Application of liquefied dimethyl ether for lipid extraction from wet biomass and amino acid crystallization

HOSHINO Rintaro

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HOSHINO Rintaro

Graduate School of Engineering

Nagoya University

Contents

Chapter 1

Introduction

1.1 Introduction

Energy consumption of the world continues to increase year by year. According to the International Energy Agency (IEA), the world energy consumption in 2035 will increase to approximately 1.3 times compared with 2011, mainly in Asia such as China and India, which accounts for much of the increase was that it is emerging [1]. In recent years, these emerging countries has achieved great economic development, is expected to continue to accelerate more and more its growth in the future. Accordingly, the demand for fossil fuel such as oil, coal and natural gas are also expected to continue to increase. However, the depletion of coal and uranium resources approaches after a lapse of about 100 years. On the other hands, the depletion of oil and natural gas approaches after a lapse of about 60 years [2, 3]. Although it is possible that will change these number, by new oil fields and mines will be discovered, or technological innovation is occurred in the future, the fossil fuels is limited. In addition, the reduction of carbon dioxide (CO_2) emission is a major cause of global warming is also a major problem.

In recent years, with the utilization of fossil fuels has been rapidly increasing, emissions of CO_2 are also increased significantly [3]. An increase in the consumption of fossil fuel due to the rapid increase of the emerging energy demand is expected. Therefore, global

warming is an urgent need to be carried out on a global scale.
Japan is the fifth largest energy consumer in the world, Japanese energy self-sufficiency rate that does not include nuclear power is only 5% [4, 5]. Most of the energy resources are dependent on imports from abroad because energy self-sufficiency rate in Japan is very low in the developed countries. Thus, a new alternative energy technology have been developed around the world in recent years because depletion of fossil fuel is concerned. Especially fuel development using biomass has attracted attention.
In general, the water was removed from biomass by using mechanical dewatering operation such as pressing and centrifugation methods, because of the water content of biomass is high [7-9]. However, in the case of pulverized biomass, the biomass becomes clay-like state after the dewatering processes, and the water content remains high. Typically, when to further reduce the water content of the clay-like state biomass, the methods of exhaust gas treating and direct contact are used. However, in this case, the problems of dust are produced or requiring a huge amount of heat because of recovery of latent heat of vaporization arises. On the other hands, an indirect heating method for recovery of evaporation latent heat of water such as vapor recompression method that is commonly utilized in the concentration of drinking water is not suitable for these biomass. This is because the dried biomass to form a coating film on the surface of the heat transfer tube, and inhibit heat transfer. Further, in the case of a woody biomass of chip-like form, sufficient heat transfer performance is not obtained, because of reduction of the contact surface with the heat transfer tube [10]. In other words, it is difficult to sufficiently recover the latent heat of evaporation of water, and it is necessary to add an energy exceeding the energy possessed by the biomass as a latent heat of vaporization of water. Therefore, construction of the water removal process from the energetically independent biomass is difficult.

1.2 Organic solvent extraction

On the other hands, water extraction method from biomass by using alternative liquid is often used [11, 12]. Thereafter, the alternate liquid was evaporated by heating in the heat transfer tubes. This method solves the heat transfer problems and energy saving of water removal process. Alcohol is a typical alternative liquid [13]. However, when using an alcohol as a solvent, separating step of the alcohol and water are required by distillation of the extract. In other words, the energy for the reduction of water content of the biomass, replaces the energy of distillation of alternative liquids. This problem is caused by the boiling point of alternative liquid and the mixing with water and. Because the boiling

point of water is 100°C, the evaporation step of the water takes heating temperature more than its boiling point. In addition, the boiling point of ethanol is 78.3°C, and completely mixed with water.

Further, in the case of 96-wt% ethanol, these components become the azeotrope. Therefore, energy for separation of ethanol becomes huge. To solve this problem, appropriate alternative liquid to replace the alcohol has been desired. On the other hand, in the food processing, the method according to the combination of high-temperature drying + hexane extraction has been used for the extraction of oil from food biomass [14]. In addition, Bligh Dyer method, which use chloroform, methanol with water mixture as solvent, is used for the determination of fats and oils in food [15]. However, these organic solvents have high boiling point, and toxicity. In addition, it need a drying and cell destruction step as a pre-processing with respect to the above-mentioned method, organic solvent extraction has been used since the olden days.

