves of *Perilla frutescans* were enhanced using ultrasound assisted SC-CO_2 extraction.

2.3.3.2 Effect of temperature

To evaluate the effect of temperature on extraction yield and curcumin content, the extraction experiments were conducted at pressure of 25 MPa, CO_2 flow rate of 3 mL/min with 10% ethanol as cosolvent, and extraction time of 90 min. Figure 2.3.4 shows that extraction yield and curcumin content increased gradually with increasing temperature of 40 to 50 ^{o}C. When temperature increased from 50 to 60 ^{o}C, the extraction yield and curcumin content was declined. At temperature of 50 ^{o}C, the extraction yield and curcumin content were 7.17% w/w, 1.69% w/w, respectively. It is notable to point that the temperature could effect on the density of SC-CO_2, the vapor pressure of solute and the desorption of the solute form material. Increasing temperature increased vapor pressure of solute from the material. Thus, the solubility of extraction yield and curcumin content increased [19, 20]. However, when higher temperature was applied in extraction, extraction yield and curcumin content in extract may decrease because temperature might be continue to increase by the high intensity of ultrasound. Also, the oxidation of some compounds may occur due to the small amount of moisture content in material [21, 22]. Moreover,

increasing temperature increased the volatility and diffusion of the solute and reduced the density of CO_2. As the result, the solubility of curcumin decreased [17].

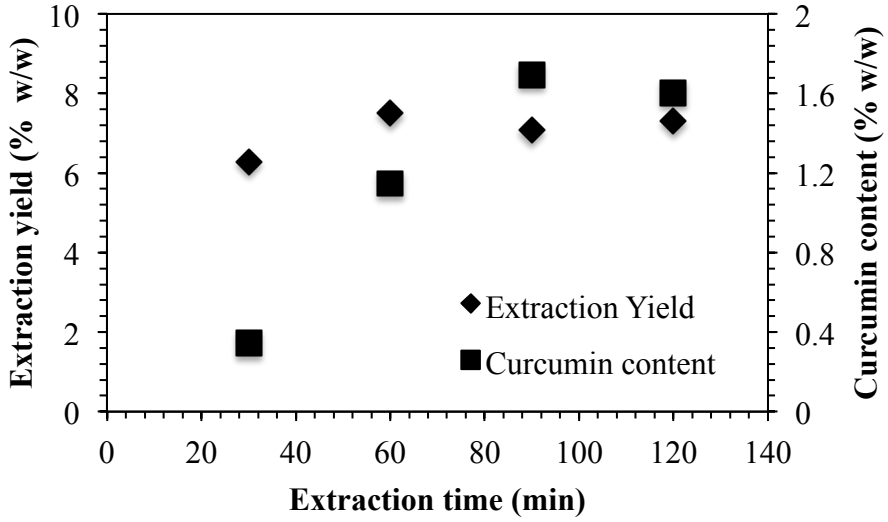


Figure 2.3.3 Effect of extraction time on extraction yield and curcumin content at 50 ^{o}C, 25 MPa, CO_2 flow rate of 3 mL/min, and 10% cosolvent

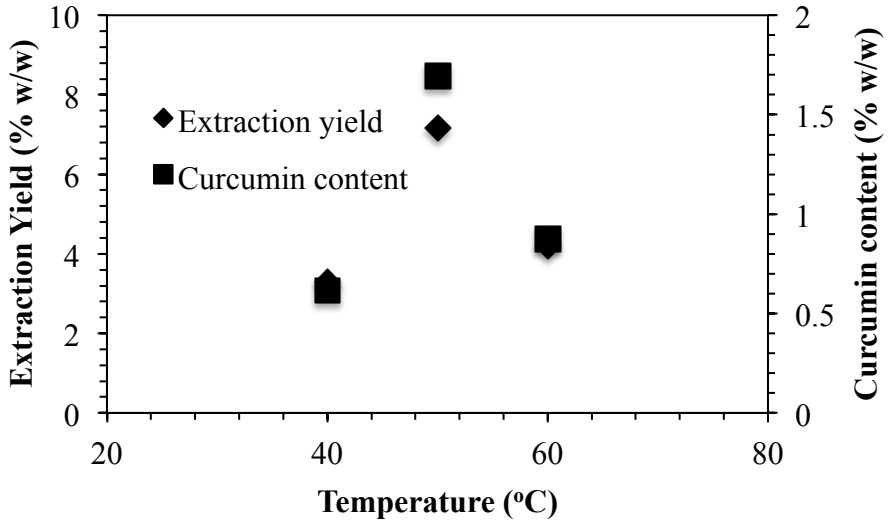


Figure 2.3.4 Effect of temperature on extraction yield and curcumin content at 25 MPa, CO_2 flow rate of 3 mL/min, 10% cosolvent and extraction time of 90 min

2.3.3.3 Effect of pressure

The effect of pressure on extraction yield and curcumin content is shown in Figure 2.3.5. The extraction was conducted at temperature of 50 ^{o}C, CO_2 flow rate of 3 mL/min with 10% ethanol as cosolvent, and extraction time of 90 min. The result shows that the low extraction yield and curcumin content were obtained at pressure of 15 and 20 MPa. When pressure increased from 20 to 25 MPa, the extraction yield and curcumin content dramatically increased. The high extraction yield and curcumin were obtained at 25 MPa. It can be explained that increasing pressure increased the density of CO_2 and decreased the distance between the molecules. This increased the interaction of the solute and CO_2. Therefore, the solubility of curcumin in USC-CO_2 increase and this enhance the efficiency of extraction [17, 18]. Similar to this study, Santos *et al*. 2016 [15], who extracted cumbaru oil from the cumbaru almond using ultrasonic assisted SC-CO_2 extraction, reported that the global yield of extraction increased with increasing pressure because increasing pressure increased the density of CO_2.

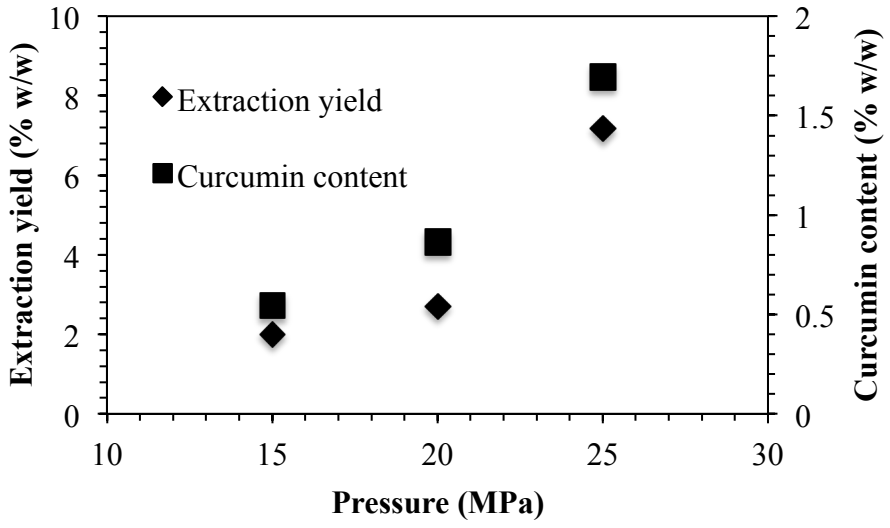


Figure 2.3.5. Effect of pressure on extraction yield and curcumin content 50 ^{o}C, CO_2 flow rate of 3 mL/min, 10% cosolvent, and extraction time of 90 min

2.3.3.4 Effect of CO_2 flow rate

Figure 2.3.6 shows the effect of CO_2 flow rate on extraction yield and curcumin content. The extraction experiments were done at temperature of 50 ^{o}C, pressure of

25 MPa, extraction time of 90 min, and cosolvent percentage constant with varying the CO_2 flow rate at three levels (2, 3, and 4 mL/min). According to Fig. 6, the extraction yield and curcumin content dramatically augmented when the CO_2 flow rate increase from 2 to 3 mL/min. Then, the extraction yield and curcumin content was slightly declined with increasing CO_2 flow rate from 3 to 4 mL/min. It is possible that increasing CO_2 flow rate increased the amount of CO_2 molecule per unit volume that enters the extractor. Therefore, it increased the interaction of CO_2 and solute. This could increase amount of extracted solute in extraction process. However, an increase in CO_2 flow rate also provides the negative effect to the dissolution of compounds because the CO_2 flow through the sample matrix by channeling when high CO_2 flow rate was applied. This caused the shorter contact time between the solute and solvent. As a consequence, the solubility of desired compounds was decreased [13, 17].

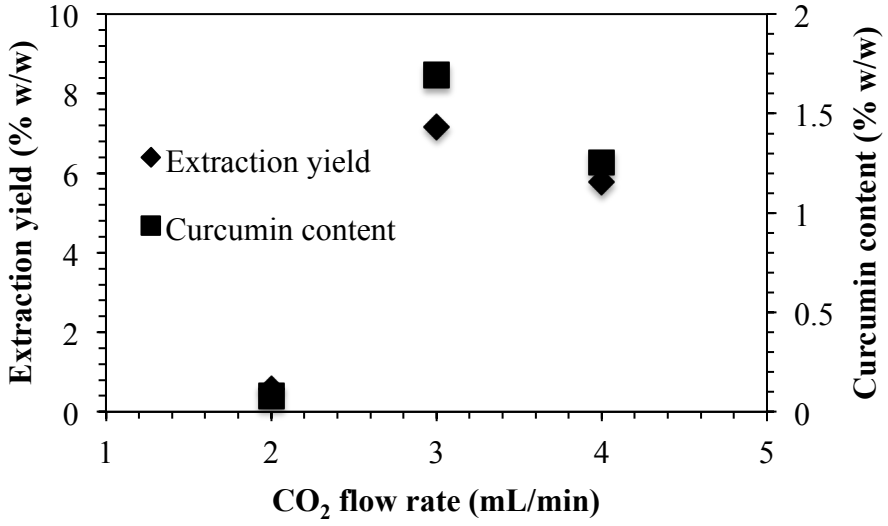


Figure 2.3.6 Effect of CO_2 flow rate on extraction yield and curcumin content 50 ^oC, 25 MPa, 10% cosolvent, and extraction time of 90 min

2.3.3.5 Effect of cosolvent

The effect of cosolvent on extraction yield and curcumin content is presented in Figure 2.3.7. The extraction experiments were conducted at 50 ^oC, pressure of 25 MPa, extraction of 90 min, CO_2 flow rate of 3 mL/min, and varying the cosolvent at three level (10, 15, 20% v/v). Figure 2.3.7 shows that the extraction yield and curcumin content seem constant with increasing of cosolvent from 10 to 20% v/v. In this study the preferred percentage of cosolvent is 10% v/v. This can be explained that addition of cosolvent increased the polarity of solvent and the contact time between solvent and solute. Thus, it could improve the solubility of polar

compounds in extraction and enhance the extraction yield. Moreover, cosolvent can make matrix swell, this may allow the supercritical fluid penetrate into the matrix, resulting in increased the diffusion of solute in extraction [13, 23, 24].

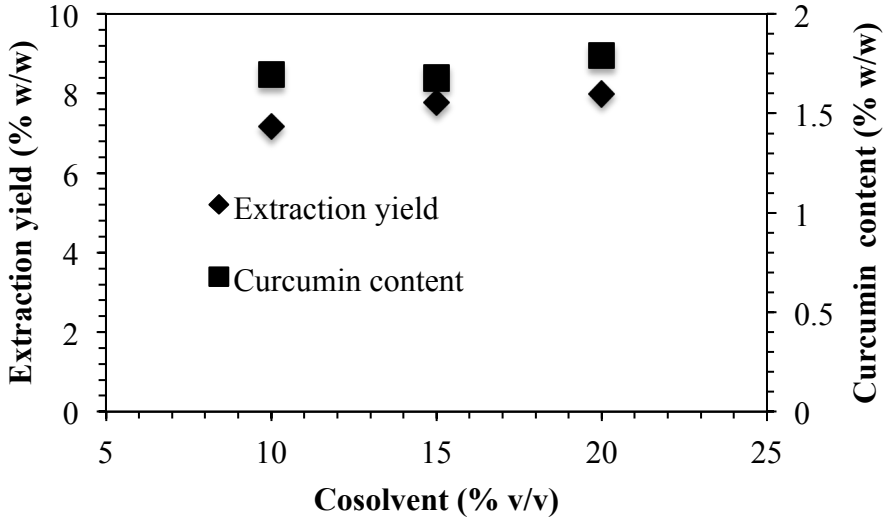


Figure 2.3.7 Effect of percentage of cosolvent on extraction yield and curcumin content 50 ^{o}C, 25 MPa, CO_2 flow rate of 3 mL/min, and extraction time of 90 min

2.3.3.6 Comparison with Soxhlet extraction

Based on experiment result above, the high extraction yield and curcumin content from turmeric using USC-CO_2 was at temperature of 50 ^{o}C, pressure of 25 MPa, extraction time of 90 min, flow rate of CO_2 of 3 mL/min with 10% cosolvent. The extraction yield and curcumin content obtained with this condition (USC-CO_2) were compared with those obtained with Soxhlet extraction using ethanol (SoxEtOH) and hexane (SoxHexane). Figure 2.3.8 (a) and (b) show that the extraction yield obtained with USC-CO_2 was comparable with that obtained with SoxEtOH. The low extraction yields was obtained by using SoxHexane. On the other hand, the curcumin content in extract of USC-CO_2 were greater than those obtained with SoxEtOH and SoxHexane. These results demonstrate that USC-CO_2 have the potential to extract curcumin from turmeric because ultrasound occurred in extraction helped to disrupt the cell water and increase mass transfer, thus reduce the amounted of solvent needed, temperature, and extraction time.

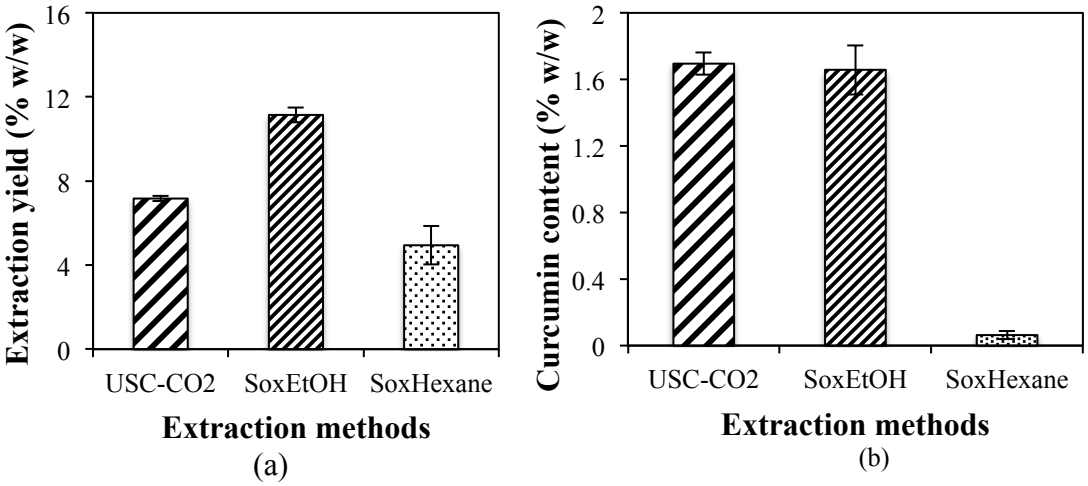
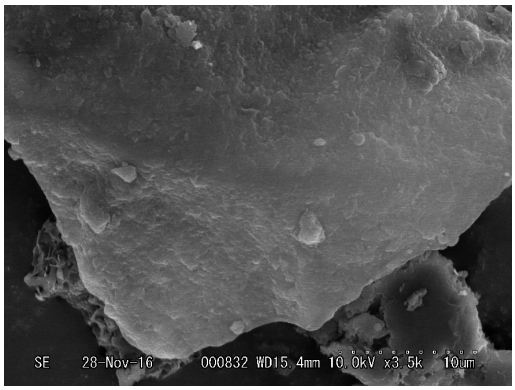


Figure 2.3.8 Comparison of extraction yield and curcumin content with USC-CO_2, SoxEtOH, and SoxHexane

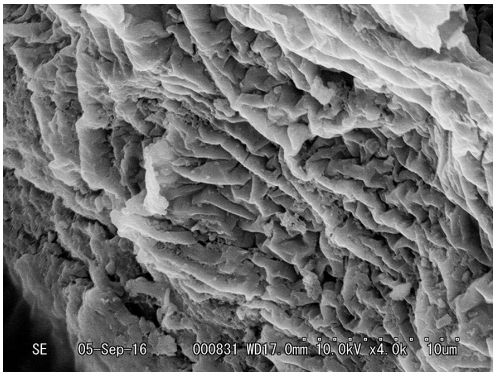
2.3.3.7 Extraction mechanism

In order to investigate the effect of USC-CO_2 on solid matrix, surface morphology of turmeric was analyzed by using SEM. Fig. 9 show the images obtained by SEM on turmeric before extraction and after USC-CO_2, SoxEtOH, and SoxHexane extraction. Based on Figure 2.3.9, it is clearly seen that sample that the surface of raw material seems hard and does not present any cracks or pores. However, sample after USC-CO_2, SoxEtOH, SoxHexane extraction had the presence of cracks and pores on its surface. These micrographs showed that the surface morphology of turmeric was changed after extraction. It is notable that there were some cracks and pores appears on surface morphology of solid reside after SoxHexane, but the pore size on this surface was smaller than that on the surface of the residue after USC-CO_2 and SoxEtOH extraction. Even though USC-CO_2 consumed less amount of solvent and shorter extraction time compared to SoxEtOH, the surface morphology of residue after USC-CO_2 and SoxEtOH extraction was similar because the ultrasound produced the cavitation, which disturbed the cell wall and open up the pore, leading to release of solute from material [10, 11]. The degradation of material in extraction process is complicated due to the component joined to fiber by physical and chemical adsorption or embodied in cell of vegetable by unknown action. Ji et al. 2006 [25] explained the mechanism of ultrasound-assisted extraction as follow: first, the ultrasound waves propagated into the plant particle, resulting in breaking of some chemical bond of solute and plant material. Second, the high-speed micro-jet

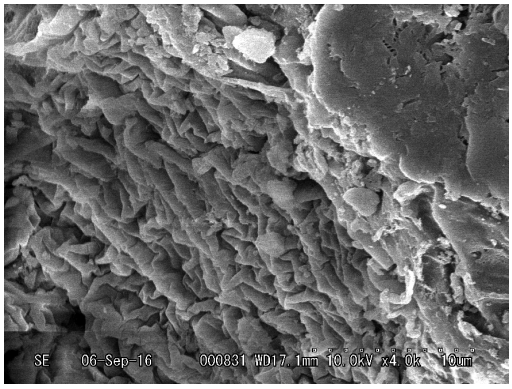
and high-pressure shock wave were generated by the collapse of cavitation, leading to make a hollow and erosion of external surface as well as the porous wall within the particle. Third, the turbulence wave created by ultrasound near the interfacial surface and porous within the particle increased mass transfer both external and internal surface. The mechanism of extraction of curcumin from turmeric by USC-CO_2 maybe similar to mechanism proposed by Ji et al. First, the ultrasound creates the cavitation that generates bubbles. Then, the tiny bubble go through the particle and break down the chemical bond of curcumin with fiber in turmeric.



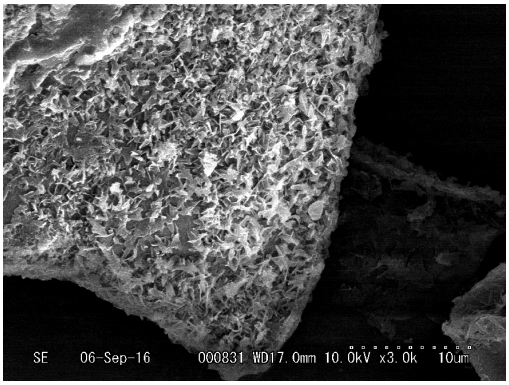
(a) Turmeric before extraction



(b) Solid residue after USC-CO_2 extraction



(c) Solid residue after SoxEtOH extraction



(d) Solid residue after SoxHexane extraction

Figure 2.3.9 SEM images of turmeric and its solid residues after USC-CO_2, SoxEtOH, and SoxHexane extraction.

The production of cavitation near surface of particle can facilitate swelling and hydration. This causes expansion in the pore of cell wall, leading to enhance mass transfer of curcumin from turmeric to solvent by diffusion and osmotic process [26]. Furthermore, ultrasound can disrupt the external and internal surface of turmeric which favors the washing out of the disrupt cell content, leading to increase the accessibility of the curcumin to solvent [10]. The mass transfer of fluid into the cavitation surface also can be accelerated by addition of ultrasound in extraction process [11]. By these reactions, the curcumin was extracted from turmeric.

2.3.3.8 Characterization of residues

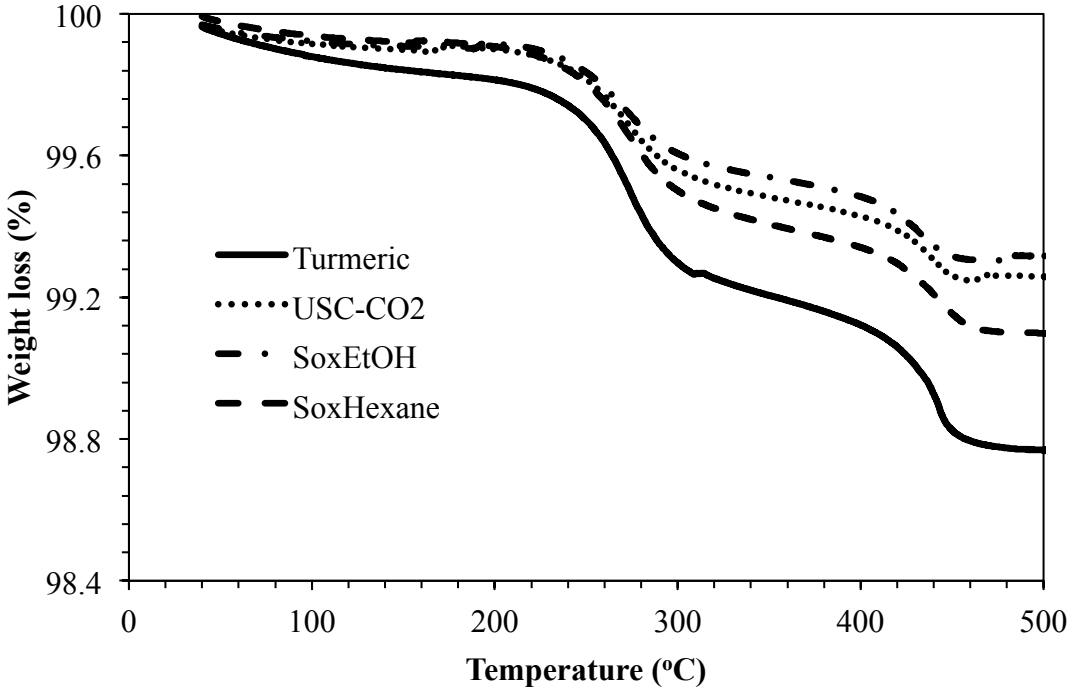


Figure 2.3.10 TG of turmeric and its solid residues after USC-CO_2, SoxEtOH, and SoxHexane extraction.