1.3 Supercritical carbon dioxide extraction

Supercritical carbon dioxide (SC-CO_2) has attracted attention as an extraction solvent to replace the organic solvent. SC-CO_2 is nontoxicity, nonflammable, and well known as environmental friendly extraction solvent in the food industry. Additionally, SC-CO_2 has properties such as low viscosity, high diffusivity, liquid-like density, and easily separated from sample by depressurization to atmospheric pressure. In other words, there is no concern of residual solvent. Furthermore, the solubility of the SC-CO_2 with target substances can be controlled by operating conditions such as temperature and pressure. [16]. For these reasons, SC-CO_2 extraction method has been mainly used for extraction of bio oil, functional ingredients, and aroma components from plants. [16-23]. On the other hand, this method requires high pressure resistant equipment with high cost.

1.4 Liquefied DME extraction

In the present study, liquefied dimethyl ether (DME) was used as an alternative solvent. Principles and methods for extracting the moisture contained in the biomass with liquid DME is patent application in 2002 by Kanda et al., Registered in 2009 [24]. The method is excellent in terms of green chemistry because it doesn't have toxicity. Liquefied DME is combustible gas. The normal boiling point is -24.8°C, the saturated vapor pressure at 20°C is 0.51MPa. The temperature dependence of the saturation vapor pressure at room temperature shown in Figure 1.1 [25]. Liquefied DME is partly mixed with water at room

temperature. The DME mole fraction in liquefied DME + water system shown in Figure 1.2 [26]. When this mole fraction in terms of weight, liquefied DME required for the extraction of water, the water weight of (1 ÷ 0.072 =) is 13.9 times.