The turmeric particle contains cellulose, hemicellulose, lignin, protein, lipid, starch and aromatic substances [27]. After extraction, the physical and chemical component of turmeric might change. Therefore, the properties of residue after extraction will be useful to understand about the effect of ultrasound in SC-CO_2 extraction. Characterization of residues was determined by using TG and FTIR. Figure 2.3.10 shows the TG curves of turmeric and its residue after USC-CO_2, SoxEtOH, and SoxHexane extraction. The decomposition of turmeric and its residue

were divided into three stages. First stage is ranging from 40 to 120 ^{o}C. At this stage, the water and volatile compounds in turmeric were evaporated. The stage II, ranging approximately from 180 to 350 ^{o}C, the weight loss was observed due to degradation carbohydrates in lignin, converting into volatile gases such as CO, CO_2, CH_4. The stage III, ranging from 350 to 500 ^{o}C, the weight losses also happened because the degradation of volatile compounds derived from lignin (Phenolic, alcohols, aldehyde acid and formation of gaseous products) was removed from sample [28, 29]. Other than that, it also could be seen that the TG curves of solid residue after USC-CO_2 was higher than that of the turmeric and its residue after SoxHexane extraction, but lower than that of the solid residue after SoxEtOH extraction. This can explained that the solid residue after USC-CO_2 had the small amount of cell wall components because some of components was extracted and degraded by ultrasound during USC-CO_2 extraction. Figure 2.3.11 illustrates the FTIR of turmeric and its residue after USC-CO_2, SoxEtOH and SoxHexane extraction. The list of typical functional group and IR signal with possible compounds was given in work of Kodama et al. 2015 [30]. Based on this figure, it is clearly understand that the peak spectrum in the region 3600-3000 cm^{-1} represents O-H stretching in cellulose was found in all samples. The intensity of these peaks decreased in solid residues after extraction. The prominent band at 2860-2970 cm^{-1} is attributable to the C-H_n stretching was obtained. This suggested that the residue also have aromatic structure. The peak at 1625 cm^{-1} representing C=C, indicating the benzene stretching ring still found in the solid residue. The peak at region 1601 cm^{-1} reflecting C=C stretching was found in turmeric and its residue after SoxHexane, however it was disappeared in the residue after USC-CO_2 and SoxEtOH extraction. This implied that the aromatic skeletal mode was taken away from solid residue after USC-CO_2 and SoxEtOH extraction. The sharp peak in region at 1510-1560 and 1375 cm^{-1} indicated to C=O stretching and C-H bending, respectively, were found in all solid residue after extraction with low intensity. The peak at 1470-1430, 1280, 1206 cm^{-1} are ascribed to O-CH_3, C-O-C stretching, C-O stretching presented in the solid residue after SoxHexane, but disappeared in the solid residues after USC-CO_2 and SoxEtOH extraction. The peak at 1152 and 1023 cm^{-1} are due to C-O-C stretching vibration and C-O deformation, respectively. The intensity of this peak fell down in all solid residues. The peaks at the region between 700-900 cm^{-1} indicating to C-H was appeared in turmeric and its solid residue after SoxHexane, however it was vanished in treated residue with USC-CO_2 and SoxEtOH extraction, indicating that the aromatic-hydrogen was removed from material after USC-CO_2 and SoxEtOH extraction. Based on current results, it can be

concluded that USC-CO_2 and SoxEtOH have power to remove more functional group from turmeric compared to SoxHexane extraction. Addition of ultrasound to SC-CO_2 could breakdown cell wall and improved the mass transfer. This could remove some functional groups from cell wall, thus improved the extraction yield.

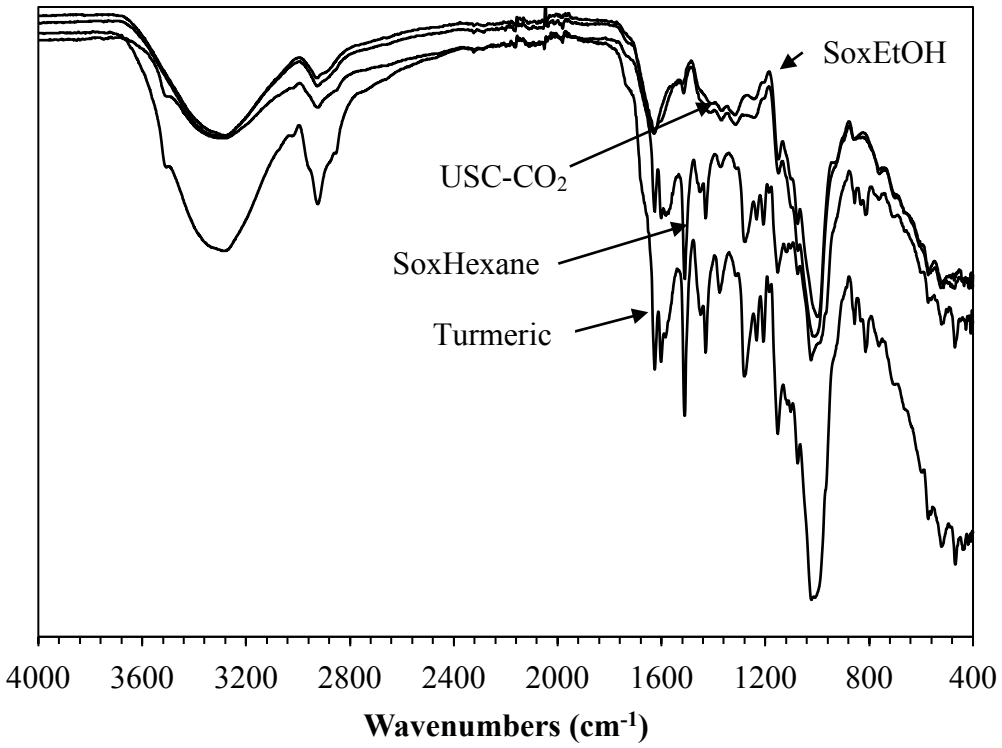


Figure 2.3.11 FTIR of turmeric and its solid residues after USC-CO_2, SoxEtOH, and SoxHexane extraction.

2.3.4 Conclusion

USC-CO_2 has been used to extract the curcumin from turmeric. The extraction was performed at 40-60 ^oC, 15-25 MPa, CO_2 flow rate of 2-4 mL/min, cosolvent of 10-20% and extraction time of 30-120 min. The temperature, pressure and CO_2 flow rate and extraction had significant effect on extraction yield and curcumin content while the cosolvent had small effect on extraction yield and curcumin content. The high extraction yield of 7.17% w/w and curcumin of 1.69% w/w as achieved at 50 ^oC, 25 MPa, CO_2 flow rate of 3 mL/min with 10% cosolvent, and extraction time of 90 min. Based on results of FTIR, the intensity of some bands was decreased and

some bands were disappeared, indicating that some components in turmeric were removed during USC-CO_2 extraction process. Compared to conventional method, USC-CO_2 could provide higher selectivity in extract. Addition of ultrasound in SC-CO_2 extraction process could improve both quality and quantity curcumin in extract from turmeric in a short extraction time.

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2.4 Extraction of phytochemical constituents of Khmer medicinal plants using supercritical carbon dioxide integrated hydrothermal process

2.4.1 Introduction

Khmer medicinal plants have been employed to treat human diseases for nearly a thousand years. The medicinal plants have gained interested among people due to their good efficacy of therapeutic performance and safety [1]. Several studies showed that the important phytochemical constituents of medicinal plants are phenolic acid, flavonoids, tannins, and alkaloid, which have diverse bioactivities such as antioxidant, anti-inflammatory, anticancer, anti-diabetic, antiallergic, and antimicrobial [2, 3]. Therefore, they can be used in food, cosmetic, and pharmaceutical applications.

The amount of phytochemical constituent of plant depends on many factors such as type of plants, cultivation area, climatic conditions, vegetation phase and genetic modifications [4]. This work aim to investigate the phytochemical constituents of five Khmer medical plants grown in Cambodia including *Dialium cochinchinense* Pierre*, Cinnamomum cambodianum* Lecomte, *Gardenia angkorensis* Pitard, *Dialium cochinchinense* Pierre, *Cananga latifolia* (Hook. f. & Thomson) Finet & Gagnep, and *Oroxylum indicum* (L.) Kurz bark.

Previous studies indicated that these plants possessed medicinal properties such as antiallergic [3], anti-diabetic [5], antifungal [6], antirheumatic, antimicrobial, antioxidant, and anticancerous [2]. The phytochemical constituents from medicinal plants have usually extracted by distillation or organic solvent, which make loss of volatile compounds and low extraction efficiency, require long extraction time and leave the toxic solvent residue in final product [7, 8].

Alternatively, an emerging combination of supercritical carbon dioxide (SC-CO_2) and hydrothermal process has been reported in the extraction of valuable bioactive compounds from natural plants. The technique of combining the two, a novel approach of intensifying a process, is a potential alternative to the conventional extraction method as well as enhancing the efficacy of SC-CO_2 extraction. Coupling SC-CO_2 and Hydrothermal triggers the reaction between carbon dioxide and water forming carbonic acid that lowers the pH of water. Some of valuable compounds in the medicinal plants with affinity to acidic medium may increase its solubility in water [9, 10].

This work is aimed to extract the phytochemical constituents of Khmer medicinal plants using supercritical carbon dioxide integrated with hydrothermal (SC-CO_2-H) process.

2.4.2 Materials and methods

2.4.2.1 Materials and chemicals

Khmer medicinal plants (*Dialium cochinchinense* Pierre*, Cinnamomum cambodianum* Lecomte, *Gardenia angkorensis* Pitard, *Dialium cochinchinense* Pierre, *Cananga latifolia* (Hook. f. & Thomson) Finet & Gagnep, and *Oroxylum indicum* (L.) Kurz bark) were purchased from local market in Phnom Penh, Cambodia. 99.9% purity of CO_2 was obtained from Sogo Kariya Sanso, Inc., Nagoya, Japan. 99.5% methanol, 99.5% hexane, and sodium carbonate were supplied by Wako Chemical, Japan. Folin–Ciocalteu agent solution and 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma–Aldrich. Pure distilled water was used in all experiments.

2.4.2.2 Supercritical carbon dioxide integrated hydrothermal extraction

SC-CO_2-H extraction system is presented schematically in Figure 2.4.1. The experiment apparatus comprises of chiller (CCA-1111, EYELA, Japan), two high-pressure pumps (PU-2080, JASCO, Japan; Isco syringe pump 260D, Teledyne, Isco, USA), CO_2-water mixer (Swagelok, USA), extractor (Thar Tech, Inc., USA), electric heater (ESPEC ST-110, Japan), back-pressure regulator (BPRs; AKICO, Japan), and a filter (SS-2TF-7, Swagelok, USA). First, one gram of ground sample was placed in the10 mL stainless-steel extractor. The void space of the extractor was filled with the glass beads whereas the plant material was packed in the central area of extractor. The extractor was placed in an electric oven. Water was introduced to extractor by means of HPLC pump until 10 MPa. The pressure was kept constant by mean of back-pressure regulator. Water was then heated up to desired temperature of 150 °C [11]. When the extractor reached desired temperature and pressure, water was introduced in the extractor at flow rate of 2 mL/min at the pump condition. At the same time, CO_2 was also applied to extractor at flow rate of 0.15 mL/min and extraction process started. The extraction time for each experiment was 4 h. After extraction, water was evaporated and extract was dissolved in methanol and kept in

refrigerator at temperature of 4 °C until analysis was carried out. Each experiment was conducted in duplicates.

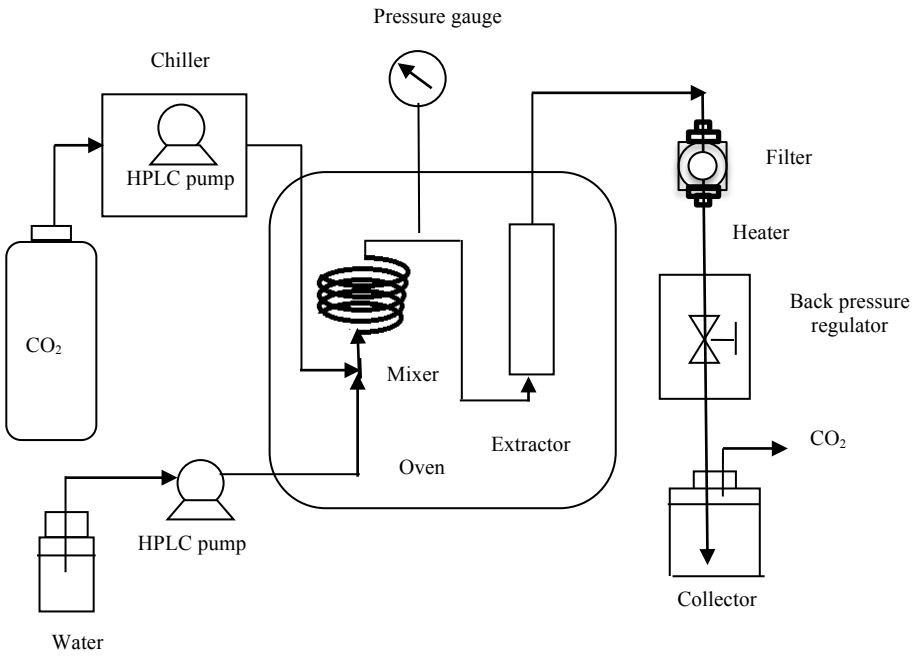

Figure 2.4.1 Schematic diagram of SC-CO_2-H process

2.4.2.3 Soxhlet extraction

Soxhlet was performed as conventional method technique in order to compare the yield and composition of the extracts with those of SC-CO_2-H. 5 g of Khmer medicinal plant was loaded in the thimble and extracted with 250 mL of methanol for 24 h in Soxhlet apparatus. Thereafter, the solvent was evaporated using rotary evaporator (SB-1200, EYELA, Japan).

2.4.2.4 Hot water extraction

1 g of Khmer medicinal plant and 20 mL of distilled water was placed in the extractor. After that, the extractor was heated at temperature of 100 ^{o}C for 6 h. After that, the solution was centrifuged by using centrifugetor (CN-1040, HSIANGTAI, Taiwan) in order to separate the extract and residues.

2.4.2.5 Total phenolic compounds content assay

Total phenolic compounds content (TPC) of the extracts were determined using Folin–Ciocalteu method, in accordance to method of Chhouk et al. [12]. 1 mL of sample were mixed with 1 mL of Folin–Ciocalteu reagent. After 3 min, 1 mL of 7.5% (w/w) sodium carbonate was added and the volume was made up to 10 ml with distilled water. The reaction was incubated for 2 h in dark place at room temperature. After that, the total phenolic contents were evaluated by measuring at 750 nm absorbance using UV–VIS spectrophotometer (V-550, Jasco, Japan).

2.4.2.6 DPPH free radical scavenging activity assay

DPPH radical scavenging activity was quantified by the method described in Chhouk et al. [12]. DPPH was measured by UV/vis spectrophotometer. The extract was diluted in methanol in the ranged of 0.01–3 mg/mL. The sample was mixed with 2.7 mL of DPPH solution (6×10^{-5} M in methanol). After being thoroughly stirred, the mixture was kept in dark place at room temperature. Sixty min later the absorbance of the mixture was measured at 517 nm wavelength.
The percentage of DPPH radical scavenging activity was calculated by the following equation:

$$\%\text{DPPH Scavenging activity} = \left[\frac{(A_{517\ \text{of control}} - A_{517\ \text{of sample}}}{A_{517\ \text{of control}}}\right] \times 100 \quad (1)$$

where,
$A_{517\ \text{of control}}$ is the absorbance of control reaction (containing the entire reagent except the sample). $A_{517\ \text{of sample}}$ is the absorbance of the reaction of sample and reagents.
IC_{50} is the concentration of the sample that is necessary for 50% scavenging of DPPH free radical. Its value was determined by the plot of %DPPH scavenging activity against the concentration.

2.4.2.7 GC-MS analysis

The identification of phytochemical constituents of medicinal plants was performed using gas chromatographic and mass spectrometry (GC-MS), equipped with an Agilent 7890A GC system connected to an Agilent 5975C mass spectrometer, and a phenyl arylene capillary column (HP-5MS; 30 m × 0.25 mm i.d,

Agilent Technology Tokyo Ltd, Japan). The chromatograph was programmed from 40 ^{o}C to 320 ^{o}C at a rate of 5 ^{o}C/min. The injection volume of sample extract was 1 μL. Helium was used as the carrier with the flow rate of 24 mL/min. The scan range of mass number was 69-502 m/z.

2.4.3 Results and discussion

2.4.3.1 Total phenolic compounds content

In this study, the total phenolic compounds content in SC-CO_2-H extract of Khmer medicinal plants were measured using the Folin-Ciocalteu method and also shown in Figure 2.4.2. The total phenolic compounds content ranged from 80.11 to 16.34 mg GAE/g of dried sample. It has been noted that *Dialium cochinchinense* Pierre had highest phenolic compounds content (80.11mg GAE/g of dried sample), followed by *Cinnamomum cambodianum* Lecomte (67.52 mg GAE/g of dried sample), *Gardenia angkorensis* Pitard (28.17 mg GAE/g of dried sample), *Cananga latifolia* (Hook.f. & Thomson) Finet & Gagnep (22.55 mg GAE/g) of dried sample. The lowest phenolic content was *Oroxylum indicum* (L.) Kurz (16.34 mg GAE/g of dried sample). Total phenolic compounds are phenolic acid, flavonoid, and tannins, which show wide biological activities such as anticancer, anti-inflammatory, anti-microbial, anti-tumor and anti-allergic. The amounts of total phenolic content in plants depend on some biological factors (genotype, organ, ontogeny, and edaphic) and environmental (temperature, salinity, water stress, light intensity, nutrient supply, growing condition, UV radiation) conditions [13, 14].

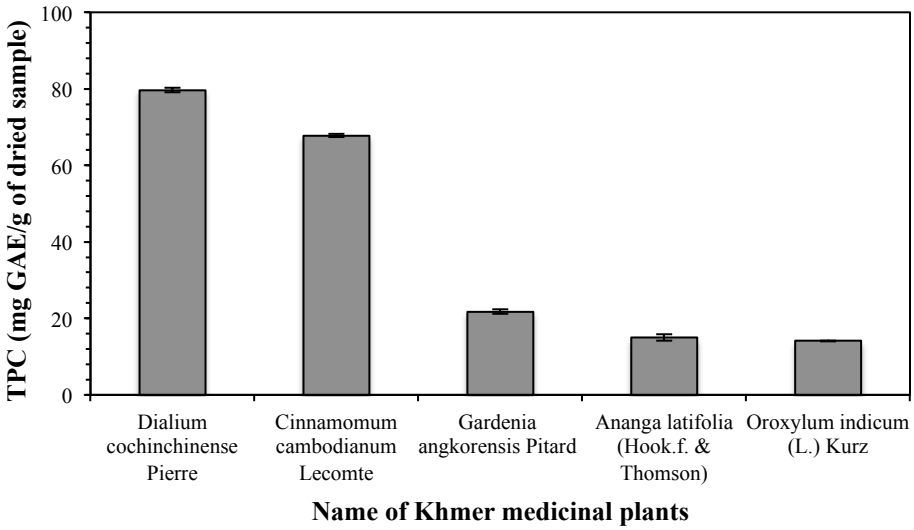

Figure 2.4.2 Total phenolic compounds content in Khmer medicinal plants

2.4.3.2 Antioxidant activity

Antioxidant activity in SC-CO_2-H extract of Khmer medicinal plants is presented in Figure 2.4.3. The IC_{50} value ranged from 0.06 to 0.92 mg/mL. It was observed that the highest IC_{50} value was found in *Oroxylum indicum* (L.) Kurz extract, while the lowest value of IC_{50} was observed in the *Dialium cochinchinense* Pierre extract. The lowest IC_{50} value indicates the highest antioxidant activity. This result suggested that there is relationship of total phenolic content and antioxidant activity in the extract obtained by SC-CO_2-H process. This may be due to the fact that the phenolic components (phenolic acid, flavonoids, and phenolic diterpenes) are main factor effect on antioxidant activity. The antioxidant activity of phenolic compounds is mainly because of their redox properties. These properties make them act as reducing as agents, hydrogen donors, and singlet oxygen quenchers. They may also process a metal chelating potential [15, 16].

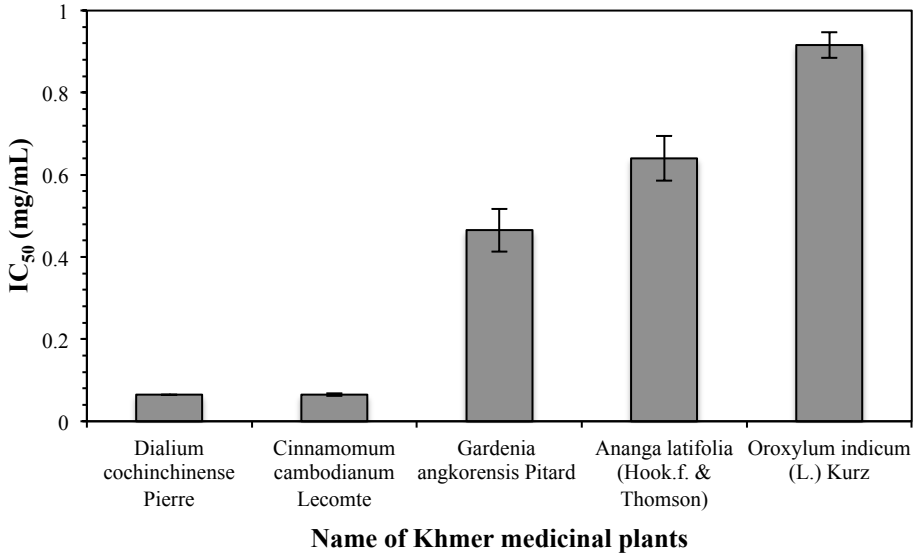

Figure 2.4.3 IC_{50} value in Khmer medicinal plants

2.4.3.3 Comparison with conventional methods

The conventional methods (Soxhlet and hot water extraction) were carried out to compare the results with that of SC-CO_2-H extraction. The total phenolic compounds content and antioxidant activity of extract obtained by SC-CO_2-H,

Soxhlet methanol (SoxM), and hot water (HW) extraction are indicated in Figure 2.4.4 and 2.4.5, respectively. The result shows that SC-CO_2-H could give higher total phenolic compounds than the Soxhlet and hot water extraction methods. This may be because when CO_2 was dissolved in water, the pH of water in return was lowered. These conditions may facilitate the extraction of total phenolic compounds in the water phase [9-11]. However, SC-CO_2-H gave a little bit lower antioxidant activity than that of SoxM extraction, but higher that of hot water. This could be explained that methanol is not only extraction phenolic compounds, but also other antioxidant compounds. Even though SoxM could provide a little bit higher antioxidant activity than SC-CO_2-H extraction, methanol may provide high toxicity in final produce and it might not suitable to use for food and pharmaceutical industry [12]. Hot water was not good extraction method in this study because it provided low antioxidant activity in extract due to low ability of hot water in extraction of bioactive compounds from plant. Thus, it could be concluded that the SC-CO_2-H was effective and safe method to extraction total phenolic compounds and antioxidant activity in Khmer medicinal plants.

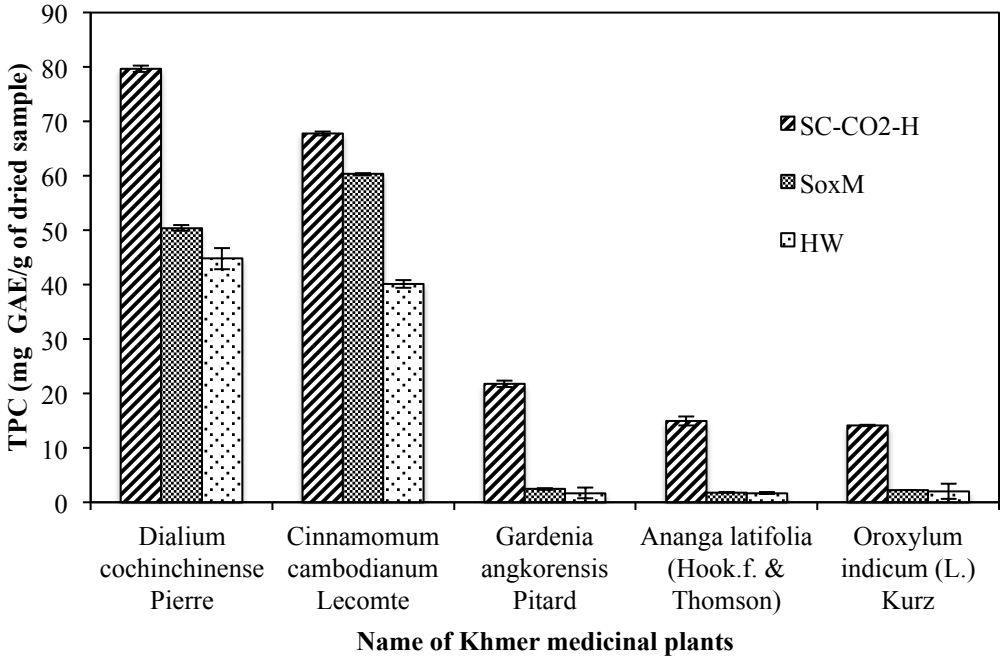

Figure 2.4.4 Total phenolic compounds content in extract by using different extraction methods

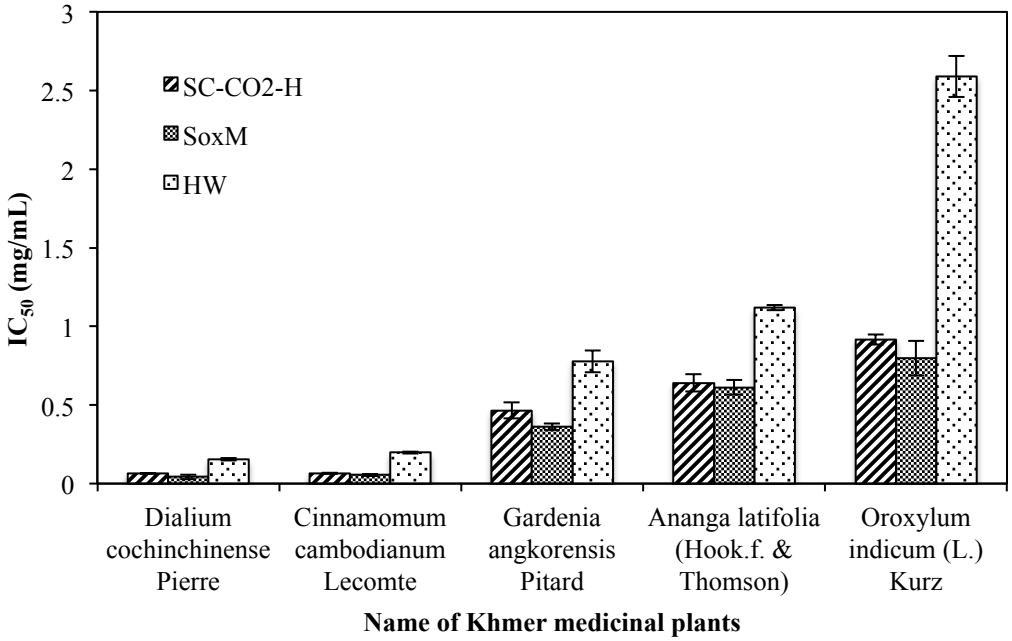

Figure 2.4.5 Antioxidant activity (IC_{50}) in extract by using different extraction methods

2.4.3.4 Chemical constituents in Khmer medicinal plants

The extract obtained by SC-CO_2-H was subjected to GC-MS analysis in order to identified chemical constituents in extract. Results of GC-MS total ion chromatogram showed chemical constituents presented in Khmer medicinal plant extracts (Figure 2.4.6-2.4.10). These compounds were corresponded with those found in the NIST database and the data are given. The chemical constituents and their activities in SC-CO_2-H extract of *Dialium cochinchinense* Pierre bark indicate in Table 2.4.1. The major chemical constituents were found to be scyllo-Inositol (55%), acetic acid (4.89%), ferulic acid (3.43%), 1,2-Benzenediol (2.75%), galactitol (2.72%), 2-o-methyl-d-xylose (2.6%), dibutyl phthalate (2.32%), octyl Isobutyrate (2.25%), 2,5-diketopiperazine (1.45%), squaric acid (1.42%), and bitoscanate (1.02%). Scyllo-Inositol, as major constituent in SC-CO_2-H extract of *Dialium cochinchinense* Pierre, is one of the stereoisomers of inositol. The pervious study has confirmed that scyllo-Inositol had ability to inhibit the cognitive deficits and improved the disease pathology. Therefore, scyllo-Inositol was considered as a potential therapeutic for treatment of Alzheimer's disease [17]. The bark of *Dialium cochinchinense* Pierre was used traditionally for treatment of diarrhea and vertigo in

Cambodia. Dunkoskung et al. [5] also have reported that the alcoholic extract of this plant could prohibit the intestinal alpha-glucosidase activity, thus it could be regarded as anti-diabetic potential.

The chemical constituents and theirs activities in the SC-CO_2-H extract of *Cinnamomum cambodianum* Lecomte bark were indicated in Table 2.4.2. The major constituents were α-terpinol (16.06%), 4-terpineol (4.50%), eucalyptol (4.06%), methoxyeugenol (3.76%), eugenol (3.26%), acetic acid (3.05%), pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (2.93%), umbelliferone (2.8%), linalool (2.49%), benzofuran-2-carboxylic acid (1.86%), α-terpinolene (1.57%), piperazine (1.45%), furfural (1.41%), 2-buten-1-imine, n-cyclohexyl-4-(2-fluoro-2-methylcyclohexyl)-, n-oxide (1.41%), amitryptyline (1.33%), ethyl pentanoate (1.28%), hexadecanal (1.23%), 2H-1,2-Oxazine, 6-(4-chlorophenyl)tetrahydro-2-methyl- (1.22%), spathulenol (1.10%), terpin hydrate (1.07%), vanillin (1.04%). Most of major compounds in this extract are essential oils, which could be employed in human consumption including functional foods, food additives, medicine, natural supplement and cosmetic product due to their antioxidant, anti-inflammatory, antifungal, and antimicrobial properties [18]. Traditionally, the bark of *Cinnamomum cambodianum* was also employed as antispasmodic and antiseptic agent. Duñg et al. [19] have found that the essential oils of the wood of *Cinnamomum cambodianum* were α-terpinol (33.4%), linalool (22.4%) and 4-terpineol (13.3%). Chae et al. was reported that the chloroform-soluble extract of *Cinnamomum cambodianum* contains pharmacological characteristics with antiallergic effect.

Table 2.4.3 shows the chemical compositions and theirs activities in SC-CO_2-H extract of *Gardenia angkorensis* Pitard bark. The major constituents were carbazole (33%), 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol (13.24%), sinapic aldehyde (6.32%), syringaldehyde (4.13%), 2,4-hexadienedioic acid, 3,4-diethyl-, dimethyl ester, (Z,Z)- (2.82%), Acetic acid (2.54%), pyrazole (2.45%), 2,3-butanedione monoxime (1.89%), sinapic acid (1.76%), piperazine (1.6%), furfural (1.41%), dibutyl phthalate (1.02%), benzenepropanoic acid, 2,5-dimethoxy- (1.01%). Carbazole alkaloid, which is the major constituent in SC-CO2-H extract of *Gardenia angkorensis* Pitard, processes a wide range of pharmacological activities such as antimalarial, antibacterial, anti-TB, anti-HIV reverse transcriptase-inhibitory, and cytotoxic activities [20]. Traditionally, the bark of *Gardenia angkorensis* Pitard was utilized as an element in diuretic preparation. The chemical composition of this was not found in the previous study [21].

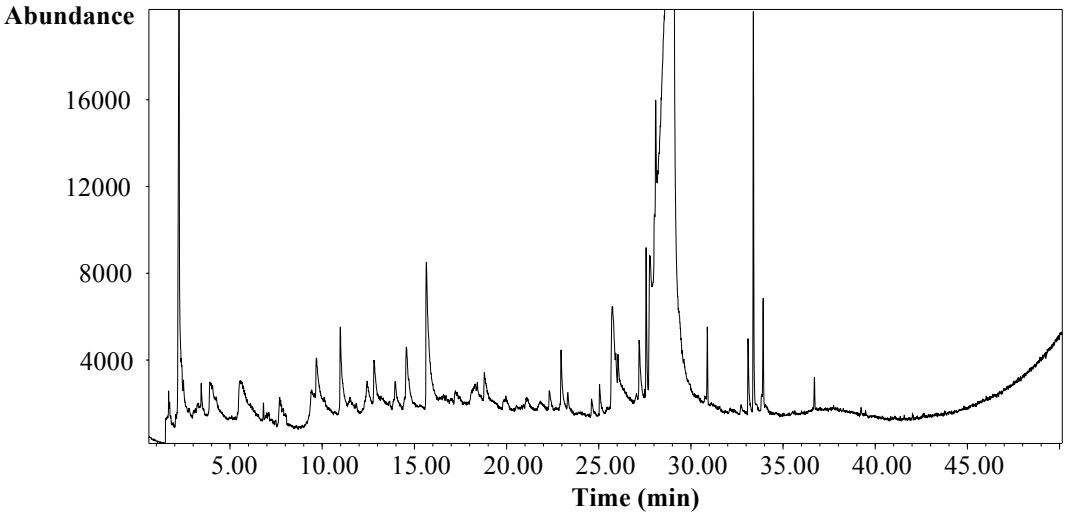

Figure 2.4.6 GC-MS total ion chromatogram of chemical constituents in SC-CO_2-H extract of *Dialium cochinchinense* bark

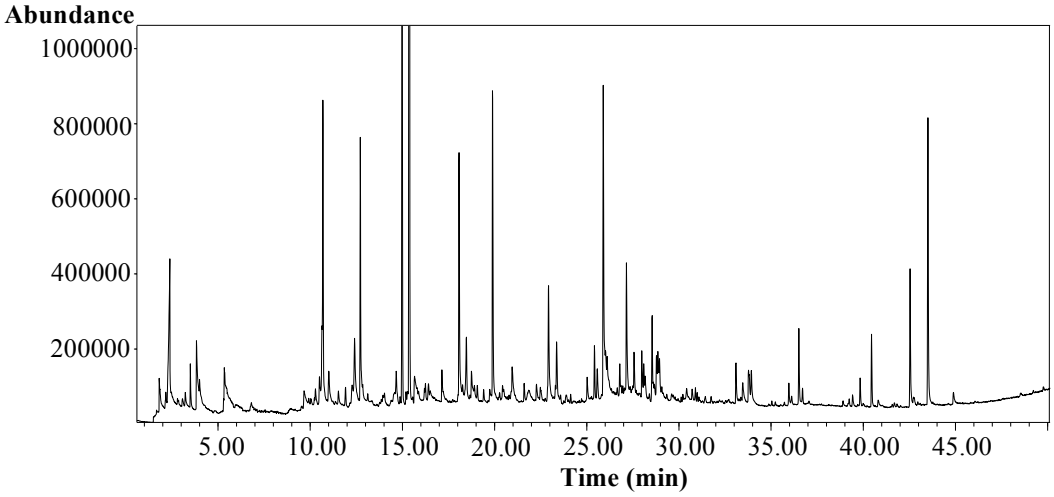

Figure 2.4.7 GC-MS total ion chromatogram of chemical constituents in SC-CO_2-H extract of *Cinnamomum cambodianum* Lecomte bark

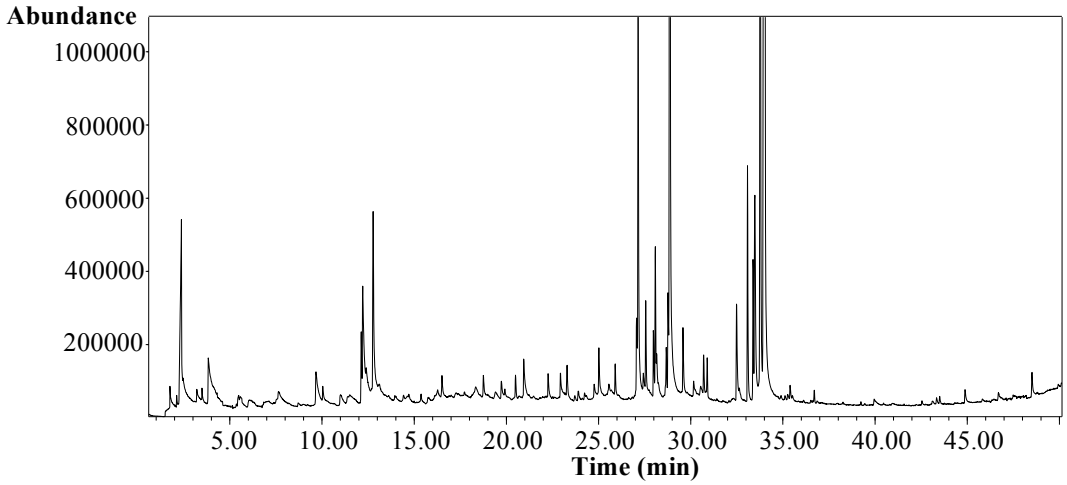

Figure 2.4.8 GC-MS total ion chromatogram of chemical constituents in SC-CO_2-H extract of *Gardenia angkorensis* Pitard bark

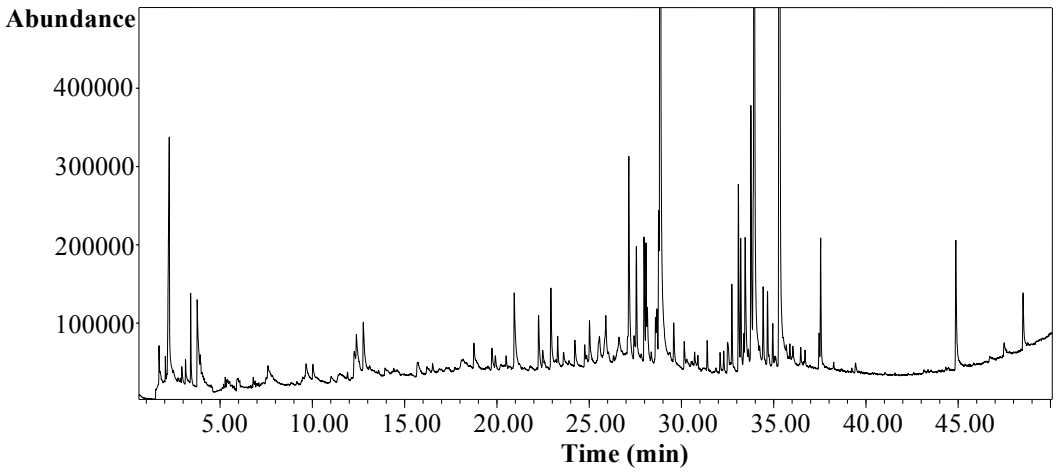

Figure 2.4.9 GC-MS total ion chromatogram of chemical constituents in SC-CO_2-H extract of *Cananga latifolia* (Hook. f. & Thomson) Finet & Gagnep bark

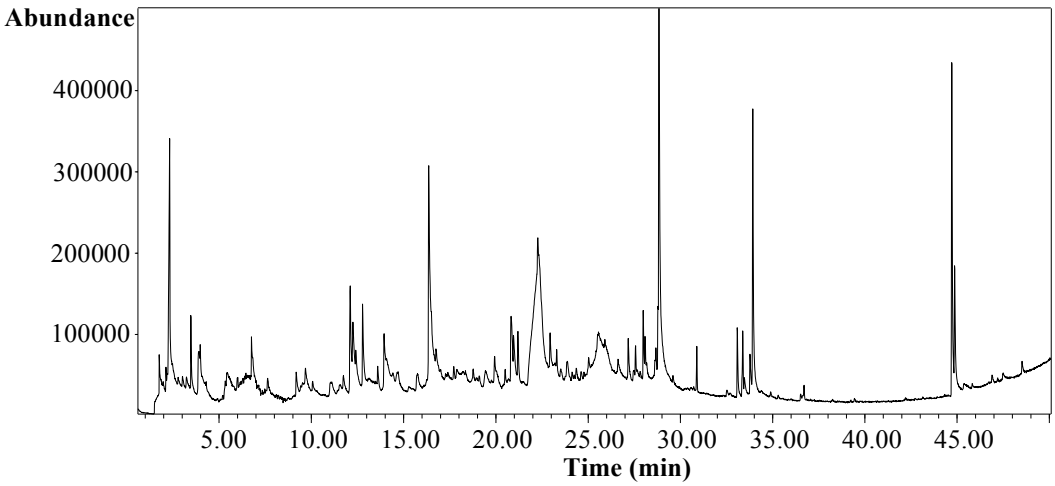

Figure 2.4.10. GC-MS total ion chromatogram of chemical constituents in SC-CO_2-H extract of *Oroxylum indicum* (L.) Kurz bark

The chemical constituents and theirs activities in SC-CO_2-H extract of *Cananga latifolia* (Hook. f. & Thomson) Finet & Gagnep bark were illustrated in Table 2.4.4. The main constituents were α-cyclocitral (26.96%), 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol (13.65%), Desaspidinol (12.03%), Acetic acid (3.28%), Syringaldehyde (2.38%), Sinapaldehyde (2.10%), 4-Azafluorenones (1.37%), Tetramethylpyrazine (1.27%), Sinapic acid (1.23%), Estradiol (1.19%). The most abundance constituent in this extract was α-cyclocitral. α-cyclocitral known as an inhibitor for leukotriene production, thus it could be used for treatment of a number of inflammatory diseases such as asthma, chronic bronchitis, and allergic rhinitis. The stem bark of this tree was also used traditionally as antifungal and for treatment of nasal polyprosis and fever [22]. The previous study reported that the hexane extract of stem bark of this tree showed cytotoxicity again the KB cancer cell with an IC_{50} of 25.00 μg/ml. Cananginones A-I f which was isolated from this extract have a weak cytotoxicity, antimalarial and antifungal activity [6].

The chemical constituents and theirs activities in SC-CO_2-H extract of *Oroxylum indicum* (L.) Kurz bark is presented in Table 2.4.5. The major constituents were maltose (14.48%), 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol (10.23%), 2-furancarboxaldehyde, 5-(hydroxymethyl)- (5.72%), Acetic acid (5.25%), 9,10-anthracenedione-1,8-dihydroxy-3-methoxy-6-methyl (3.91%), desaspidinol (3.8%),

ethyl 2-methylbutanoate (3.34%), 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (2.35%), estradiol (2.01%), 1H-3a,7-methanoazulene, octahydro-1,4,9,9-tetramethyl- (2.02%), 6-methyluracil (1.7%), benserazide (1.64%), sucrose (1.48%), 1-nonen-4-ol (1.6%), 2,4-diaminopyrimidine (1.47%), benzofuran-2-carboxylic acid (1.3%), vanillin (1.17%), erythritol (1.1%), 6-octen-2-one, (Z)- (1.12%), and 1-tetradecene (1.02%). Phenolic acid, flavonoids, aldehyde, steroid, fatty acid and sugar were mostly found in this extract. These compounds showed variety of biological activities such as antioxidant, antibacterial, antiinflmmatory, and anticancer activity [23-27]. Traditionally, *Oroxylum indicum* (L.) Kurz was employed as medicine for prevention and treatment of jaundice, arthritic and rheumatic problem, gastric ulcers, tumors, respiratory disease, diabetes, diarrhea and dysentery [28]. Samatha et al. [2] found that methanol, benzene, chloroform and water extract of the stem bark of *Oroxylum indicum* (L.) Kurz contained tanins, saponins, sterols, phenols, fats and oil. Dina et al. [28] reported that the major flavonoid in the stem bark of *Oroxylum indicum* (L.) Kurz were baicalein, chrysin and oroxylin A. This stem bark had many pharmaceutical activities such as antibacterial, antiinflammatory, anticancer, antiulcer, immunostimulant, antidiarrheal, antidiabetic, hepto-protective, antioxidant, analgesic, proprotein convertase inhibitory, and antilesishmanial.