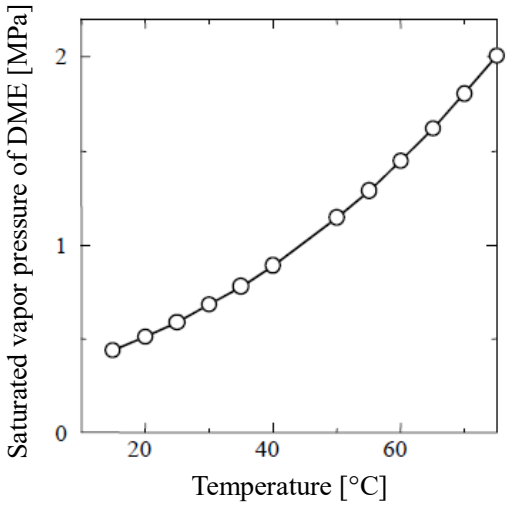

Figure 1.1 Saturated vapor pressure curve of DME

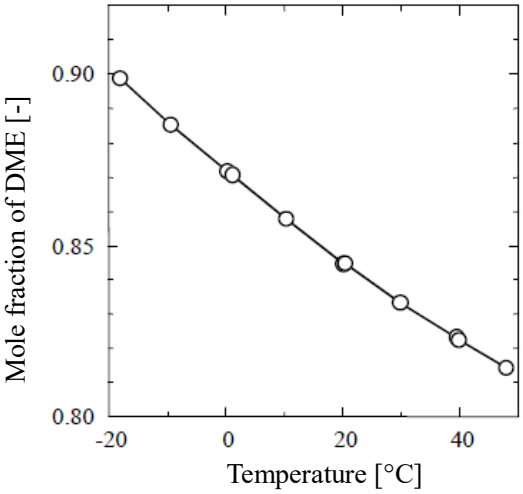

Figure 1.2 Equilibrium diagram of liquefied DME + water

DME is originally an artificial fuel that has been developed as a diesel alternative fuel, from the feature that soot is not generated during combustion, are popular in China as a clean fuel [27]. Further, the liquid DME is neither causative agent for destruction of greenhouse and ozone layer, even without forming peroxides in long-term storage unlike other ethers, toxicity lower than hexane. European Food Safety Authority, shows the view that may be introduced liquefied DME in the extraction of the food production process [28]. Subsequently, illustrating the principle and method for extracting water-organic substances contained in the biomass using a liquefied DME. First extracts the water contained in the biomass, the DME liquefied at ambient temperature. At that time, soluble organic matter also extracted at the same time to liquefied DME. After the extraction operation, the flash distillation a mixture of water and organic matter and liquefied DME. DME is flashed off from the extract of the water and organic matter. The extract can be separated easily in oil-water separator, is taken out remains liquid. On the other hand, DME steam can be used again as a liquefied DME by cooling condensation. It illustrates this principle and method in Figure 1.3. The operating pressure is the same as the saturated vapor pressure of the operating temperature.

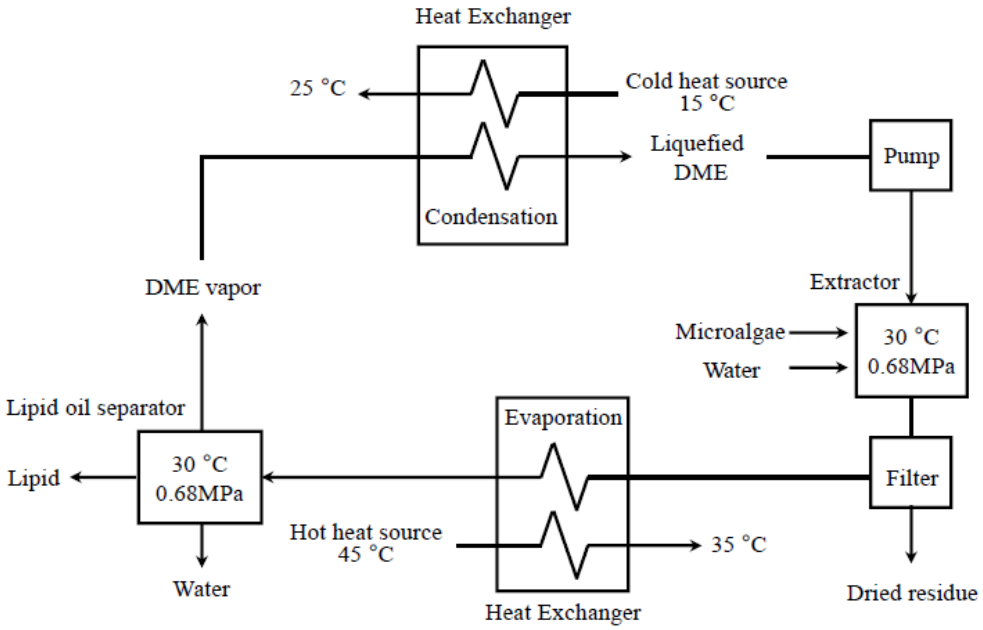

Figure 1.3 Extraction principle of moisture and organic material of biomass by liquefied DME

Further, the operating temperature and pressure (30°C, 0.7MPa) shown in Figure 1.3 is one example. With increasing operating temperature, the greater the amount of heat released to the outside of the apparatus. On the other hands, the liquid DME + mole fraction of water is increased in the aqueous, the amount of liquid DME required for extraction is reduced. From these conflicting two factors, the operating temperature is determined to be around room temperature. In this method, by adjusting the saturated vapor pressure of DME, it can be set boiling point of DME at room temperature. Therefore, as the heat source for the evaporation and condensation of DME, it made available a low-level waste heat unused.

For example, in this process can utilize a heat source such as factory waste heat (50°C), sewage heat (20°C) are stable throughout the year and, geothermal heat (15°C). These heat source, depending on the season, by combining as appropriate, such as the atmosphere, river water, seawater, it is possible build a circulation cycle of evaporation and condensation of DME. In this case, the extraction operation is possible only by the power of the sending pump of liquefied DME.

The energy consumption of liquefied DME-sending pump was calculated by Kanda et al 2015 [29]. As an example, the official basic specs of a large-scale commercially available LPG-sending pump (Model number; E-516F2M-0810U1-E/F, Tokyo Boeki Mechanics

Ltd., Tokyo, Japan) are as follows: flow rate, 800 L/min; power requirement, 15 kJ/s; and receiving end efficiency (which is the ratio of electric energy received by the pump based on the HHV of fossil fuel consumed in a thermal power plant) in Japan, 0.369. Thus, 0.570 g (extracted lipid = 32.5% of *E. gracilis*, the water content = 80.3 wt%) of lipids obtained were extracted by 0.544 L of liquefied DME (359.5 g, density of liquefied DME at 20 °C is 0.661 g/mL). Here, the energy consumption (E) of the liquefied DME-sending pump per kg of lipid is calculated by the following equation (1):

$$E = V/L \times W/F/R = 2.96\text{MJ/kg} \quad (1)$$

V is the volume of the liquefied DME (0.554 L); L is the weight of lipids extracted by liquefied DME V (0.570 g), W is the energy requirement (15 kJ/ s); F is the flow rate of liquefied DME (13.33 L/s); and R is the receiving end efficiency in Japan (0.369). As a results, energy consumption of the liquefied DME-sending pump would be much smaller than 2.96 MJ/kg. This implies that the energy consumption of the liquefied DME-sending pump is clearly smaller than the HHV of the extracted lipid. Accordingly, this method is an excellent process in energy efficiency compared to conventional methods such as a high-temperature drying method and a waste heat recovery system.

1.5 Purpose of this works

In this study, the water and bio-oil from a variety of land-based plant-based and marine biomass were extracted by using the liquefied dimethyl ether (DME). In addition, the characteristics of the moisture and volatile substances of extracts for each sample were clarified.

Change of the fuel properties and suitability as a coal alternative fuel of biomass residue after extraction was revealed for each sample. In addition, the properties of the extracted bio-oil have been revealed. From these, the usefulness of this method for the use of vegetation quality-based biomass has been demonstrated.

Moreover, the functional substances contained in the bio-oil was investigated. We aimed at the reducing cost of the biomass fuel production by co-producing a biomass fuel and functional materials.

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Chapter 2

Utilization of plant biomass as a bioactive compounds resources by using liquefied DME

2.1 Simultaneous extraction of water and essential oils from citrus leaves and peels using liquefied DME

2.1.1 Introduction

Nearly one million tons of citrus fruits are cultivated in Japan every year; these are used as the raw material for juice processing [1]. However, most of the pomace obtained after juice processing is disposed of, although it contains valuable substances such as flavonoids and essential oils [2]. Waste containing high moisture content is not only difficult to transport during disposal but also creates problems with regard to hygiene because it decomposes easily. Moreover, during the combustion process, the temperature of the combustion gases significantly drops inside the combustion furnace owing to the