Table 2.4.1 Chemical constituents and their activities in SC-CO_2-H extract of *Dialium cochinchinense* Pierre bark

No	Name of Compounds	Molecular formula	MW	Area (%)	RT	Activity
1	Acetic acid	$C_2H_4O_2$	60	4.89	2.24	Antibacterial, antifungal activity
2	D-Serine	$C_3H_7NO_3$	105	0.41	2.48	Antipsychotic activity
3	Piperazine	$C_4H_{10}N_2$	86	0.12	3.92	Antiparasitic activity
4	Furfural	$C_5H_4O_2$	96	0.79	5.56	Antibacterial activity
5	2-Pentanethiol	$C_5H_{12}S$	104	0.58	9.44	Antimicrobial, anti-tumor, immuno - modulatory,

						antioxidant activity
6	Squaric acid	$C_4H_2O_4$	114	1.42	9.97	Immunotherapeutic activity
7	2,5-Diketopiperazine	$C_4H_6N_2O_2$	114	1.45	10.9	The inhibition of plasminogen activator inhibitor-1 (PAI-1), alteration of cardiovascular and blood-clotting functions, antitumour, antiviral, antifungal, antibacterial activity, antihyperglycaemic agents and affinities for calcium channels and opioid, GABAergic, serotoninergic 5-HT1A and oxytocin receptors.
8	1,2-Benzenediol	$C_6H_6O_2$	110	2.75	15.66	Anticancer (breast), antioxidant, pesticide activity
9	Hexamethyl benzene	$C_{12}H_{18}$	162	0.76	22.96	Pituitary-sensitizer
10	Octyl Isobutyrate	$C_{12}H_{24}O_2$	200	2.25	25.75	Herbaceous, fruity odor, flavoring agent
11	Coumalic acid	$C_6H_4O_4$	140	0.49	25.93	Antioxidant

						activity
12	4-Methoxy-3,5-dihydroxybenzoic acid	$C_8H_8O_5$	184	0.61	26.05	Antibacterial activity
13	Syringaldehyde	$C_9H_{10}O_4$	182	0.81	27.21	Antioxidant, antifungal, anti-oncogenic properties, mediator, inhibitor of enzymatic hydrolysis, organic marker in wood smoke, biological control activity, moderate antiplasmodial activity (antimalaria)
14	Bitoscanate	$C_8H_4N_2S_2$	192	1.02	27.58	Anthelmintic activity
15	2-O-Methyl-d-xylose	$C_6H_{12}O$	164	2.6	27.78	No activity found
16	Galactitol	$C_6H_{14}O_6$	182	2.72	28.03	Antitumor activity
17	Ferulic acid	$C_{10}H_{10}O_4$	194	3.43	28.11	Anti-inflammatory, antiatherogenic, antidiabetic, antiageing, neuroprotective, radioprotective, hepatoprotective activity
18	Scyllo-Inositol	$C_6H_{12}O_6$	180	42.86	28.98	Treatment of Alzheimer's disease

19	Scyllo-Inositol	$C_6H_{12}O_6$	180	13.88	29.04	Treatment of Alzheimer's disease
20	Tetradecamethyl-hexasiloxane	$C_{14}H_{42}O_5Si_6$	459	0.44	30.91	Nematiside, antiantrogenic, anticoranary, antieczemic activity
21	Sinapic acid	$C_{11}H_{12}O_5$	224	0.66	33.12	Antioxidant, antimicrobial, anti-inflammatory, anticancer, and anti-anxiety activity
22	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	2.32	33.39	Plasticizer compound, antimicrobial, antifouling activity
23	Hexasiloxane, tetradecamethyl-	$C_{14}H_{42}O_5Si_6$	459	0.75	33.93	Nematiside, antiantrogenic, anticoranary, antieczemic activity
24	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.21	36.72	Antimicrobial, antioxidant activity

Table 2.4.2 Chemical constituents and theirs activities in SC-CO_2-H extract of *Cinnamomum cambodianum* Lecomte bark

No	Name of Compounds	Molecular formula	MW	Area (%)	RT	Activity
1	N,N-Dimethyltrypta-mine	$C_{12}H_{16}N_2$	188	0.33	1.68	Psychotropic properties
2	Amitryptyline	$C_{20}H_{23}N$	277	1.33	1.82	Antidepressant activity
3	Acetic acid	$C_2H_4O_2$	60	3.05	2.39	Antibacterial, antifungal activity
4	2H-1,2-Oxazine, 6-(4-chlorophenyl)tet-rahydro-2-methyl-	$C_{11}H_{14}CINO$	212	1.22	2.48	Antioxidant activity
5	D-Serine	$C_3H_7NO_3$	105	0.26	2.81	Antipsychotic activity
6	Hexanoic acid	$C_6H_{12}O_6$	116	0.39	2.86	Antibacterial activity
7	D-Serine	$C_3H_7NO_3$	105	0.47	3.08	Antipsychotic activity
8	Benactyzine	$C_{20}H_{25}NO_3$	327	0.78	3.23	Anticholinergic activity
9	Isosorbide Dinitrate	$C_6H_8N_2O_8$	236	0.81	3.51	Treatment of heart failure
10	Piperazine	$C_4H_{10}N_2$	86	1.45	3.84	Anthelmintic activity
11	Ethyl pentanoate	$C_7H_{14}O_2$	130	1.28	4.01	Flavor agent
12	Furfural	$C_5H_4O_2$	96	1.41	5.35	Antibacterial activity
13	Furfural	$C_5H_4O_2$	96	0.34	5.64	Antibacterial activity
14	Imidodicarbonic diamide, N-formyl-	$C_3H_5N_3O_3$	131	0.18	6.82	Antimicrobial activity

15	5-Methylfurfural	$C_6H_6O_2$	110	0.16	8.98	Antioxidant activity
16	2,5-Diketopiperazine	$C_4H_6N_2O_2$	114	0.61	9.68	The inhibition of plasminogen activator inhibitor-1 (PAI-1), alteration of cardiovascular and blood-clotting functions, antitumour, antiviral, antifungal, antibacterial, and antihyperglycaemic agents and affinities for calcium channels and opioid, GABAergic, serotoninergic 5-HT1A and oxytocin receptors.
17	Oxazolidine, 2,2-diethyl-3-methyl-	$C_8H_{17}NO$	143	0.22	9.93	Antipetitmal, anti-grandmal activity
18	Parachlorophen-ol	C_6H_5ClO	129	0.26	10.04	Disinfectant
19	Citronellal	$C_{10}H_{18}O$	154	0.15	10.23	Antifungal and antibacterial activity
20	α-Terpinene	$C_{10}H_{16}$	136	0.40	10.29	Antifungal activity, flavor agent
21	o-Cymene	$C_{10}H_{14}$	134	0.53	10.51	Antioxidant, antifungal activity

22	Eucalyptol	$C_{10}H_{18}O$	154	4.06	10.70	Antimicrobial activity, flavor agent
23	2-Methyl-3-furanthiol	C_5H_6OS	114	0.87	11.02	Meaty aroma volatile
24	γ-Terpinene	$C_{10}H_{16}$	136	0.38	11.53	Antifungal activity
25	Myrcenol	$C_{10}H_{18}O$	154	0.27	11.92	Antimicrobial activity, fragrant component
26	α-Terpinolene	$C_{10}H_{16}$	136	1.57	12.41	Antifungal activity
27	Linalool	$C_{10}H_{18}O$	154	2.49	12.72	Antioxidant, antifungal, antibacterial activity, neroprotective agent
28	Dodecanal	$C_{12}H_{24}O$	184	0.53	12.85	Sex attractants
29	β-Terpineol	$C_{10}H_{18}O$	154	0.25	14.03	Flavor agent
30	Undecylenic Acid	$C_{11}H_{20}O_2$	184	0.07	14.40	Antifungal activity
31	Isopulegol	$C_{10}H_{18}O$	154	0.35	14.60	Anticonvulsant, antioxidant activity
32	α-terpinol	$C_{10}H_{18}O$	154	0.57	14.67	Antioxidant, antifungal, antibacterial activity
33	Isopulegol	$C_{10}H_{18}O$	154	0.09	14.89	Anticonvulsant, antioxidant activity
34	4-Terpineol	$C_{10}H_{18}O$	154	4.50	14.99	Antibacterial activity
35	Thymol	$C_{14}H_{10}O$	150	0.12	15.20	Antioxidant, antifungal, antibacterial

						activity
36	α-terpinol	$C_{10}H_{18}O$	154	0.15	15.29	Antioxidant, antifungal, antibacterial activity
37	α-terpinol	$C_{10}H_{18}O$	154	16.06	15.37	Antioxidant, antifungal, antibacterial activity
38	1,2-Benzenediol	$C_6H_6O_2$	110	0.6	15.66	Anticancer (breast), antioxidant, pesticide activity
39	1,2-Benzenediol	$C_6H_6O_2$	110	0.17	15.80	Anticancer (breast), antioxidant, pesticide
40	1,2-Benzenediol	$C_6H_6O_2$	110	0.17	15.89	Anticancer (breast), antioxidant, pesticide activity
41	1,2-Benzenediol	$C_6H_6O_2$	110	0.27	16.25	Anticancer (breast), antioxidant, pesticide activity
42	Nerol	$C_{10}H_{18}O$	154	0.2	16.42	Antioxidant, antifungal, antibacterial activity
43	Maltol	$C_6H_6O_3$	126	0.15	16.52	Marginal feeding deterrent, antioxidant, antifungal, antibacterial activity
44	Geraniol	$C_{10}H_{18}O$	154	0.57	17.15	Antioxidant, antifungal,

						antibacterial, antinflammatory, anti-apoptotic, anti-tumour, anticarcinogenic activity
45	Umbelliferone	$C_9H_6O_3$	162	2.8	18.07	Antihyperglycemic effect , antioxidant, anti-asthmatic, anti-inflammatory activity, immunomodulatory, vasodilating.
46	Terpin Hydrate	$C_{10}H_{22}O_3$	190	1.07	18.47	Mucolytic activity
47	Thymol	$C_{14}H_{10}O$	150	0.58	18.75	Antioxidant, antifungal, antibacterial activity
48	1-Docosene	$C_{22}H_{24}$	308	0.19	19.42	Antimicrobial, anticarcinoma activity
49	Syringol	$C_8H_{10}O_3$	154	0.25	19.74	Antioxidant activity
50	Eugenol	$C_{10}H_{12}O_2$	164	3.26	19.90	Antioxidant, anti-inflammatory, fungicidal, antiseptic, anesthetic activity
51	Vanillin	$C_8H_8O_3$	152	1.04	20.96	Antioxidant, anticancer, antibacterial, antimutagenic, antitumor activity
52	Hydroquinone	$C_6H_6O_2$	110	0.27	21.61	Depigmentation, antimalarial

53	Undecanal	$C_{11}H_{22}O$	170	0.65	21.86	Antibacterial activity
54	Eugenol	$C_{10}H_{12}O_2$	164	0.38	22.27	Antioxidant, anti-inflammatory activities, fungicidal, antiseptic, anesthetic activity
55	Benzofuran-2-carboxylic acid	$C_9H_6O_3$	162	1.86	22.93	Potent Pim-1 inhibitors, leukotriene biosynthesis Inhibitor
56	methyl cinnamate	$C_{10}H_{10}O_2$	162	0.17	23.31	Antibacterial, antifungal and antiaflatoxigenic activity
57	τ-Muurolol	$C_{12}H_{26}O_2$	222	0.09	24.13	Antifungal activity
58	Theophylline	$C_7H_8N_4O_2$	180	0.41	25.03	Treatment of cardiorespiratory disorders
59	(-)-Spathulenol	$C_{15}H_{24}O$	220	0.66	25.43	Anticancer, antitumor, antibiosis1Hcyclo prop and azulene for analgesia
60	Spathulenol	$C_{15}H_{24}O$	220	0.56	25.57	Antimicrobial, immunomodulat-ory, antitumor activities
61	2-Nonen-1-ol	$C_9H_{18}O$	142	0.16	25.82	Flavouring agent
62	Methoxyeugenol	$C_{11}H_{14}O_3$	194	3.76	25.90	Antifungal, antimicrobial, insect attractant activity
63	Hexadecanal	$C_{16}H_{23}O$	240	1.23	26.11	Flavouring agent

64	(-)-Spathulenol	$C_{15}H_{24}O$	220	0.28	26.66	Anticancer, antitumor activity, antibiosis1Hcyclo prop and azulene for analgesia
65	Spathulenol	$C_{15}H_{24}O$	220	0.23	26.80	Antimicrobial, immunomodulat-ory, antitumor activity
66	Globulol	$C_{15}H_{26}O$	222	0.23	26.86	Antibacterial, antifungal activity
67	Thujopsene	$C_{15}H_{24}$	204	0.24	26.96	Antibacterial, antifungal activity
68	Syringaldehyde	$C_9H_{10}O_4$	182	0.81	27.16	Antioxidant, antifungal, anti-oncogenic, mediator, inhibitor of enzymatic hydrolysis, organic marker in wood smoke, biological control activity, moderate antiplasmodial activity (antimalaria)
69	Myristicin	$C_{11}H_{12}O_3$	192	0.71	27.57	Antibacterial, antiinflammatory, hepatoprotective activity, flavor agent
70	Methoxyeugenol	$C_{11}H_{14}O_3$	194	0.64	28.00	Antifungal, antimicrobial, insect attractant activity

71	Ferulic acid	$C_{10}H_{10}O_4$	194	0.46	28.10	Antiinflammatory antiatherogenic, antidiabetic, antiageing, neuroprotective, radioprotective and hepatoprotective activity
72	Thioguanine	$C_5H_5N_5S$	167	0.49	28.17	Treatment of certain autoimmune, immunologic, psoriasis, leukemias
73	DL-Phenylephrine	$C_9H_{13}NO_2$	167	0.17	28.41	Treatment of decongestant
74	Spathulenol	$C_{15}H_{24}O$	220	1.10	28.55	Antimicrobial, immunomodulat-ory, antitumor activity
75	Ethyl 3,4-dihydroxybenzo-ate	$C_9H_{10}O$	182	0.25	28.68	Antioxidant, anticancer, cardioprotective, neuroprotective, antimicrobial, antiinflammatory, myoprotective, anti-ulcer activity
76	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	0.71	28.86	Antimicrobial, Antioxidant Antiinflammatory Analgesic activity
77	Spathulenol	$C_{15}H_{24}O$	220	0.65	28.95	Antimicrobial, immunomodulat-ory, antitumor activity

78	β-ionone	$C_{13}H_{20}O$	192	0.24	29.07	Cancer chemopreventive, antitumor activity
79	Thioguanine	$C_5H_5N_5S$	167	0.18	29.61	Treatment of certain autoimmune, immunologic, psoriasis, leukemias
80	Calarene epoxide	$C_{15}H_{24}O$	220	0.06	30.13	Antioxidant, anti-inflammatory, anti-cancer activity
81	Spathulenol	$C_{15}H_{24}O$	220	0.11	30.22	Antimicrobial, immunomodulat-ory, antitumor activity
82	4-Azafluorenones	$C_{12}H_7NO$	181	0.24	30.72	Antimicrobial, antimalarial, and DNA-damaging activity
83	Octadecamethyl cyclononasiloxa-ne	$C_{18}H_{54}O_9Si_9$	667	0.19	30.89	Antioxidant, antioksidaan and antimicrobial activity
84	Sinapic acid	$C_{11}H_{12}O_5$	224	0.49	33.09	Antioxidant, antimicrobial, antiinflammatory, anticancer, and anti-anxiety activity
85	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	0.11	33.36	Antioxidant, hypocholesterole mic, nematicide, pesticide, antiandrogenic, flavor, hemolytic

						and 5-Alpha reductase inhibitor
86	Thioguanine	$C_5H_5N_5S$	167	0.5	33.47	Treatment of certain autoimmune, immunologic, psoriasis, leukemias
87	Caffeic acid methyl ester	$C_{10}H_{10}O_4$	194	0.65	33.78	Antiinflammatory antioxidant inhibition on the growth of HIV-replication in PBMC cells
88	Octadecamethyl cyclononasiloxa-ne	$C_{18}H_{54}O_9Si_9$	667	0.67	33.93	Antioxidant, antioksidaan, antimicrobial
89	Camphor	$C_1H_{10}O_4$	152	0.25	35.97	Insecticidal, antimicrobial, antiviral, anticoccidial, anti-nociceptive, anticancer, antitussive activity, skin penetration enhancer
90	1-Hexadecene	$C_{16}H_{32}$	224	0.13	36.12	Antibacterial, antifungal, antioxidant activity
91	2,5-Diketopiperazine	$C_4H_6N_2O_2$	114	0.76	36.51	The inhibition of plasminogen activator inhibitor-1 (PAI-1), alteration of

						cardiovascular and blood-clotting functions, antitumour, antiviral, antifungal, antibacterial, and antihyperglycaemic agents and affinities for calcium channels and opioid, GABAergic, serotoninergic 5-HT1A and oxytocin receptors
92	Octadecamethyl cyclononasiloxa-ne	$C_{18}H_{54}O_9Si_9$	667	0.18	36.71	Antioxidant, antioksidaan, antimicrobial activity
93	Octadecamethyl cyclononasiloxa-ne	$C_{18}H_{54}O_9Si_9$	667	0.11	39.24	Antioxidant, antioksidaan, antimicrobial activity
94	2-Buten-1-imine, N-cyclohexyl-4-(2-fluoro-2-methylcyclohexyl)-, N-oxide	$C_{17}H_{28}FNO$	281	1.41	42.54	Antimicrobial, narcotic, antitumor, myo-neuro-stimulant activity
95	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	210	2.93	43.52	Antimicrobial activity
96	Butorphanol	$C_{16}H_{32}O$	240	0.22	44.89	Pain-reducing effect

97	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.03	49.24	Antimicrobial, antioxidant activity

Table 2.4.3 Chemical constituents and theirs activities in SC-CO_2-H extract of *Gardenia angkorensis* Pitard bark

No	Name of Compounds	Molecular formula	MW	Area (%)	RT	Activity
1	Diazoprogestero-ne	$C_{21}H_{30}N_4$	338	0.54	1.77	Antimicrobial activity
2	D-Glycero-d-galacto-heptose	$C_7H_{14}O_7$	210	0.08	2.12	Preservative
3	Caprylic acid	$C_8H_{16}O_2$	114	0.01	2.21	Antibacterial activity
4	Acetic acid	$C_2H_4O_2$	60	2.54	2.38	Antibacterial and antifungal activity
5	Caprylic acid	$C_8H_{16}O_2$	114	0.48	2.46	Antibacterial activity
6	Lidocaine	$C_{14}H_{22}N_2O$	234	0.23	3.22	Effective antiarrhythmic agent
7	Hexanoic acid	$C_6H_{12}O_6$	116	0.14	3.49	Antibacterial, antifungal activity
8	Piperazine	$C_4H_{10}N_2$	86	1.6	3.84	Anthelmintic activity
9	Piperazine	$C_4H_{10}N_2$	86	0.17	4.31	Anthelmintic activity
10	Selegiline	$C_{13}H_{17}N$	187	0.15	5.49	Treatment of Parkinson's disease, major depressive disorder
11	Furfural	$C_5H_4O_2$	96	1.41	5.59	Antibacterial activity
12	2-Furanmethanol	$C_5H_6O_2$	98	0.13	6.09	Antioxidant,

						antibacterial activity
13	2-Furanmethanol	$C_5H_6O_2$	98	0.04	6.85	Antioxidant, antibacterial activity
14	Cycloheptane	C_7H_{14}	98	0.42	7.65	Antifungal activity
15	Squaric acid	$C_4H_2O_4$	114	0.79	9.68	Immunotherapeutic activity
16	Furaneol	$C_6H_8O_3$	128	0.17	10.05	Antioxidant, anticancer, antibacterial activity
17	2,5-Diketopiperazine	$C_4H_6N_2O_2$	114	0.27	11.02	The inhibition of plasminogen activator inhibitor-1 (PAI-1), alteration of cardiovascular and blood-clotting functions, antitumour, antiviral, antifungal, antibacterial, and antihyperglycaemic agents and affinities for calcium channels and opioid, GABAergic, serotoninergic 5-HT1A and oxytocin receptors.
18	Muscimol	$C_4H_6N_2O_2$	114	0.09	11.41	A gamma-aminobutyric acid

						(GABA) agonist
19	Benzyl nitrile	C_8H_7N	117	0.52	12.12	Anti-aphrodisiac activity
20	2,3-Butanedione monoxime	$C_4H_7NO_2$	101	1.89	12.22	A cardioplegic agent, non-competitive inhibitor of skeletal muscle myosin-II
21	3-Piperidinol	$C_5H_{11}NO$	101	0.79	12.40	Anesthetic, analgesic, antibiotic activity
22	Pyrazole	$C_8H_4N_2$	68	2.45	12.77	CNS depressant, neuroleptic, tuberculostatic, antihypertensive, antileishmanial, analgesic, antidiabetic, antitumor and antimicrobial activity
23	Undecylenic Acid	$C_{11}H_{20}O_2$	184	0.37	13.11	Antifungal, antibacterial, antiviral activity
24	Pyrazine diazohydroxide	$C_4H_4N_4O$	124	0.09	14.43	Antifungal, antitumor activity
25	Bicyclo [3.1.1]heptane, 2,6,6-trimethyl-, [1S-(1.alpha.,2.beta.,5 .alpha.)]-	$C_{10}H_{18}$	138	0.14	14.72	Fragrance and flavor agent in the pharmaceutical and chemical industries.
26	α-terpinol	$C_{10}H_{18}O$	154	0.16	15.37	Antifungal, antibacterial activity
27	1,2-Benzenediol	$C_6H_6O_2$	110	0.14	15.76	Anticancer

						(breast), antioxidant, pesticide
28	1,10-Decanediol	$C_{10}H_{22}O_2$	174	0.07	16.11	Antimutagenic, anticarcinogenic, antipsoriatic, antiglaucomic, antiosteoporotic, antiinflammatory psychostimulant CNS active muscle relaxant, antiviral (Poxvirus) chemopreventive, antiamyloidogen-ic, antimyopathiesn, antiinflammatory, cytoprotectant, antinociceptive, antineurotoxic, antianemic antiamyloidogen-ic, antimutagenic, antialcoholic, antimyopathies activity
29	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	0.3	18.76	Antimicrobial, antioxidant, anti-inflammatory, analgesic activity
30	Ricinoleic acid	$C_{18}H_{34}O_3$	298	0.18	19.41	Antimicrobial, anti-inflammatory
31	Syringo	$C_8H_{10}O_3$	154	0.23	19.73	Antioxidant
32	Eugenol	$C_{10}H_{12}O_2$	164	0.1	19.92	Antioxidant, anti-inflammatory

						activities, fungicidal, antiseptic, anesthetic activity
33	Allantoin	$C_4H_6N_4O_3$	158	0.18	20.51	Moisturizing and keratolytic effect, increasing the water content of the extracellular matrix and enhancing desquamation of upper layers of dead skin cells, the increase of the smoothness of the skin, promoting cell proliferation and wound healing, antiirritant, protective effects on the skin
34	Vanillin	$C_8H_8O_3$	152	0.53	20.95	Antioxidant, anticancer, antibacterial, antimutagenic, antitumor activity
35	Eugenol	$C_{10}H_{12}O_2$	164	0.44	22.94	Antioxidant, anti-inflammatory, fungicidal, antiseptic, anesthetic activity
36	Methyl cinnamate	$C_{10}H_{10}O_2$	162	0.46	23.30	Antibacterial, antifungal, antiaflatoxigenic activity

37	Benadryl	$C_{17}H_{21}NO$	255	0.17	23.72	Antihistamine activity
38	Thymoquinone	$C_{10}H_{12}O_2$	164	0.13	23.91	Antioxidant, anti-inflammatory, hepato-protective activity
39	4-Propylguaiacol	$C_{10}H_{14}O_2$	166	0.04	24.25	Antioxidant activity
40	Amphetamine	$C_9H_{13}N$	135	0.26	24.77	Central nervous system stimulant
41	Theobromine	$C_7H_8N_4O_2$	180	0.69	25.02	Increasing serum HDL cholesterol, stimulates heart muscle, relaxes bronchial smooth muscles, role in the transmission of intracellular signal and antioxidant activity
42	Ferulic acid	$C_{10}H_{10}O_4$	194	0.28	25.91	Anti-inflammatory, antiatherogenic, antidiabetic, antiageing, neuroprotective, radioprotective and hepatoprotective activity
43	Gallic acid	$C_7H_6O_5$	170	0.8	27.06	Anti-oxidant, anti-inflammatory, anti-microbial, and anti-cancer activity

44	Syringaldehyde	$C_9H_{10}O_4$	182	4.13	27.15	Antioxidant, antifungal, anti-oncogenic properties, mediator, inhibitor of enzymatic hydrolysis, organic marker in wood smoke, biological control activity, moderate antiplasmodial activity (antimalaria)
45	Phenol, 4-(3-hydroxy-1-propenyl)-2-methoxy-	$C_{10}H_{12}O_3$	180	0.38	27.44	Antimicrobial, antioxidant, anti-inflammatory activity
46	Myristicin	$C_{11}H_{12}O_3$	192	0.98	27.56	Antibacterial, anti-inflammatory activity, hepatoprotective activity, flavor agent
47	Ferulic acid	$C_{10}H_{10}O_4$	194	0.57	27.98	Anti-inflammatory, antiatherogenic, antidiabetic, antiageing, neuroprotective, radioprotective, hepatoprotective activity
48	Ferulic acid	$C_{10}H_{10}O_4$	194	0.81	28.16	Anti-inflammatory, antiatherogenic,

						antidiabetic, antiageing, neuroprotective, radioprotective, hepatoprotective activity
49	Thioguanine	$C_5H_5N_5S$	167	0.81	28.16	Treatment of certain autoimmune, immunologic and related diseases, treatment in psoriasis, leukemias
50	Fervenulin	$C_7H_7N_5O_2$	193	0.56	28.68	Antibacterial, antifungal, antiparasitic, and antitumor activity
51	Coniferyl aldehyde	$C_{10}H_{10}O_3$	178	0.83	28.77	Pesticidal and antibacterial activity
52	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	13.24	28.87	Antimicrobial, antioxidant antiinflammatory, analgesic activity
53	Carbazole	$C_{12}H_9N$	167	0.96	29.58	Anti-cancer, antimicrobial, antiviral, antiinflammatory and antioxidant activity.
54	Mescaline	$C_{11}H17NO_3$	211	0.4	30.16	Psychedelic drug
55	Syringic acid	$C_9H_{10}O_5$	198	0.30	30.55	Antioxidant, antibacterial and antifungal activity
56	Aspidinol	$C_{12}H_{16}O_4$	224	0.45	30.71	Anthelmintic activity

57	Octadecamethyl cyclononasiloxa-ne	$C_{18}H_{54}O_9Si_9$	667	0.37	30.91	Antioxidant, antioksidaan and antimicrobial activity
58	Benzenepropano-ic acid, 2,5-dimethoxy-	$C_{11}H_{14}O_4$	210	1.01	32.49	Antimicrobial activity
59	4-Azafluorenones	$C_{12}H_7NO$	181	0.21	32.64	Antimicrobial, antimalarial, and DNA-damaging activity
60	Sinapic acid	$C_{11}H_{12}O_5$	224	1.76	33.08	Antioxidant, antimicrobial, anti-inflammatory, anticancer, and anti-anxiety activity
61	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	1.02	33.39	Plasticizer compound, antimicrobial, antifouling activity
62	2,4-Hexadienedioic acid, 3,4-diethyl-, dimethyl ester, (Z,Z)-	$C_{12}H_{18}O_4$	226	2.82	33.48	Antagonist activity
63	Sinapic aldehyde	$C_{11}H_{12}O_4$	208	6.32	33.78	Antimicrobial, Antiinflammatory Anticancer, Anesthetic, Antiulcer Antiviral, Hypoglycemic activity
64	Carbazole	$C_{12}H_9N$	167	33.00	34.02	Antimalarial,

						antibacterial, anti-TB, anti-HIV reverse transcriptase-inhibitory, and cytotoxic activity
65	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	0.21	35.39	Antiinflammatory hypocholesterol-emic, Cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic, antieczemic, antiacne, 5-Alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary, insectifuge activity
66	Tetradecamethyl cycloheptasiloxa-ne	$C_{14}H_{42}O_7Si_7$	519	0.1	36.71	Antioxidant, flavor, hypocholesterol-emic activity
67	2,5-Dimethoxybenzoi c acid	$C_9H_{10}O_4$	182	0.09	43.35	Antifungal activity
68	3-Pyrrolidin-2-yl-propionic acid	$C_7H_{13}NO_2$	143	0.08	43.51	Antimicrobial activity
69	Nitroscanate	$C_{13}H_8N_2O_3S$	272	0.17	44.89	Anthelmintic activity
70	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.06	47.50	Antimicrobial, antioxidant activity

71	Ellagic acid	$C_{14}H_6O_8$	302	0.31	48.51	Anti-oxidant, anti-inflammatory and anti-fibrosis activity
72	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.05	48.83	Antimicrobial, antioxidant activity
73	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.03	49.37	Antimicrobial, antioxidant activity
74	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.01	49.50	Antimicrobial, antioxidant activity
75	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.06	49.95	Antimicrobial, antioxidant activity

Table 2.4.4 Chemical constituent and theirs activities in SC-CO_2-H extract of *Cananga latifolia* (Hook. f. & Thomson) Finet & Gagnep bark

No	Name of Compounds	Molecular formula	MW	Area (%)	RT	Activity
1	Bicyclo[2.2.1]hep-tan-2-one, 4,7,7-trimethyl-, semicarbazone	$C_{11}H_{19}N$	209	0.95	1.70	Antimicrobial activity
2	Sorbitol	$C_6H_{14}O_6$	182	0.19	1.98	Sweetener agent, axative effect, preservative,
3	Metoprolol	$C_{15}H_{25}NO_3$	267	0.23	2.04	β 1-selective adrenoceptor blocking drug to treat high blood pressure, heart failure, and Prophylactic

4	Acetic acid	$C_2H_4O_2$	60	3.28	2.24	Antibacterial and antifungal activity
5	Benserazide	$C_{10}H_{15}N_3O_5$	257	0.53	2.46	Antitumor agents for cancer therapy
6	D-Serine	$C_3H_7NO_3$	105	0.30	2.93	Antipsychotic activity
7	Lidocaine	$C_{14}H_{22}N_2O$	234	0.56	3.13	Effective antiarrhythmic agent
8	Piperazine	$C_4H_{10}N_2$	86	0.87	3.76	Anthelmintic activity
9	Piperazine	$C_4H_{10}N_2$	86	0.16	3.93	Anthelmintic activity
10	Xylitol	$C_5H_{12}O_5$	152	0.04	5.28	Anticariogenic activity
11	3,3'-Iminobispropylamine	C_6H_7N	131	0.12	5.99	Antimicrobial, anti-inflammatory
12	2-Furanmethanol	$C_5H_6O_2$	98	0.19	7.60	Antioxidant, antibacterial activity
13	4-Hydroxy-3,5,5-trimethylcyclohex-2-enone	$C_9H_{14}O_2$	154	0.37	9.66	Antityrosinase activity
14	N,N-Dimethyltryptamine	$C_{12}H_{16}N_2$	188	0.16	10.03	Psychotropic properties
15	Furaneol	$C_6H_8O_3$	128	0.31	12.27	Antioxidant, anticancer, antibacterial activity
16	1,2-Benzenediol	$C_6H_6O_2$	110	0.27	15.72	Anticancer (breast), antioxidant, pesticides
17	Norfenefrine	$C_8H_{11}NO_2$	153	0.13	18.20	Alpha-adrenergic

						agent
18	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	0.41	18.76	Antimicrobial, antioxidant, antiinflammatory, analgesic activity
19	Syringol	$C_8H_{10}O_3$	154	0.24	19.74	Antioxidant activity
20	Eugenol	$C_{10}H_{12}O_2$	164	0.15	19.92	Antioxidant, anti-inflammatory, fungicidal, antiseptic, anesthetic activity
21	1,2-Cyclopentanediol, 3-methyl-	$C_6H_{12}O_2$	116	0.11	20.51	Antimicrobial activity
22	Vanillin	$C_8H_8O_3$	152	0.87	20.95	Antioxidant, anticancer, antibacterial, antimutagenic, antitumor activity
23	Eugenol	$C_{10}H_{12}O_2$	164	0.52	22.26	Antioxidant, anti-inflammatory, fungicidal, antiseptic and anesthetic activity
24	Phenol, 2-methoxy-4-propyl-	$C_{10}H_{14}O_2$	166	0.31	22.50	Antimicrobial, anti-inflammatory, antioxidant activity
25	4-Methoxycinnama-ldehyde	$C_{10}H_{10}O_2$	162	0.78	22.93	Antimicrobial, antiviral activity
26	Naphthalene, 5-ethyl-1,2,3,4-tetrahydro-	$C_{12}H_{16}$	160	0.31	23.30	Active against the insects
27	Etilefrine	$C_{10}H_{15}NO_2$	181	0.12	23.63	Antihypotensive a

						gent
28	Phenol, 2-methoxy-4-propyl-	$C_{10}H_{14}O_2$	166	0.31	24.24	Antimicrobial anti-inflammatory, antioxidant
29	Trifluorothymidine	$C_{10}H_{11}F_3N_2O_5$	296	0.19	24.77	Anti-herpesvirus agents
30	Phenazine	$C_4H_{10}N_2$	86	0.66	25.03	Anthelmintic activity
31	1,2-Benzisothiazol-3(2H)-one	C_7H_5NOS	151	0.67	25.56	Antibacterial, antifungal activity
32	Isoferulic acid	$C_{10}H_{10}O_4$	194	0.99	25.91	Antioxidant, anti-inflammatory, antihyperglycemic, anti-glycation agent
33	Undecanal	$C_{11}H_{22}O$	170	0.93	26.63	Antibacterial activity
34	Syringaldehyde	$C_9H_{10}O_4$	182	2.38	27.15	Antioxidant, antifungal, anti-oncogenic properties, mediator, inhibitor of enzymatic hydrolysis, organic marker in wood smoke, biological control activity, moderate antiplasmodial activity (antimalaria)
35	Phenol, 4-(3-hydroxy-1-propenyl)-2-	$C_{10}H_{12}O_3$	180	0.39	27.44	Antimicrobial, antioxidant, anti-inflammatory

	methoxy-					activity
36	Tetramethylpyraz-ine	$C_8H_{12}N_2$	136	1.27	27.56	Anti-apoptotic, anti-inflammatory and antioxidant activity
37	Ferulic acid	$C_{10}H_{10}O_4$	194	0.98	27.98	Anti-inflammatory, antiatherogenic, antidiabetic, antiageing, neuroprotective, radioprotective and hepatoprotective activity
38	Ferulic acid	$C_{10}H_{10}O_4$	194	0.86	28.08	Anti-inflammatory, antiatherogenic, antidiabetic, antiageing, neuroprotective, radioprotective and hepatoprotective activity
39	Thioguanine	$C_5H_5N_5S$	167	0.73	28.16	Treatment of certain autoimmune, immunologic and related diseases, treatment in psoriasis, leukemias
40	4-Propylguaiacol	$C_{10}H_{14}O_2$	166	0.47	28.61	Antimicrobial, antiinflammatory, antioxidant

						activity
41	4-Propylguaiacol	$C_{10}H_{14}O_2$	166	0.52	28.68	Antimicrobial, antiinflammatory, Antioxidant activity
42	Phenanthrene	$C_{10}H_{14}$	178	0.95	28.77	Antimicrobial, spasmolytic, anti-inflammatory, antiplatelet aggregation, antiallergic activity
43	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	13.65	28.85	antimicrobial, antioxidant antiinflammatory analgesic
44	Carbazole	$C_{12}H_9N$	167	0.55	29.59	Anti-cancer, antimicrobial, antiviral, antiinflammatory and antioxidant activity
45	DL-Phenylephrine	$C_9H_{13}NO_2$	167	0.25	30.16	Decongestant ag-ent
46	Xanthoxylin	$C_{10}H_{12}O_4$	196	0.22	30.73	Antifungal, antioxidant, inhibition of prostaglandin synthetase, 5-lipoxygenase and the viability of Ehrlich ascite tumour cells, heart reagent, germicidal, antimicrobial and mildew- proof

						activities, and have direct inhibition effect upon uterus, intestinal and bladder muscle contraction caused by some drugs
47	Cyclohexasiloxane dodecamethyl-	$C_{12}H_{36}O_6Si_6$	445	0.18	30.89	Presevative
48	DL-Camphor	$C_{10}H_{16}O$	152	0.19	31.40	Camphor ordor, pleasant and freshing ordor
49	1-Heptatriacotanol	$C_{37}H_{76}O$	537	0.16	32.11	Antimicrobial activity
50	Benzenepropanoic acid, 2,5-dimethoxy-	$C_{11}H_{14}O_4$	210	0.40	32.51	Antimicrobial activity
51	Isophytol	$C_{20}H_{40}O$	297	0.68	32.74	Antimicrobial, antidiabetic, antiarthiritis activity
52	Sinapic acid	$C_{11}H_{12}O_5$	224	1.23	33.09	Antioxidant, antimicrobial, antiinflammatory, anticancer, anti-anxiety activity
53	1 R-α-pinene	$C_{10}H_{16}$	136	0.99	33.22	Antimicrobial activity
54	1,2-benzenedicarboxy-lic acid dinonyl ester	$C_{26}H_{42}O_4$	419	0.29	33.39	Reduce Aβ-induced neurotoxicity, possibly by reducing oxidative stress
55	4-Azafluorenones	$C_{12}H_7NO$	181	1.37	33.46	Antimicrobial,

						antimalarial, DNA-damaging activity
56	Thioguanine	$C_5H_5N_5S$	167	0.20	33.64	Treatment of certain autoimmune, immunologic and related diseases, treatment in psoriasis, leukemias
57	Sinapaldehyde	$C_{11}H_{12}O_4$	208	2.10	33.77	Antimicrobial, antiinflammatory, anticancer, anesthetic, antiulcer antiviral, hypoglycemic activity
58	Desaspidinol	$C_{11}H_{14}O_4$	210	12.03	33.49	Antibacterial, antioxidant activity
59	2,6-Dimethylphenol	$C_8H_{10}O$	122	0.83	34.44	Antioxidant, an antifungal age nt and a modified phenol resin
60	Thymol	$C_{14}H_{10}O$	150	0.83	34.44	Antioxidant, antifungal activity, antibacterial activity
61	α-cyclocitral	$C_{10}H_{16}O$	152	26.96	35.32	An inhibitor of leukotriene production
62	Solasonine	$C_{45}H_{73}NO_{16}$	884	0.36	35.69	Antifungal, insecticidal,

						molluscicidal activity
63	5-Isopropyl-6-methyl-hepta-3,5-dien-2-ol	$C_{11}H_{20}$	168	0.38	36.05	Antimicrobial activity
64	Estradiol	$C_{18}H_{24}O_2$	272	0.21	36.47	Promotes viability and survival of neurons in primary neuronal cultures, neuroprotective effects
65	Cycloheptasiloxa-ne, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	519	0.16	36.70	Antioxidant, flavor agent, hypocholesterol-emic effect
66	Cis-Pinane	$C_{10}H_{18}$	138	0.85	37.55	Fragrance and flavor agent used in pharmaceutical and chemical industry
67	Estradiol	$C_{18}H_{24}O_2$	272	1.19	44.87	Promotes viability and survival of neurons in primary neuronal cultures, neuroprotective effects
68	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.09	47.49	Antimicrobial, antioxidant activity
69	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.59	48.51	Antimicrobial, antioxidant activity

Table 2.4.5 Chemical constituents and their activities in SC-CO_2-H extract of *Oroxylum indicum* (L.) Kurz bark

No	Name of Compounds	Molecular formula	MW	Area (%)	RT	Activity
1	1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl-	$C_{15}H_{26}$	206	2.02	1.76	Antimicrobial, antiinflammatory, anti-hyperlipidemic effect
2	Isoprenaline	$C_{11}H_{17}NO_3$	211	0.65	1.98	A beta-adrenergic agonist that used for treatment of bradycardia, heart block, hypotensive conditions and rarely for asthma
3	Metoprolol	$C_{15}H_{25}NO_3$	267	0.41	2.13	β 1-selective adrenoceptor blocking drug to treat high blood pressure, heart failure, and Prophylactic
4	Acetic acid	$C_2H_4O_2$	60	5.25	2.32	Antibacterial, antifungal activity
5	Benserazide	$C_2H_4O_2$	60	1.64	2.48	Antitumor agents for cancer therapy.
6	Hexanoic acid	$C_6H_{12}O_6$	116	0.71	3.02	Antimicrobial, Antifungal activity
7	Albuterol	$C_{13}H_{21}NO_3$	239	0.69	3.24	Used to treat acute episodes of asthma
8	Erythritol	$C_2H_4O_2$	122	1.1	3.47	Sweeteners,

						food additive
9	Piperazine	$C_4H_{10}N_2$	86	0.74	3.99	Antiparasitic activity
10	D-Allose	$C_6H_{12}O_6$	180	0.23	5.32	Anticancer, antioxidant activity
11	Furfural	$C_5H_4O_2$	96	0.45	5.35	Antibacterial activity
12	Furfural	$C_5H_4O_2$	96	0.17	5.54	Antibacterial activity
13	2-Furanmethanol	$C_5H_6O_2$	98	0.18	6.02	Antioxidant, antibacterial activity
14	Mannosamine	$C_6H_{13}NO_5$	179	0.95	6.76	Inhibitor of glycosylphosphat-idylinositol incorporation into proteins which is useful for treatment of parasite disease
15	7-Hexadecene, (Z)-	$C1_6H_{32}$	224	0.28	7.63	Thioredoxin inhibitor, pulmonary edema, irritation, tetany, diarrhea
16	D-Allose	$C_6H_{12}O_6$	180	0.52	9.19	Anticancer, antioxidant activity
17	D-Glucuronolactone	$C_6H_8O_6$	176	0.15	9.43	Detoxicant
18	2-Deoxy-D-galactose	$C_6H_{12}O$	164	0.18	9.53	Antitumor activity
19	N,N-Dimethyltryptam-ine	$C_{12}H_{16}N_2$	188	0.79	9.69	Psychotropic properties
20	2-Methyl-3-	C_5H_6OS	114	0.26	11.04	Meaty aroma

	furanthiol					volatile
21	Acetophenone	C_8H_8O	120	0.50	11.74	Flavor, hypnotic, anticonvulsant
22	6-Methyluracil	$C_5H_6N_2O_2$	126	1.70	12.11	Antiulcer agent and in the treatment of hepatitis and pancreatitis agent
23	1-Nonen-4-ol	$C_9H_{18}O$	142	1.6	12.25	Food additive, flavor and fragrance
24	6-Octen-2-one, (Z)-	$C_8H_{14}O$	126	1.12	12.79	Flavor agent
25	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	2.35	13.94	Antimicrobial, antiinflammatory, antiproliferative antioxidant, automatic nerve activity
26	N-Acetyl-.alpha.-D-glucosamine	$C_8H_{15}NO_6$	221	0.44	14.42	Therapies for oste oarthritis
27	Undecanoic acid	$C_{11}H_{22}O_2$	186	0.24	14.70	Antimycotic activity
28	1,2-Benzenediol	$C_6H_6O_2$	110	0.54	15.75	Anticancer (breast), antioxidant, pesticide
29	2-Furancarboxaldeh-yde, 5-(hydroxymethyl)-	$C_6H_6O_3$	126	5.72	16.36	Antimicrobial, preservative clastogenic activity, uterotonic
30	2-Furancarboxaldeh-yde, 5-(hydroxymethyl)-	$C_6H_6O_3$	126	0.61	16.76	Antimicrobial, preservative clastogenic activity, uterotonic