high moisture content of the diapers, and waste gases containing dioxins pollute the environment [3]. The disposal cost of the pomace is expensive because it contains a large amount of water [4]. Essential oils and citrus flavonoids are mainly contained in the pomace obtained from the peel and leaves of citrus [5]. These valuable substances are traded at a high price in the market. Essential oils are used in aromatherapy and high-quality perfumes. Citrus flavonoids are used as raw materials for health supplements; these flavonoids are also known to have hypolipidemic effects and apoptosis-inducing behavior in cancer cells [6]. Commercially, essential oils are obtained from the citrus peels by the cold press and steam distillation (SD) method [7, 8]. However, the extraction yield by the cold press method is low, because large amounts of valuable oils remain in the residue [9]. On the other hand, pure essential oils can be obtained using the SD method. The distillation step is carried out over a temperature range of 130 to 150°C. The essential oils evaporate with the steam during the distillation process. However, the disadvantage of the SD method is that the quality of the obtained essential oils degrades as a result of the distillation heat [10]. Citrus flavonoids cannot be extracted using the SD method because it involves high boiling temperatures [11]. Additionally, a large amount of the residue with high water content is disposed of after the aforementioned processing methods. Therefore, the flavonoids are extracted using organic solvents such as menthol, dimethyl sulfoxide (DMSO), and hexane [12]. However, such extractions are toxic, expensive, and hazardous. Moreover, these methods require several preprocessing steps such as drying, grinding, and homogenizing the raw material [13]. Recently, alternative extraction methods are in demand in the food industry. SC-CO_2 and liquefied dimethyl ether (DME) are receiving increased attention as the extraction solvent because of their desirable properties. SC-CO_2 extraction has showed high selectivity and the possibility to fractionate the components based on temperature and pressure control [14]. However, it is necessary to use a special apparatus to withstand the high pressure of SC-CO_2 [15]. DME has been developed as a synthetic fuel for use in both liquid and gaseous forms [16-18]. In China, DME is synthesized using small-scale coalfields of low commercial value and produced as a fuel at a cost equivalent to that of the imported liquefied petroleum gas. The standard boiling point of DME is -24.8°C and its saturated vapor pressure at 20°C is 0.51 MPa [19]. Because DME has weak hydrogen bonds, water dissolves into the liquefied DME to the extent of 7–8 wt% at room temperature. The quantity of gaseous DME dissolving in water is also low, and it can be easily separated from the water without distillation [20]. Furthermore, liquefied DME has low toxicity, and thus, could be investigated as a prospective solvent for food processing [21, 22]. DME differs from typical ethers such as ethyl ether and does not form peroxides [23]. In addition, liquefied

DME can extract not only water (dewatering) from brown coal [24], but also bio-crude (the organic components contained in the vegetal biomass) from biological materials such as microalgae [25]. The DME method exhibited robust dewatering ability and efficiency in removing the oils from the high moisture orange peels [26]. Moreover, it can be operated at a relatively lower temperature and pressure, and easily separated from the extract at the ambient pressure [27]. In this work, water, essential oils, and flavonoids were extracted from the citrus pomace using liquefied DME. The peel and leaves of *Citrus junos* (Yuzu) and the peel of *Citrus tangerine* (Ponkan) were used as raw materials, and the dewatering rate and yield of essential oils and citrus flavonoids were investigated.

2.1.2 Materials and methods

2.1.2.1 Outline of extraction method

Figure 2.1.1 shows a schematic of the laboratory-scale DME extraction apparatus. Two storage tanks, one containing liquefied DME (tank A, volume: 100 cm^3; TVS-1-100, Taiatsu Techno Corp., Saitama, Japan) and an extraction column and the other holding the mixture of liquefied DME, water, and essential oils (tank B, HPG-96-3, Taiatsu Techno Corp., Saitama, Japan), were connected in series. The extraction column (diameter: 11.6 mm, length: 190 mm; HPG-10-5, Taiatsu Techno Corp., Saitama, Japan) was employed for the experiment. Additionally, bigger column (diameter: 35 mm, length: 190 mm; HPG-96-3, Taiatsu Techno Corp., Saitama, Japan) was used as extractor because a large amount of essential oils was needed for the analysis. Citrus fruit peels comprise flavedo (exocarp) and albedo (endocarp). The essential oils are present in the oil glands of the flavedo. In these experiments, the peels of citrus fruits containing the albedo were used as the raw material. The essential oils in citrus peel were present in trace amounts (1–3%), the amount being smaller than the amount of water extracted from the citrus peel. The extraction of water and essential oils was performed three times, respectively. The test samples were loaded into the extraction column, and glass beads (diameter between 1.5 and 2.5 mm; BZ-2, ASONE Co., Inc., Osaka, Japan) were loaded at the top and bottom ends of the column [28]. In addition, for the removal of water, the loaded average amounts of Yuzu peel, Ponkan peel, and Yuzu leaves were 4.4 ± 0.1 g (82.7 wt%), 5.0 ± 0.1 g (72.4 wt%), and 2.6 ± 0.2 g (82.6 wt%), respectively. On the other hand, for the extraction of essential oils, the loaded average amounts of Yuzu and Ponkan peels were 20.1 ± 0.2 g (85.6 wt%) and 18.3 ± 0.3 g (72.4 wt%), respectively. Furthermore, the thickness of the Yuzu and Ponkan peels were approximately 5 mm and 2 mm, respectively. The values in the parentheses indicate the initial water contents, which were determined by drying at

107°C.