31	2,6-Dihydroxyacetoph-enone	$C_8H_8O_3$	152	0.32	17.72	Antioxidant activity
32	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	0.32	18.77	Antimicrobial, antioxidant, antiinflammatory, analgesic activity
33	1-Tetradecene	$C_{14}H_{28}$	196	1.02	19.94	Anti-tuberculosis activity
34	2-Furanmethanol	$C_5H_6O_2$	98	0.35	20.51	Antioxidant, antibacterial activity
35	2,4-Diaminopyrimidi-ne	$C_4H_6N_4$	110	1.47	20.83	Potent human dihydrofolate reductase (DHFR) inhibitors
36	Vanillin	$C_8H_8O_3$	152	1.17	20.96	Antioxidant, anticancer, antibacterial, antimutagenic, and antitumor activity
37	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	0.76	21.20	Antiviral substance
38	Maltose	$C_{12}H_{22}O_{11}$	342	14.84	22.27	Sweetener, bulking agent, excipient
39	Benzofuran-2-carboxylic acid	$C_9H_6O_3$	162	1.30	22.94	Potent Pim-1 inhibitors, leukotriene biosynthesis Inhibitor
40	N-Tetracosanol-1	$C_{24}H_{50}O$	354	0.15	23.11	Antibacterial activity
41	N-Acetylcysteine	$C_5H_9NO_3S$	163	0.49	23.21	Antioxidant activity

42	Methyl cinnamate	$C_{10}H_{10}O_2$	162	0.63	23.30	Antifungal and antiaflatoxigenic activity
43	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	$C_6H_6O_3$	126	0.65	23.88	Antimicrobial, preservative clastogenic, uterotonic
44	Isovanillic acid	$C_8H_8O_4$	168	0.52	25.04	Antibacterial, antioxidant activity
45	Ethyl 2-methylbutanoate	$C_7H_{14}O_2$	130	3.34	25.56	Flavor agent
46	3-Deoxy-d-mannoic lactone	$C_6H1_{10}O_5$	162	0.50	25.81	Antibacterial, antifungal activity
47	Sucrose	$C_{12}H1_{22}O_{11}$	342	1.48	25.92	Antihiccup, antiophthalmitc, antioxidant, atherogenic, collyrium demulcent, flatugenic, hydpercholesterolemic, preservative, triglycerigenic, uricogenic, ulnerary
48	3-Deoxy-d-mannoic lactone	$C_6H1_{10}O_5$	162	0.48	26.63	Antibacterial, antifungal activity
49	4-Azafluorenones	$C_{12}H_7NO$	181	0.64	27.18	Antimicrobial, immunomodulatory, antitumor activity
50	Myristicin	$C_{11}H_{12}O_3$	192	0.42	27.57	Antibacterial, antiinflammatory, hepatoprotective activity, flavor

						agent
51	Ferulic acid	$C_{10}H_{10}O_4$	194	0.79	27.99	Antiinflammatory antiatherogenic, antidiabetic, antiageing, neuroprotective, radioprotective, hepatoprotective
52	Ferulic acid	$C_{10}H_{10}O_4$	194	0.53	28.09	Antiinflammatory antiatherogenic, antidiabetic, antiageing, neuroprotective, radioprotective, hepatoprotective
53	Dihydroferulic acid	$C_{10}H_{12}O_4$	196	0.75	28.69	Antioxidant activity
54	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	10.23	28.85	Antimicrobial, antioxidant, antiinflammatory, analgesic activity
55	Octadecamethyl Cyclononasiloxa-ne	$C_{18}H_{54}O_9Si_9$	667	0.84	30.89	Antioxidant, antioksidaan and antimicrobial activity
56	Sinapic acid	$C_{11}H_{12}O_5$	224	0.84	33.09	Antioxidant, antimicrobial, antiinflammatory, anticancer, and anti-anxiety activity
57	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	0.73	33.39	Plasticizer compound, antimicrobial, antifouling activity
58	Thioguanine	$C_5H_5N_5S$	167	0.59	33.78	Treatment of

						certain autoimmune, immunologic, psoriasis, leukemias
59	Fraxetin	$C_{10}H_8O_5$	208	0.59	33.78	Antioxidant, antimicrobial activity
60	Desaspidinol	$C_{11}H_{14}O_4$	210	3.8	33.93	Antibacterial, antioxidant activity
61	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.22	36.70	Antimicrobial, antioxidant activity
62	9,10-anthracenedione-1,8-dihydroxy-3-methoxy-6-methyl	$C_{16}H_{12}O_5$	284	3.91	44.71	Anticancer, anti-inflammatory, antimicrobial hepatoprotective activity
63	Estradiol	$C_{18}H_{24}O_2$	272	2.01	44.87	Promotes viability and survival of neurons in primary neuronal cultures, neuroprotective effects
64	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222	0.18	48.53	Antimicrobial, antioxidant activity

2.4.3.5 Characterization residues after SC-CO_2-H extraction

SEM images of Khmer medicinal plants before and after SC-CO_2-H extraction are illustrated in Figure 2.4.11. The SEM observation shows that the SC-CO_2-H extraction made physical change in Khmer medicinal plants. The surface

morphology of original Khmer medicinal plants were smooth and have no present of any crake or pore. However, there were some crakes and pores appeared on the surface morphology of Khmer medicinal plants after SC-CO_2-H extraction. This indicated SC-CO_2-H could break down the cell wall of plants and extract the their components. FTIR spectra of Khmer medicinal plant before and after SC-CO_2-H extraction are shown in Figure 2.4.12. FTIR results also demonstrated that the behavior of FTIR spectra and intensity of solid residues after SC-CO_2-H extraction were completely diffent from those of Khmer medicinal plants before extraction. This suggested some functional compounds were removed from plant materials during extraction. The change of surface morphology and the behavior of FTIR spectra of Khmer medical plants before and after SC-CO_2-H extraction could be explained by the mechanism of SC-CO_2-H extraction. At hydrothermal condition, water acts not only as solvent but also as catalyst, thus it can be used to extract the polar compounds or decompose lignin material to produce bioactive compounds [29, 30]. When CO_2 is introduced in water in its supercritical state and then CO_2 dissolves in water and reacts to form carbonic acid. The acidification of the water phase influence the extraction of solutes from the solid matrix especially those with affinity in acid medium. The structure of solid material was disrupted and swollen by the fluid, which makes the fluid facilitate to penetrate into the solid material in contact with the solute. Finally, the solute distributes and diffuses out of the solid material into the extraction fluid [9-11]. Similar structure change was earlier reported by for barley grain treated with SC-CO_2-H [11].

2.4.4 Conclusion

The results of present investigation report the total phenolic compounds contents and antioxidant activity in the five Khmer medicinal plants extracts using SC-CO_2-H extraction. The total phenolic compounds contents in SC-CO_2-H extract showed positive correlations with antioxidant activity. Compared to conventional methods, the total phenolic compounds contents in SC-CO_2-H extracts were higher than those in SoxM and HW extract and the antioxidant activity in SC-CO_2-H extract was similar with those of SoxM. The result of GC-MS suggested that five Khmer medicinal plants had many functional compounds with a wide range of biological activities that could be applied in food, pharmaceutical and cosmetic industries.

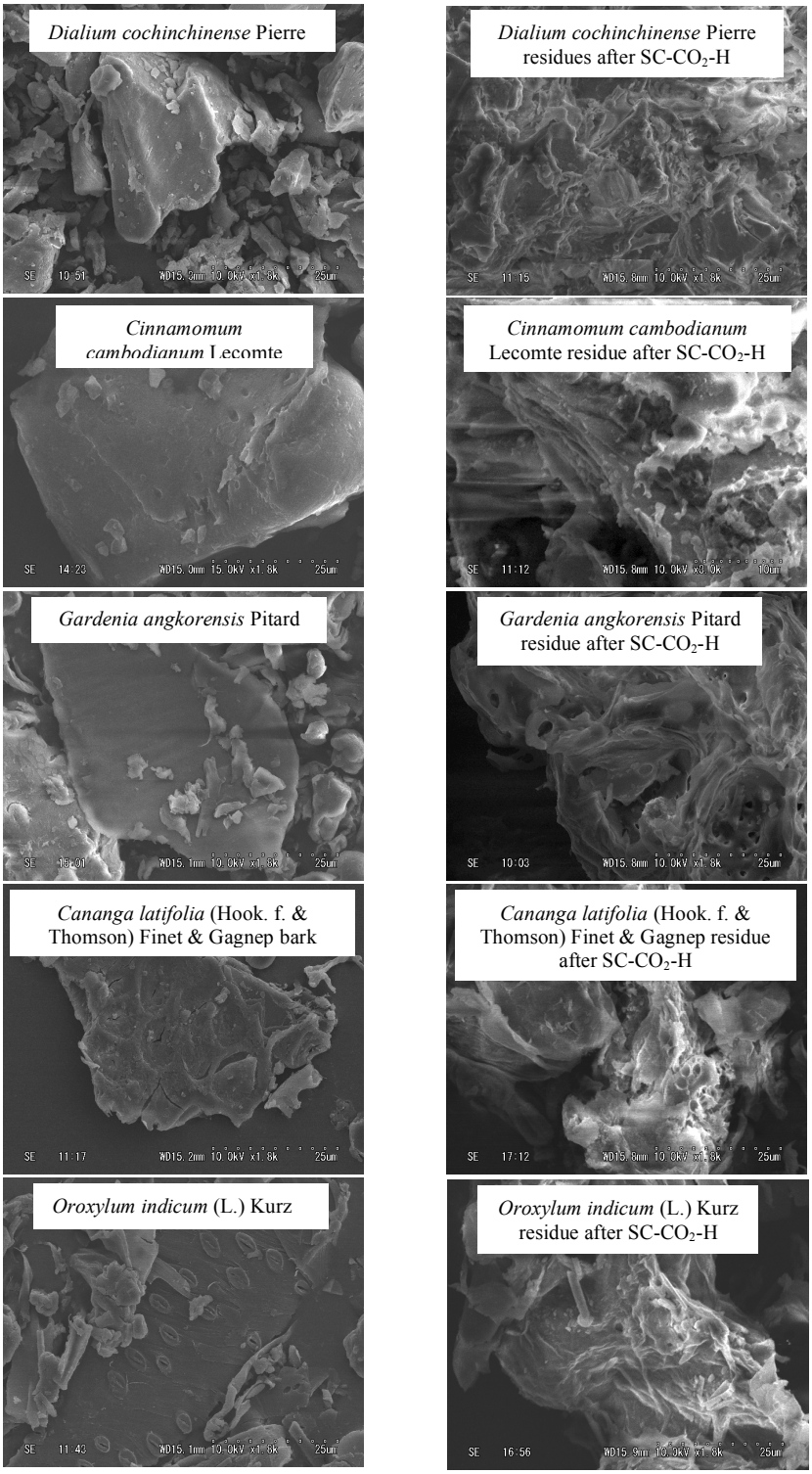

Figure 2.4.11 SEM image of Khmer medicinal plants before and after SC-CO_2-H

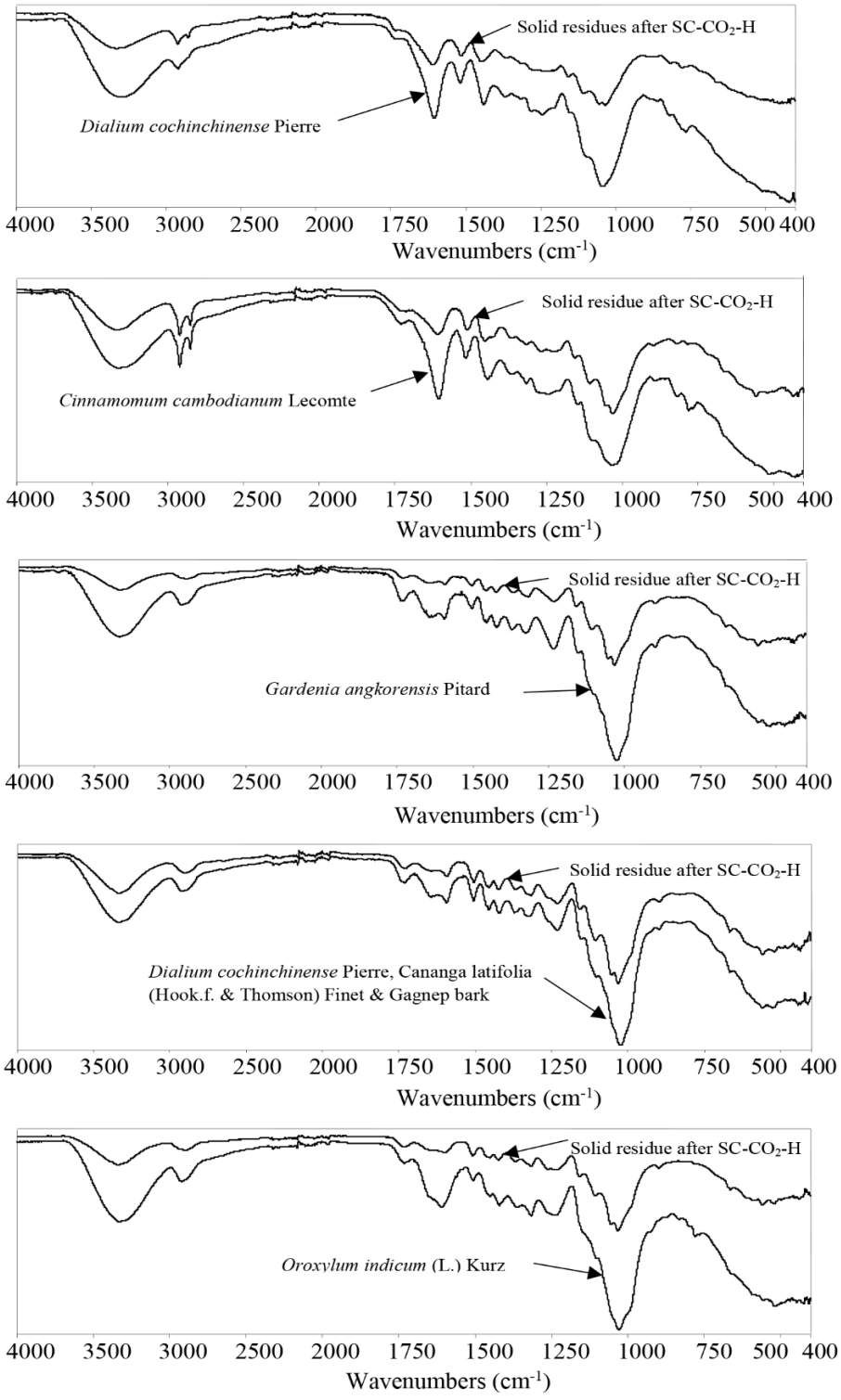

Figure 2.4.12 FTIR of Khmer medicinal plants before and after SC-CO_2-H extraction

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Chapter 3

Micronization of curcumin with biodegradable polymer by supercritical anti-solvent using micro swirl mixer

3.1 Introduction

Curcumin is a hydrophobic phenolic compound derived from turmeric has been used for centuries as a remedy to treat many diseases. Recently, curcumin has attracted increasing interest from many scientific researchers and pharmaceutical industries due to its health-promoting benefits. Curcumin has showed to exhibit antioxidant, anti-inflammatory, anti-bacterial, antifungal, antimicrobial, anti-carcinogenic activities, also contributing to the treatment of multiple myeloma, Alzheimer's, psoriasis, myelodysplastic syndrome, and anti-human immunodeficiency virus (Anti-HIV) cycle replication [1].

In spite of the medical and therapeutic properties of curcumin, its complex structure is also responsible for the problem of curcumin bioavailability such as poor absorption, rapid metabolism and elimination due to its high hydrophobicity and low solubility in water [2]. As a result, the use of curcumin is limited in functional food and pharmaceutical application. Because of the curcumin benefices and the limited use of curcumin, there is a great interest in developing new technology to modify the structure and/or new formulation of molecule in order to enhance the bioavailability of this interesting phenolic compound.

Micronization of bioactive compounds with biopolymer has been found to be successful in enhancement of the therapeutic performance in the preparation of drug delivery systems. This method can decrease the particle size, increase the surface area, and enhance the solubility and bioavailibity of bioactive compounds. Moreover, it can control the drug release system including protection from rapid degradation, controlled release rate and the prolonged duration of bioactive compounds [3,4]. Various methods have been used to produce microparticles of curcumin or curcumin-polymer composite. The traditional methods such as spray drying, oil-in-water emulsion, solvent-nonsolvent precipitation, sol-gel-based polymerization, coarcevation were used to precipitate the curcumin. However, the aforementioned techniques have some disadvantages. For example, the bioactive compounds can be degraded due to use of high temperature. Also, the particle size and morphology is difficult to control. The large amount of organic solvent, surfactants and other additives are required in the process, which may cause the toxic residues in final product [5].

To overcome the drawbacks associated with conventional method, supercritical anti-solvent (SAS) has been used. This method has been found to successfully apply to the encapsulation of food ingredient, pesticides, drugs, genes, and other bioactive agent in cosmetic, electronic, biomedicine, and other industries. The characteristic of particle (particle size, crystalline structure) can control by changing the process parameters. Furthermore, the degradation of compounds can be avoided due to mild temperature processing [6-14]. Recently, encapsulation of material with biodegradable polymer by SAS process also have been used to enhance the dissolution rate of poorly water-soluble material such as celeoxib [7], diflunisal [8], Folic acid [9], curcumin [6,10], corticosteroid [11], ellagic acid [12], and nimesulide [13].

Due to the low solubility of curcumin in supercritical carbon dioxide (CO_2), supercritical CO_2 techniques have been studied for the micronization of curcumin. Adami et al. [5] used supercritical assisted atomization to fabricate the curcumin

with polyvinylpyrrolidone (PVP). The authors produced the particle size smaller than 400 nm. Zabihi et al. [6] used fluidization assisted SAS process to produce the curcumin nanoparticle with poly (lactic-co-glycolic acid) (PLGA). The average size of PLGA-curcumin of 40 nm was obtained. Xie et al. [15] produced curcumin with silk-fibroin using SAS process. They produced the particle size less than 100 nm. Jia et al., [16] prepared the curcumin proliposom by using ultrasound-assisted supercritical anti-solvent method. The smallest curcumin proliposom particle size was 85 nm. Pedro et al. [17] encapsulated curcumin in solid particle by Gas Saturated Solution technology with small particle size distribution. Baldino et al. [18] used SAS process to fabricate the nanostructure of curcumin in biodegradable cellulose acetate.

Various types of SAS process have been developed including a batch type and continuous type. In most of the processes, high pressure precipitation vessel is required to produce particles. The important part of the SAS device is mixing of organic solvent and supercritical fluid. The objective of this study is to investigate micronization of curcumin with PVP using SAS process with swirl mixer. Recently, many types of mixer such as Y-shaped, nozzle-type, central-collision-type, and swirl mixer type have been developed in order to avoid the formation of large particle and wide size distribution during nanoparticle production. Swirl mixer is one of effective mixer can produce small size nanoparticles with a narrow size distribution [19]. Kawasaki et al. [19] used micro swirl mixer for producing fine metal oxide nanoparticle by continuous supercritical hydrothermal method. The result showed that using micro swirl mixer with diameter of 0.5 mm produced the average particle size of 20 nm. In this study, the effect of different parameters such as curcumin/PVP ratio, feed concentration, temperature, pressure, flow rate of carbon dioxide (CO_2) on particle formation using SAS process with swirl mixer was determined.

3.2 Materials and methods

3.2.1 Materials and chemicals

Crystalline curcumin and PVP (average molecular weight 10000) were purchased from Wako and Sigma-Aldrich, Japan. Ethanol (>99.5%) and acetone (>99.7%) were provided by Wako, Japan. CO_2 (>99.9%) was obtained from Sogo Kariya Sanso, Inc., Japan.

3.2.2 Method

The micronization of curcumin with polymer was prepared by SAS with swirl mixer. Figure 3.1 shows a schematic diagram of SAS apparatus with swirl mixer. The apparatus consists of a CO_2 chiller (TBG020AA, Toyo Seisakusho Kaisha, Ltd, Japan), a pump for CO_2 (PU-1586 Intelligent HPLC pump, JASCO, Japan), a pump for solvent (LC-6AD, SHIMADZU, Japan), a heater chamber (WFO-400W, EYELA, Japan), swirl mixer (4-1/16YSM-0.8-0.5-S, Sugiyama Shoji Co., Ltd., Japan), a collector (SS-2TF-05, Swagelok, USA), and a back-pressure regulator (BPR; AKICO, Japan).

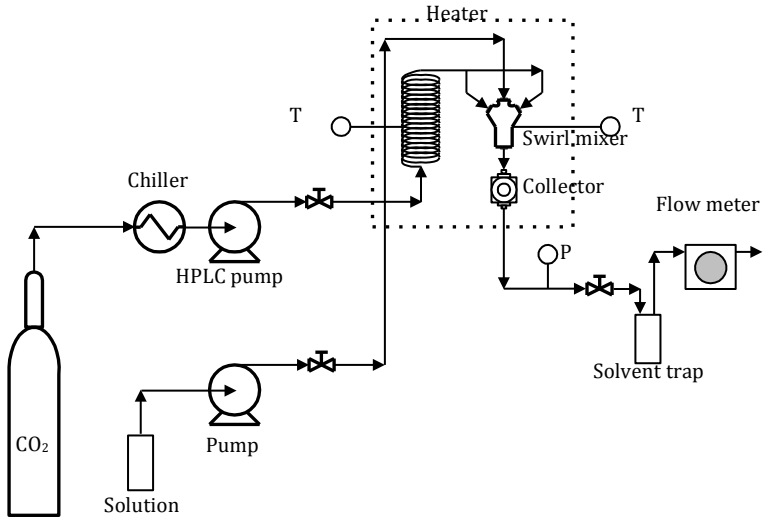


Figure 3.1 Schematic diagram of SAS process with swirl mixer

In this process, the heater chamber was first set to the desired temperature. Then, CO_2 was added to heater chamber at desired flow rate until the desired pressure was reached. When the desired operational condition (temperature, pressure, CO_2 flow rate) was achieved, curcumin with PVP solution was injected to swirl mixer for 60 min. After that, pure CO_2 was pumped to wash the precipitated particle for 120 min to eliminate the remaining solvent. When the washing process was completed, the pressure was released slowly by using back-pressure regulator. The micronized particle was collected on the filter membrane and kept in desiccator in the absence of light until analysis to avoid the degradation of curcumin. The experiment was carried out at temperature of 30-50 ^{o}C, pressure of 10-20 MPa, and CO_2 flow rate of 10-20 mL/min. The curcumin and PVP ratio was varies from 1:10 to 1:30. As a

solvent to dissolve curcumin/PVP mixture, the mixture of acetone-ethanol with ratio 90:10 (v/v) was used. Feed concentration was 1-10 mg/mL. The flow rate of curcumin with PVP solution was 0.1 mL/min.

3.2.3 Surface morphology, particle size, and particle size distribution

The surface morphology of curcumin/PVP particle was determined by scanning electron microscopy (SEM; S-4300, Hitachi, Japan). Before SEM analysis, sample was placed in the aluminum holder and coated with gold at the high pressure evaporator. The particle size and the particle size distribution were determined using Image J software from SEM photograph for at least 250 particles collected at each experiment.

3.2.4 FTIR analysis

Raw curcumin, PVP, and curcumin/PVP particles were analyzed using a Spectrum Two FT-IR spectrophotometer (PerkinElmer Ltd., England) in order to determine their structure. Data was attained between 4000 to 400 cm^{-1}.

3.2.5 Dissolution study

The dissolution of the original curcumin and the curcumin/PVP particles was measured using an UV/vis spectrophotometer (V-550, JASCO, Japan). An accurate amount of sample containing equivalent of amount of curcumin for drug/polymer ratio was dispersed in 20 mL of distilled water. After 12 h, the sample was filtered with 0.2 μm disposal membrane filter. Thereafter, the absorbance of solution at 430 nm wavelength was measured.

3.3 Results and discussion

3.3.1 Curcumin/PVP particle formation by SAS

To study the particle formation using SAS process with swirl mixer, the morphology of curcumin and PVP before and after process was observed by using SEM (Figure 3.2). The original curcumin particles (Figure 3.2(A)) were irregular with mean size approximately 6 μm, while the original PVP particles (Figure 3.2(B)) were almost spherical with mean of particle size of 11 μm. On the other hand, the spherical curcumin/PVP microparticles (Figure 3.2(C)) were obtained when both solutes was

precipitated simultaneously at temperature of 40 ^{o}C, pressure of 15 MPa, CO_2 flow rate of 15 mL/min, feed concentration of 5 mg/mL and curcumin/PVP ratio of 1:30. The particle size was between 25 and 342 nm. According to these images, it could be concluded that the precipitation of curcumin and PVP was successfully performed using SAS process with swirl mixer. Effect of operational parameters (curcumin/PVP ratio, concentration, temperature, pressure, CO_2 flow rate) was studied for the efficacy of SAS process with swirl mixer.

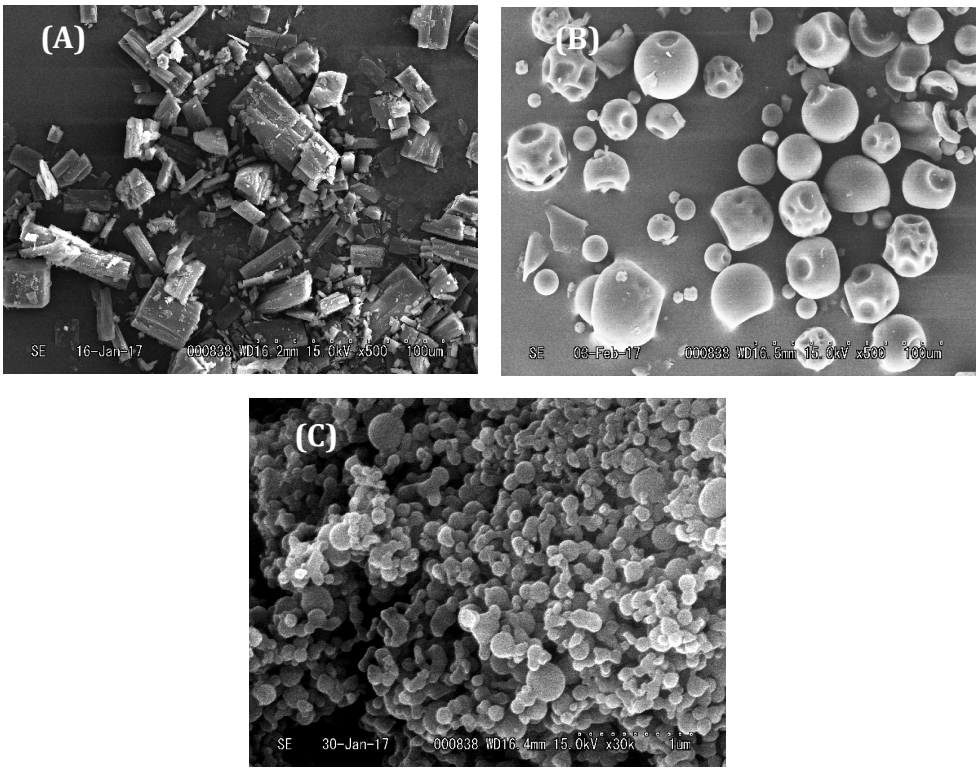


Figure 3.2 SEM images of (A) raw curcumin, (B) PVP, and (C) curcumin/PVP particles

3.3.2 Effect of curcumin/PVP ratio in acetone:ethanol solution

Both nature of drug and percent drug loading significantly affect the morphology of particles and particle size distribution. In this experiment, the curcumin/PVP ratio in acetone:ethanol solution was varied from 1:10 to 1:30 at temperature of 40 ^{o}C, pressure of 15 MPa, CO_2 flow rate of 15 mL/min, and feed concentration of 5 mg/mL. According to Figure 3.3 (A), it could be seen that a small decrease in mean particle sizes ranging from 104 to 90 nm with increasing curcumin/PVP ratio from

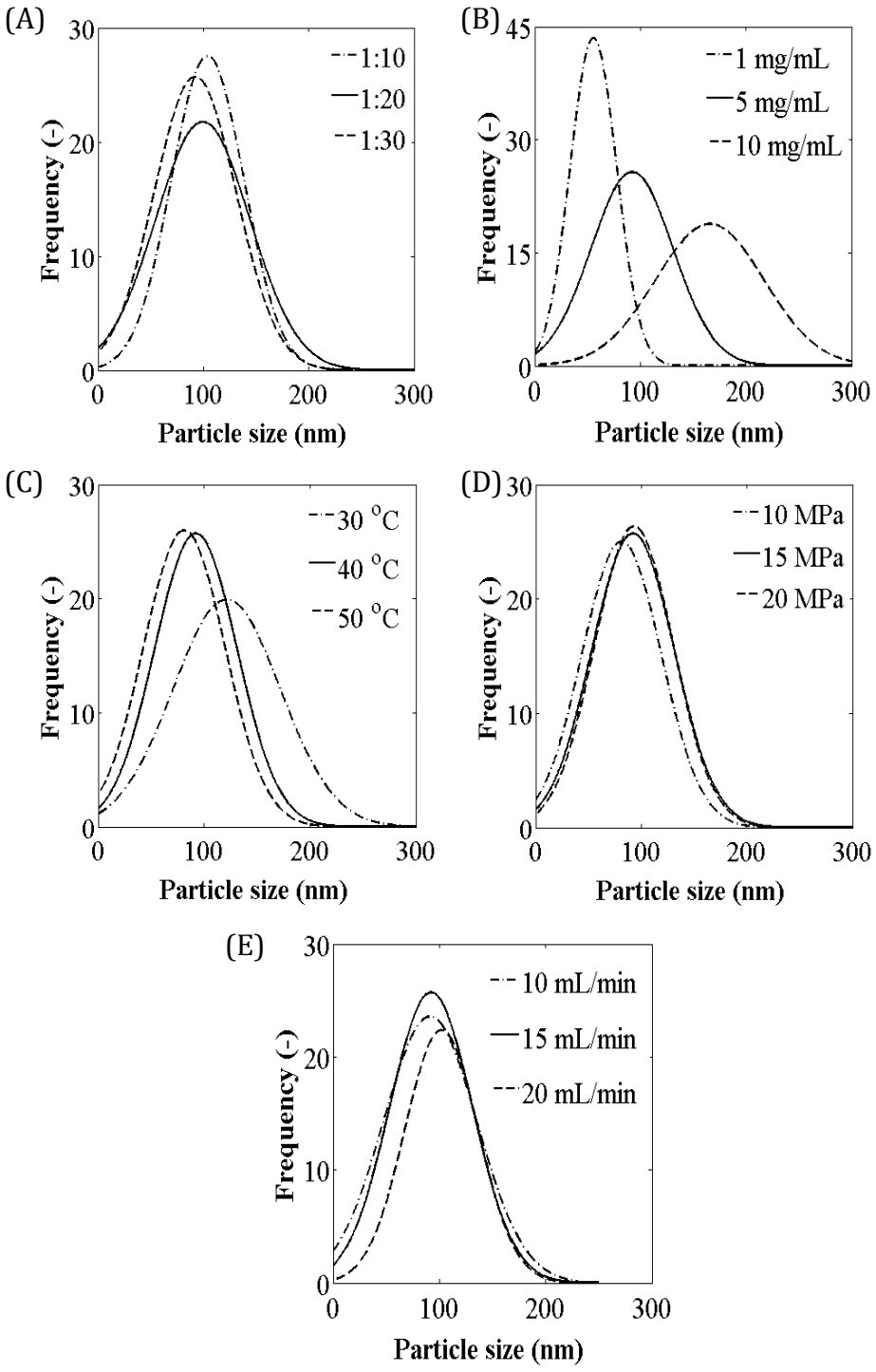


Figure 3.3 Particle size distribution at different operating conditions (A) curcumin:PVP ratio, (B) concentration, (C) temperature, (D) pressure, and (E) flow rate of CO_2

1:10 to 1:30. This result could be supported by the fact that the presence of curcumin increases the viscosity of the feed solution. When the lower viscosity of feed solution is used in SAS process, the smaller droplet and particle size are precipitated [5]. Moreover, the much agglomerated and irregular microparticles were found at the low curcumin/PVP ratio of 1:10 and 1:20 (Figure 3.4). This may be due to the insufficient amount of polymer for hardening the droplet into spherical particles. Similar result was obtained by Patomchaiviwat et al. [20]. They found that the Rifampicin-L-PLA began to form the agglomerated and irregular microparticles at polymer drug ratio of 5:5 or below because the amount of polymer was not enough to form the spherical particles.

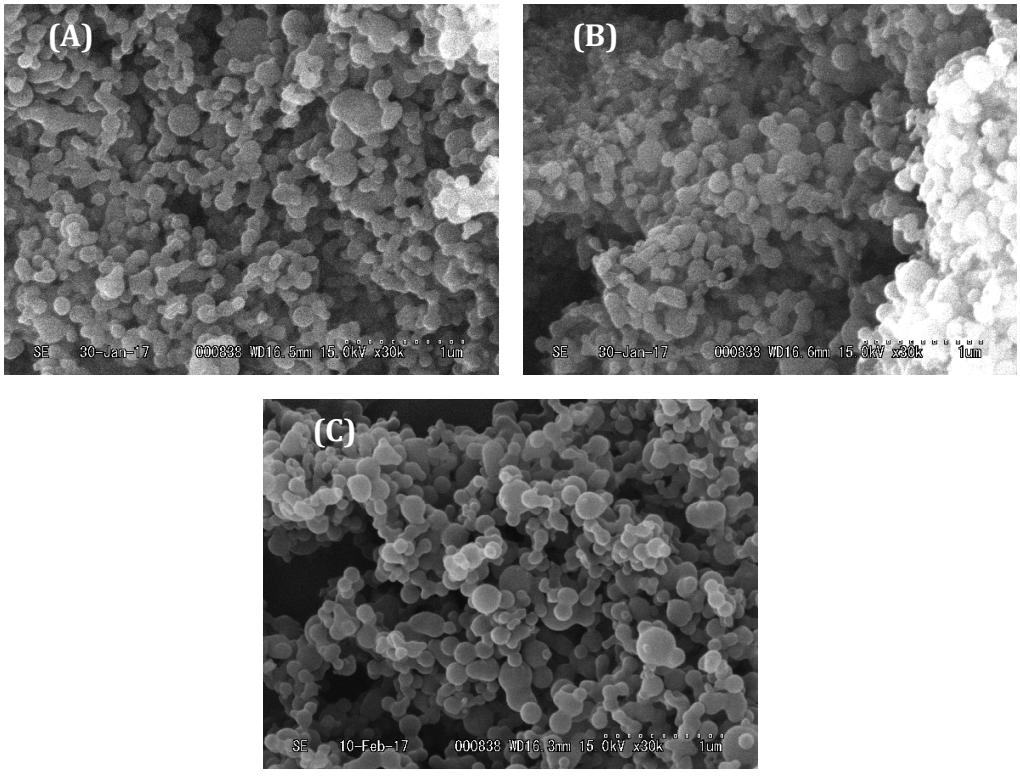


Figure 3.4 SEM images of curcumin/PVP particles at curcumin:PVP ratio of (A) 1:10, (B) 1:20, (C) 1:30

3.3.3 Effect of feed solution

The effect of feed concentration on the morphology and the particle size distribution was investigated at temperature of 40 ^{o}C, pressure of 15 MPa, CO_2 flow rate of 15 mL/min, and curcumin/PVP ratio of 1:30 as shown in Figure 3.5 and 3.3 (B).

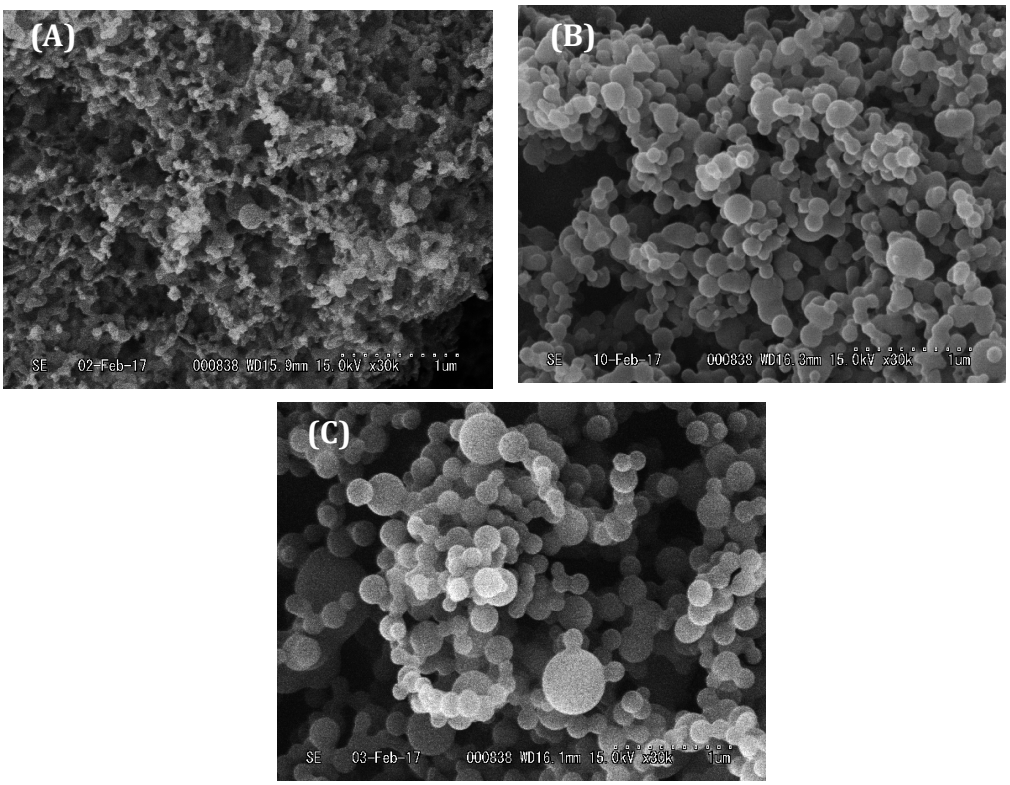


Figure 3.5 SEM images of curcumin/PVP particles at feed concentration of (A) 1 mg/mL, (B) 5 mg/ml, (C) 10 mg/mL

The mean of particle size drastically increased from 55 to 164 nm with increasing the feed concentration range from 1 to 10 mg/mL. The curcumin/PVP particles precipitated in form of sub microparticles with agglomeration for the concentration of 1mg/mL and in form microparticles for the concentration of 5 and 10 mg·mL^{-1}. This result indicated that the concentration of feed solution influenced on the particle size. The low concentration may produce the fine particles due to the nucleation and growth process. Reverchon et al. [21] suggested that when the low concentration of solution was used, the saturation and then precipitation of solute occurred very late during the droplet expansion process. As the result, the nucleation is the prevailing mechanism at these conditions, thus the small particles with small

size distribution were produced. On the other hand, when the concentrate solution was used, solute precipitation of solute obtained earlier during droplet expansion process and the mechanism of particle growth intersected with nucleation, resulting in the production of the large particle with wide particle size distribution. Similar result was reported by De Marco and Reverchon [22]. They found that increasing concentration of sample in liquid solution could increase the mean particle size and enlarged the particle size distribution.

3.3.4 Effect of temperature

The effect of temperature on the morphology and particle size distribution of the curcumin/PVP particles was studied at pressure of 15 MPa, CO_2 flow rate of 15 mL/min, feed concentration of 5 mg/mL, and curcumin/PVP ratio of 1:30 (Figure 3.6 and 3.3 (C)).

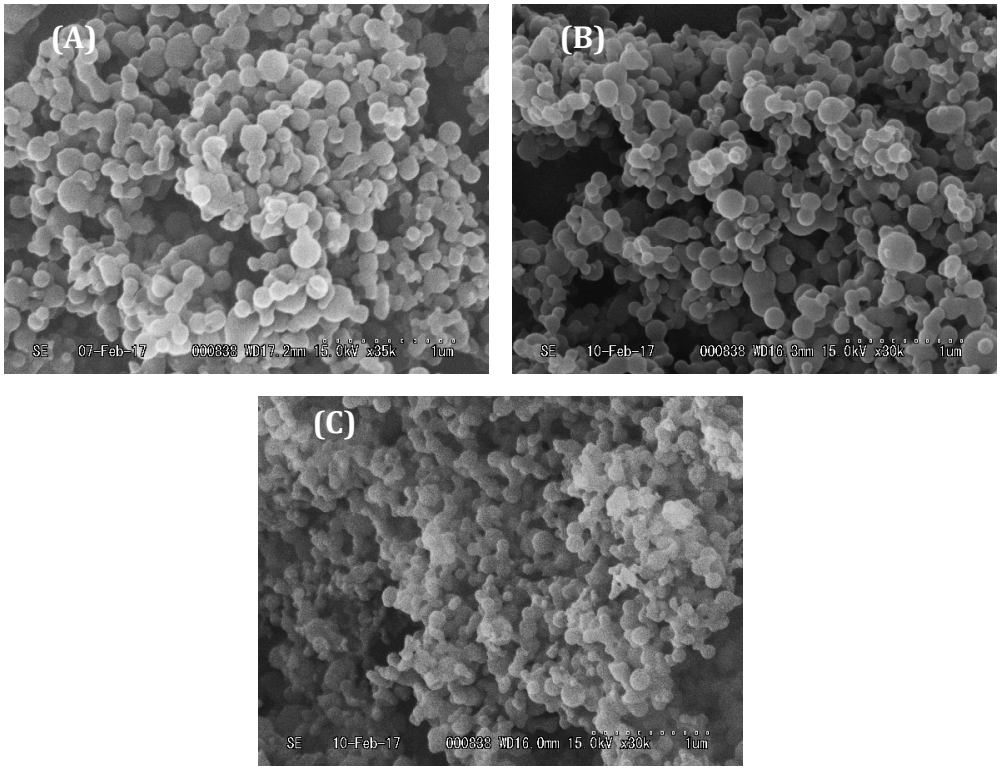


Figure 3.6 SEM images of curcumin/PVP particles at temperature of (A) 30 ^{o}C, (B) 40 ^{o}C, (C) 50 ^{o}C

Mean of particle size decreased from 114 to 78 nm with increasing temperature from 30 to 50 ^{o}C. It was also notable that the much agglomeration of particles was found in particle at 30 and 50 ^{o}C. This tendency was explained by the fact that increasing temperature increased the mass transfer between the solvent and supercritical CO_2, which lead to the formation of high supersaturation and small particles were produced. However, when the temperature was higher than certain value, the supercritical CO_2 might decrease the grass transition temperature of biodegradable polymers. At low grass transition temperature, polymers tend to form sticky and aggregated particles [23-25]. Miguel et al. [26] found that increasing temperature decreased particle size of lycopene because increasing temperature the decreased solubility, which triggered higher supersaturation.

3.3.5 Effect of pressure

Figure 3.7 and 3.3 (D) shows the effect of pressure on the morphology and particle size distribution of the curcumin/PVP particles at temperature of 40 ^{o}C, CO_2 flow rate of 15 mL/min, feed concentration of 5 mg/mL, and curcumin/PVP ratio of 1:30. On increasing pressure from 10 to 15 MPa, mean of particle size slightly increased from 81 to 90 nm. The irregular of particle size was observed at 10 MPa. When pressure rose from 15 to 20 MPa, no significant change of particle size was observed. At low pressure, density of CO_2 was decreased, resulting the low solubility of organic solvent in SC-CO_2 [27, 28]. Because there was limitation of mass transfer between solvent and CO_2 by interface of the droplets, some particles were generated by droplets drying during the precipitation process. Consequently, some big particle sizes were produced [27]. At high pressure, the volumetric expansion of liquid phase occurred due to high amount of CO_2 solubilized in it. As the result, the diffusion driving force and solubility of solvent increased, it caused the diminution of the partial molar volume and cohesive energy density of the solvent, lowing the solvent power for the solid solute and generated the high supersaturation. Thus, the small mean particle size with narrower particle size was precipitated [29]. On the other hand, Reverchon [30] suggested that there was no significant the effect of pressure in particle size and particle size distribution precipitated if the pressure was larger than the asymptotic volume expansion.