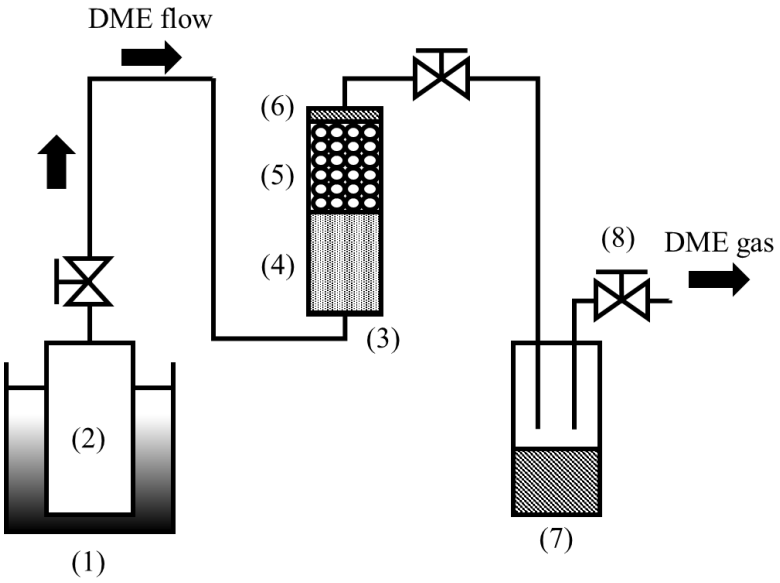

Figure 2.1.1 Lab-scale DME extraction apparatus: (1) water bath, (2) liquefied DME tank (tank A), (3) extraction column, (4) wet sample, (5) glass beads, (6) filter, (7) extract collector (tank B), (8) pressure-reducing valve

The liquefied DME in tank A was controlled at 35 ± 2°C in a water bath, while the saturated vapor pressure of DME in tank A was 0.78 ± 0.03 MPa. The liquefied DME was supplied to the extraction column, and cooled down to room temperature using the long tube, which connected tank A and the extraction column. The temperature of the DME in the tube and extraction column was 15 ± 1°C, which was detected using an infrared thermometer. DME flowed owing to the pressure difference between tank A and the extraction column while the DME flow rate was maintained at 10 mL/min using a pressure reducing valve in the outlet of the extraction column. After the liquefied DME was passed through the extraction column at different time intervals, DME was evaporated by opening the pressure-reducing valve of tank B. The amount of the extracts remaining in tank B was equal to the total amount of essential oils and water extracted from the test samples. The extract was weighed after collection, and the yield of (1) essential oil and (2) water were determined using the equation shown below. In addition, the amount of citrus flavonoids in the water phase was analyzed using high-performance liquid chromatography (HPLC). The dry weights of the samples in the equation 1 were

determined by drying at 107°C up to constant weight [26]. In other words, the dry weight was determined by subtracting both essential oil and water from the wet weight of the samples. Initial water amounts in the equation (2) were determined by difference between weight reduction by the drying and extracted essential oil amount.

$$\text{Yield of essential oil} = \frac{Extracted\ essential\ oil\ [g]}{Dry\ sample\ [g]} \times 100\% \quad (1)$$

$$\text{Yield of water} = \frac{Extracted\ water\ [g]}{Initial\ amount\ of\ water\ in\ samples\ [g]} \times 100\% \quad (2)$$

2.1.2.2 Steam distillation method

50 g of citrus peel was cut using scissors, and placed into a 1 L round bottom flask containing 500 mL of distilled water. Then, the flask was connected to a distilling receiver with Liebig condenser. Distillation was carried out for 4 hour at atmospheric pressure. The essential oils evaporated with steam during the distillation process, and were separated from the condensates and collected in the distillate receiver. After reaching a certain volume, the water was refluxed in the distiller, and the distillation process continued [9].