3.3.6 Effect of CO_2 flow rate

The effect CO_2 flow rate on the morphology and particle size distribution of the curcumin/PVP particles at temperature of 40 ^{o}C, pressure of 15 MPa, feed concentration of 5 mg/mL, and curcumin/PVP ratio of 1:30 is shown in Figure 3.8 and 3.3 (E). Under this condition, mean of particle size increased from 88 to 114 nm with increasing CO_2 flow rate range from 10 to 20 mg/mL. The much agglomeration

of small particles was observed at the low CO_2 flow rate of 10 mg/mL. At high CO_2 flow rate, the big particles with spherical shape were obtained while the small particle size with much agglomeration was found at the low CO_2 flow rate. This result could be explained that the polymer might not able to cover the curcumin completely at low CO_2 flow rate [31]. At high CO_2 flow rate, the solution might achieve supersaturation restrictedly and some nascent were generated firstly, thus the small amount of particles with big size was produced [25]. Base on the results above, it could be concluded that the suitable condition for production curcumin/PVP particles by SAS process with swirl mixer was at temperature of 40 ^{o}C, pressure of 15 MPa, and CO_2 flow rate of 15 mL/min. The particles under this condition were used to analyze the chemical structure and dissolution in aqueous solution.

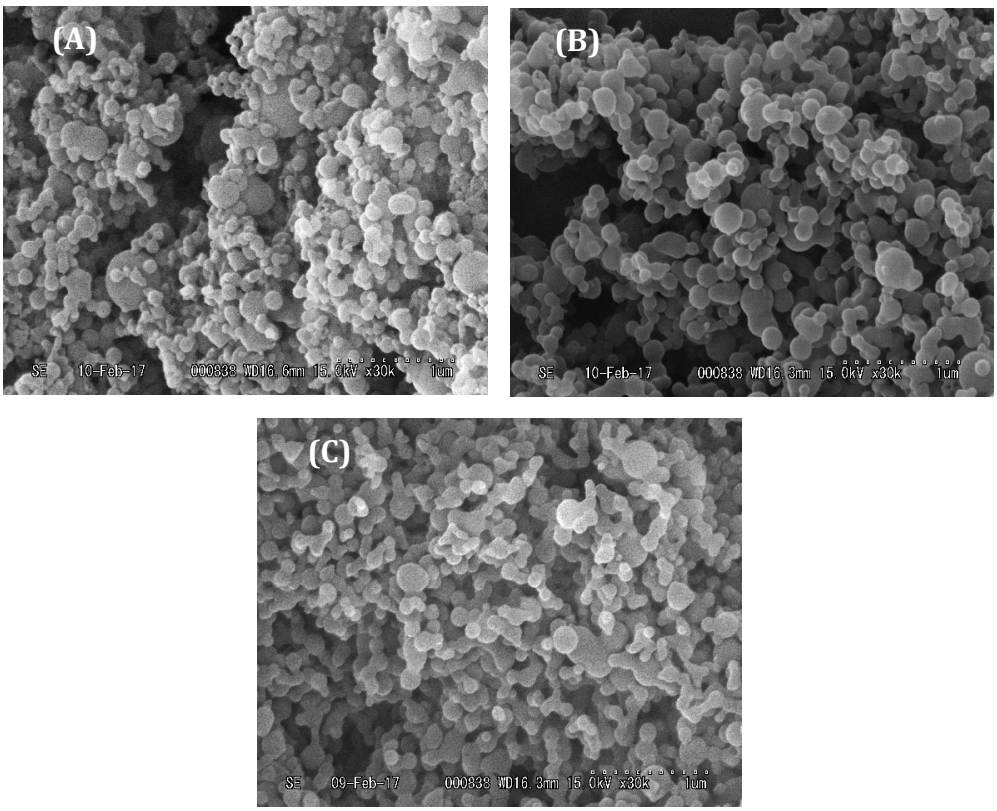


Figure 3.7 SEM images of curcumin/PVP particles at pressure of (A) 10 MPa, (B) 15 MPa, (C) 20 MPa

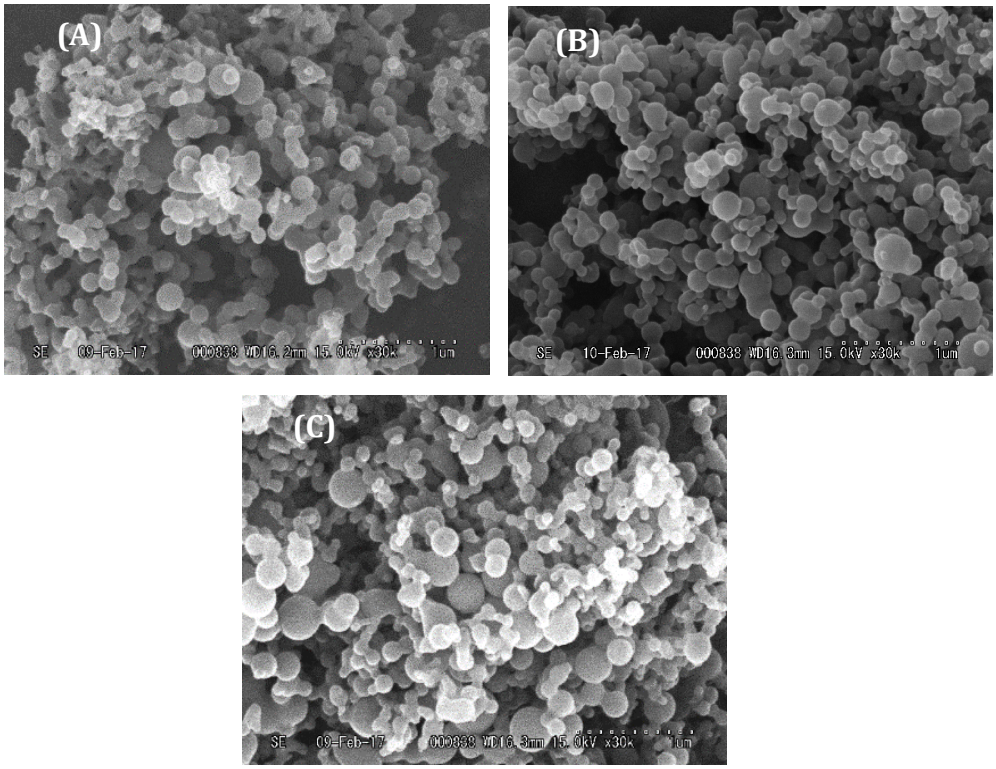


Figure 3.8 SEM images of curcumin/PVP particles at CO_2 flow rate of (A) 10 mL/min, (B) 15 mL/min, (C) 20 mL/min

3.3.7 FTIR analysis

Characterization of particles was determined by using FTIR. Figure 3.9 shows FTIR of raw curcumin, PVP, and curcumin/PVP particles. The FTIR of raw curcumin showed the characteristic absorption band at 3529 cm^{-1} related to O-H stretching vibration. In addition, shape absorption band at 1620 cm^{-1}, 1511 cm^{-1}, 1435 cm^{-1}, 1292 cm^{-1}, 1030 cm^{-1}, 857 cm^{-1} correspond to C=C benzene stretching ring, C=O stretching, C-H bending, C-O stretching, C-O-C stretching vibration and C-H aromatic hydrogen, respectively, was also found in raw curcumin. The FTIR of PVP showed characteristic peak at 1667 cm^{-1} attributed to C=O stretching vibration. Furthermore, the absorption band at 2968 cm^{-1}, 3469 cm^{-1} due to, C-H stretching, O-H bending was obtained in raw PVP. The spectra of curcumin/PVP showed the similar characteristic band of PVP, but the intensity of absorption band of particles was completely different. This could be caused by the formation of intermolecular hydrogen bond between O-H band of curcumin and the C=O band of PVP inside

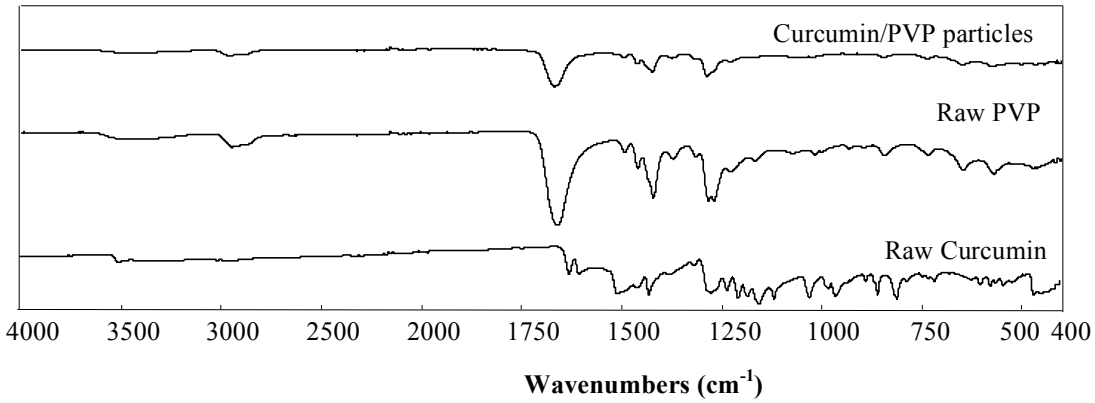
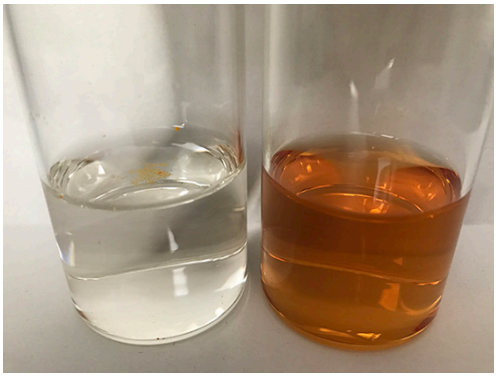


Figure 3.9 FTIR spectrum of raw curcumin, PVP and curcumin/PVP particles

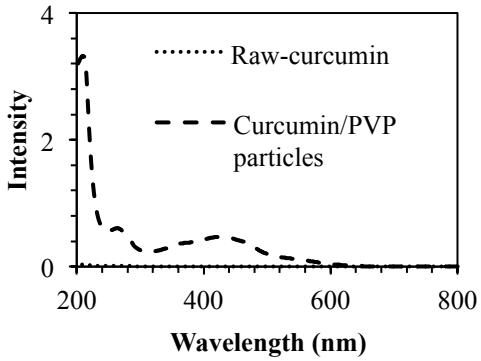
curcumin/PVP particle. Therefore, it could be concluded that the presence of curcumin in the PVP matrix (encapsulation). Moreover, this result also gave information that there was no other chemical bond was formed in curcumin/PVP particles after SAS process [32]. Similar observation was reported by Yen et al. [32]. They produced the curcumin nanoparticle using nanoprecipitation method and found that the wavenumber of C=O absorption band of PVP was reduced and the O-H absorption of curcumin was disappeared in curcumin nanoparticle due to the interaction of hydrogen bond between O-H band of curcumin and the C=O band of PVP in particles.

3.3.8 Dissolution study

In order to study the dissolution of curcumin/PVP particles, raw curcumin and curcumin/PVP particles were dissolved in 20 mL aqueous solution for 12 h. The concentration of curcumin in both solutions was measured by using UV spectrophotometry (Figure 3.10). The result shows that the curcumin/PVP particle completely dissolved in aqueous solution. As the result, the clear yellow solution was obtained. In contrast, raw curcumin, which is hydrophobic phenolic compounds, was less soluble in the aqueous solution. The dissolution of curcumin/PVP particles was 2.34 μg/mL, while the dissolution rate of raw curcumin was 0.006 μg/mL after 12 h. The dissolution of curcumin/PVP particles was faster than that of raw curcumin because PVP is water-soluble polymer, so it can modify the surface properties of the particle and enhance the dissolution of curcumin in aqueous solution [33]. Additionally, the mean curcumin with PVP particles size, which produced by SAS process with swirl mixer, is less than 150 nm, so particles is easy to disperse and dissolve in aqueous solution [34].



(A)



(B)

Figure 3.10 Image of (A) raw curcumin and curcumin/PVP particles in aquoeus solution, (B) UV spectrum of raw curcumin and curcumin/PVP particles in aqueous solution after 12 h

3.4 Conclusion

Curcumin/PVP particles were successfully fabricated by using SAS process with swirl mixer. The morphology of particle and particle size distribution could be controlled by curcumin/PVP ratio, concentration, temperature, pressure, and flow rate of CO_2. The suitable condition for production of curcumin/PVP particles (mean particle size of 90 nm) by SAS process with swirl mixer was at curcumin/PVP ratio 1:30, feed concentration of 5 mg/mL, temperature of 40 ^{o}C, pressure of 15 MPa, and CO_2 flow rate of 15 mL/min. The FTIR results indicated that the amount of curcumin contained inside the particles. In addition, the dissolution test showed that the dissolution of curcumin/PVP particles was faster than that of raw curcumin. Therefore, it could be concluded that micronization of curcumin with PVP by using SAS process with swirl mixer is an effective method to enhance the bioavailability of curcumin.

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Chapter 4

Summary and future perspective

Supercritical fluid method has not only been used to extract bioactive or valuable compounds from plants, industrial by products, algae and microalgae, and extract the toxic compounds and the metal from solid and liquid environmental matrices, but also used for micronization and particle formation. Therefore, this method recently has been applied in food and natural products extraction, pharmaceutics, and environment. In this work, the application of process integration of supercritical fluid with novel techniques in extraction and microencapsulation of bioactive compounds from plant materials was investigated.

Extraction of phenolic compounds and antioxidant activity from garlic husk using carbon dioxide expanded ethanol

The garlic husk, which contributes 25% of garlic bulk, was regarded as agricultural waste. Recent studies have found that the extract from garlic husk contains phenolic compounds with antioxidant and anti-bacterial activities, which can be applied in food and pharmaceutical industries. In order to overcome the drawback of conventional methods, carbon dioxide expanded ethanol (CXE) was used to extract phenolic compounds from garlic husk. The extraction was carried out in ranges of temperature (50-200 °C), CO_2 flow rate (0.5-2 mL/min) at constant pressure

(10 MPa). The high amount of total phenolic compounds of 56.26 mg GAE/g of dried garlic husk and antioxidant activity (IC_{50}) of 0.41 mg/mL was achieved at temperature of 200 °C and CO_2 flow rate of 0.5 mL/min. The five major phenolic compounds identified from CXE method were garlic acid, 4-hydrobenzoic acid, caffeic acid, p-coumaric acid, and trans-ferulic acid. Compared to the pressurized ethanol, CXE not only improved the extraction yield, but also increased the total phenolic compounds and antioxidant activity in extract because CXE had ability to enhance the extraction efficiency compared to organic solvent by reducing viscosity, decreasing the interval interfacial tension, and increasing diffusivity of solvent. Moreover, the amount of total phenolic compounds and antioxidant activity extracted with CXE surpassed those obtained with Soxhlet extraction. It can be concluded that CXE is an efficient method to extract phenolic compounds and improve antioxidant activity of extract from garlic husk.

Extraction of curcumin from turmeric using ultrasound assisted supercritical carbon dioxide

Curcumin, one of phenolic compounds, has been recently shown to have useful pharmacological properties such as anti-inflammatory, anti-bacterial, anti-carcinogenic, antifungal, and antimicrobial activities. The objective of this research is to extract the curcumin from turmeric using ultrasound assisted supercritical carbon dioxide extraction (USC-CO_2). The extraction was performed at 50 ^{o}C, 25 MPa, CO_2 flow rate of 3 mL/min with 10% cosolvent. The result of extraction, thermogravimetry (TG), Fourier transform infrared spectroscopy (FTIR) and scanning electron microscope (SEM) showed that ultrasound power could disrupt cell wall and release the target compounds from turmeric. USC-CO_2 could provide higher curcumin content in the extracts and faster extraction compared to SC-CO_2 extraction without ultrasound.

Comparison of conventional and ultrasound assisted supercritical carbon dioxide extraction of curcumin from turmeric (*Curcuma longa* L.)

Recently, ultrasound assisted supercritical fluid is used for extraction of the valuable compounds from a number of plant materials as an alternative to conventional method because it can enhance the extraction rate and yield. Curcumin is an important component of turmeric (*Curcuma longa* L.) with many useful functions to

human health. The objective of this study is using USC-CO_2 to extract curcumin from turmeric and comparing the result of USC-CO_2 with that of conventional method. The effect of operating conditions on extraction, including temperature (40-60 ^oC), pressure (15-25 MPa), extraction time (30-120 min), CO_2 flow rate (2-4 mL/min) and percentage of cosolvent (10-20% v/v) were also studied. The result showed that the high extraction yield of 7.17% w/w and curcumin content of 1.69% w/w were achieved at temperature of 50 ^oC, pressure of 25 MPa, extraction time of 90 min, CO_2 flow rate of 3 mL/min with 10% cosolvent. Compared to conventional method, USC-CO_2 could provide higher curcumin content in extraction yield in a shorter extraction time. SEM, TG, and FTIR were used to analyze turmeric undergoing USC-CO_2 and conventional extraction and showed that ultrasound could break down the cell walls and remove some functional groups from plant materials, resulting in an increase in the selectivity of compounds in extraction yield.

Extraction of phytochemical constituents of Khmer medicinal plants using supercritical carbon dioxide integrated hydrothermal process

Khmer medicinal plants have been employed to treat human diseases for nearly a thousand years. Several studies have showed that the important phytochemical constituents of medicinal plants are phenolic acid, flavonoids, tannins, and alkaloid, which have diverse bioactivities such as antioxidant, anti-inflammatory, anticancer, anti-diabetic, antiallergic, and antimicrobial. The present study was carried out to extract phytochemical constituents in Khmer medicinal plants (*Dialium cochinchinense* Pierre*, Cinnamomum cambodianum* Lecomte, *Gardenia angkorensis* Pitard, *Dialium cochinchinense* Pierre, *Cananga latifolia* (Hook. f. & Thomson) Finet & Gagnep, and *Oroxylum indicum* (L.) Kurz bark) using supercritical carbon dioxide integrated hydrothermal process (SC-CO_2-H) at temperature of 150 ^oC, pressure of 10 MPa, water flow rate of 2 mL/min, CO_2 flow rate of 0.15 mL/min, and extraction time of 4 h . The total phenolic compounds and antioxidant activities of Khmer medicinal plants were determined by using Folin Ciocalteu and DPPH scavenging activity method. The phytochemical constituents in Khmer medicinal plants were identified using GC-MS. The result showed that the extract of Khmer medicinal plants obtained by SC-CO_2-H contained the high amount of total phenolic compounds and high antioxidant activity. The result of GC-MS also suggested that Khmer medicinal plants had many bioactive compounds with a board range of biological activities that could be applied in food,

pharmaceutical and cosmetic industries.

Micronization of curcumin with biodegradable polymer by supercritical anti-solvent using micro swirl mixer

Curcumin is a hydrophobic polyphenol compound exhibiting a wide range of biological activities such as anti-inflammatory, anti-bacterial, anti-fungal, anti-carcinogenic, anti-HIV, and anti-microbial activity. In this work, swirl mixer was employed to produce the micronized curcumin with polyvinylpyrrolidone (PVP) by supercritical anti-solvent (SAS) process in order to improve the bioavailability of this compound. The effect of swirl mixer operating parameters such as curcumin/PVP ratio, feed concentration, temperature, pressure, and CO_2 flow rate was investigated. The characterization and solubility of particles were determined by using SEM, FTIR, and UV spectrophotometry. The result showed that the best condition for production of curcumin/PVP particles by SAS process with swirl mixer was at curcumin/PVP ratio of 1:30, feed concentration of 5 mg/mL, temperature of 40 ^{o}C, pressure of 15 MPa, and CO_2 flow rate of 15 mL/min. Moreover, the dissolution of curcumin/PVP particles was faster than that of raw curcumin.

Future perspective

The traditional methods for extraction of bioactive compounds from plant materials are Soxhlet, maceration, and hydrodistillation. Most of these methods usually require the large amount of organic solvent and long extraction time. Currently, the demand of bioactive compounds is increasing due to their application in functional food, pharmaceutical and cosmetic industries. To meet this demand, the new extraction method needs to develop in order to provide high purity of bioactive compounds in extract. The development of new extraction method needs to consider some current issues and follow the green chemistry principles including: (1) the toxic-chemical solvent residue and pollution control in government regulation; (2) the global yield attained in a proposed extraction process; (3) the quality and purity of bioactive compounds in extract. In this work, the extraction of bioactive compounds form plant materials using process integration of the supercritical fluid with novel techniques is discussed. Also, the use of supercritical fluid in microencapsulation of bioactive compounds is presented. Meanwhile, the process integration of supercritical fluid with novel techniques is now considered as a fast, efficient, and environmental friendly method and has been successfully conducted to

extract the bioactive compounds from plant materials in laboratory. However, more research is needed to exploit industrial application of these combined methods. The special effort should be acquired to solve the theoretical and technical problems such as thermodynamic constraints of solubility and selectivity, kinetic constraints of mass transfer rate, modeling of combined extraction methods for better understanding of extraction mechanism and optimization.

List of publications

1. K. Chhouk, C. Uemori, Wahyudiono, H. Kanda, M. Goto, Extraction of phenolic compounds and antioxidant activity from garlic husk using carbon dioxide expanded ethanol, *Chem. Eng. Process*., 117, 113-119 (2017).

2. K. Chhouk, Wahyudiono, H. Kanda, M. Goto, Extraction of curcumin from *Curcuma longa* L. using ultrasound assisted supercritical carbon dioxide, in: International Seminar on Fundamental and Application of Chemical Engineering 2016 (ISFAChE 2016), AIP Conference Proceedings, vol. 1840, pp. 100001-1-100001-9, 2017.

3. K. Chhouk, Wahyudiono, H. Kanda, M. Goto, Comparison of conventional and ultrasound assisted supercritical carbon dioxide extraction of curcumin from turmeric (*Curcuma longa* L.), *Eng. J*., 21, 53-65 (2017).

4. K. Chhouk, Wahyudiono, H. Kanda, M. Goto, Micronization of curcumin with biodegradable polymer by supercritical anti-solvent using micro swirl mixer, *Front. Chem. Sci. Eng*. (2017). DOI: 10.1007/s11705-017-1678-3

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Kimthet CHHOUK