2.1.2.3 HPLC analysis

The amount of citrus flavonoids in the water phase was analyzed by high performance liquid chromatography (HPLC) using the external reference method. The standards, hesperidin (<91.3%) and naringin (<98.6%), were purchased from Kanto Chemical Co., Inc, Japan, while neohesperidin (<98.0%), nobiletin (<95.0%), tangeretin (<95.0%), HPLC-grade acetonitrile and acetic acid, used as the mobile phase, were purchased from Wako Pure Chemical Industries, Ltd., Japan. The flavonoids were identified by comparing their spectra and retention times with the standards. The content of each flavonoid was calculated from the integrated peak area of the sample and the corresponding standard. The flavonoid extracts were analyzed using an HPLC gradient system (LC-20AD, Shimadzu Corp., Kyoto, Japan) equipped with a diode array detector

(SDP-M10A, Shimadzu Corp., Kyoto, Japan). The flavonoids were monitored at 285 nm. ODS column (Intertsil® ODS-3, GL Sciences Inc., Tokyo, Japan) was used for separation at 35°C. The mobile phase consisted of solvent A, 0.1% acetic acid in water, and solvent B, 0.1% acetic acid in acetonitrile (acetonitrile/water = 75/25, v/v). The flow rate was 1.0 mL/min. The gradient elution was carried out as follows: 0 min A–B (88:12); 5 min A–B (78:22); 15 min A–B (72:28); 22 min A–B (62:38); 32 min A–B (52:48); 37 min A–B (32:68); 42 min A–B (0:100); 45 min A–B (0:100); 50 min A–B (88:12); and 60 min, A–B (88:12) [29]. The yields of (3) citrus flavonoids were determined using the following equation.

$$\text{Yield of citrus flavonoid} = \frac{Extracted\ citrus\ flavonoids\ [mg]}{Dry\ samples\ [g]} \times 100\% \quad (3)$$

2.1.3 Results and discussion

2.1.3.1 Removal of water from citrus leaves and peel by using liquefied DME

Figure 2.1.2 shows the amounts of water removed from the samples using liquefied DME. Here, owing to differing water contents in each sample, the DME consumption (abscissa) was expressed as the ratio between the consumption of DME and the total initial amount of water in the tested sample. The magnitudes of maximum dewatering in the Yuzu peel, Ponkan peel, and Yuzu leaves were as follows: 78.1 ± 1.0%, 76.2 ± 1.0%, and 83.9 ± 0.5%, respectively. As the Yuzu leaves were thin, the amounts of the extracted water were larger compared to those extracted from the other samples. Such large differences in the amounts of the extracted water among the tested samples were not confirmed. Therefore, it was possible to remove about 70–80% moisture from the raw material using liquefied DME. Moreover, the color of the extraction residue was relatively paler after DME extraction.

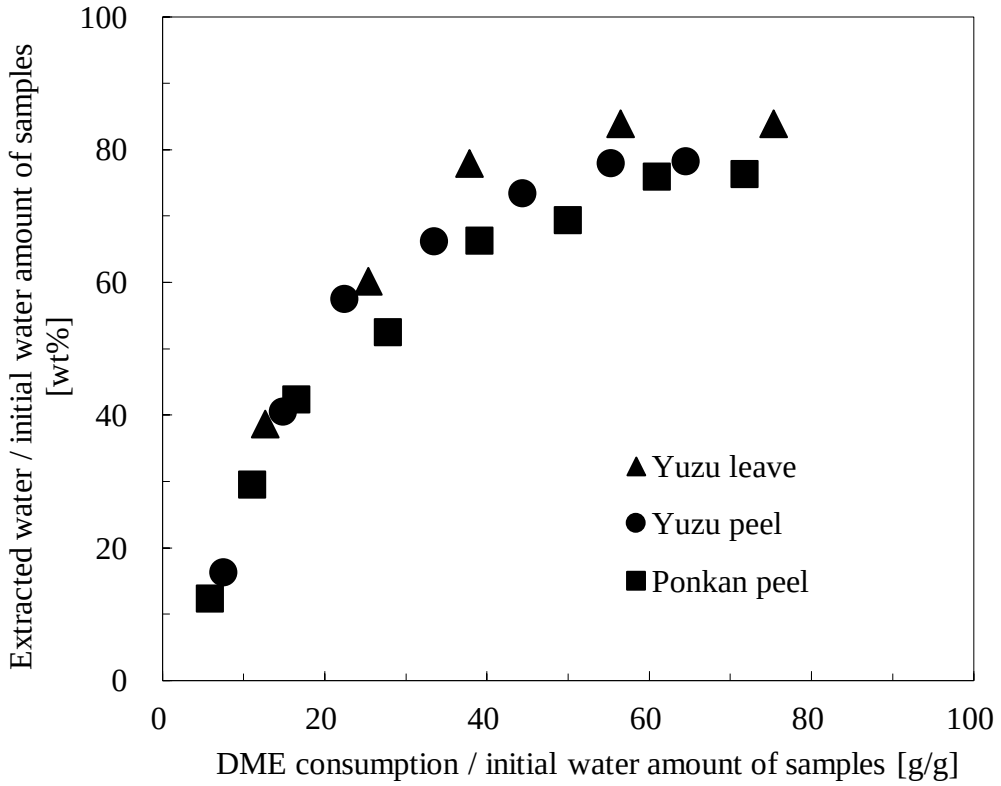

Figure 2.1.2 Dewatering rates of the DME method for Yuzu peel, Yuzu leave, and Ponkan peel

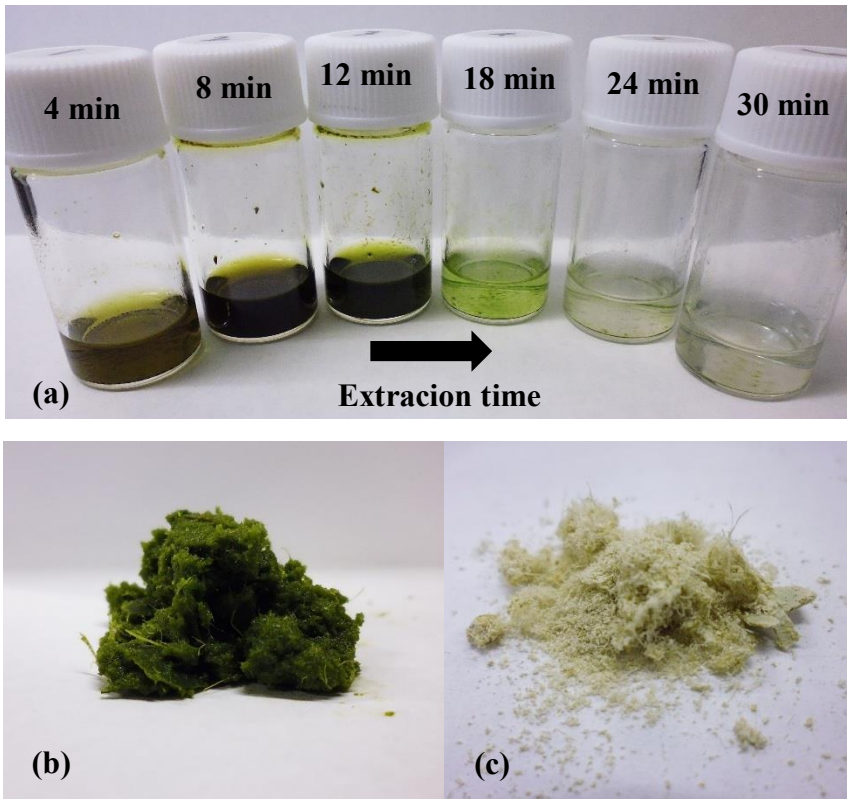

Figure 2.1.3 Appearances of extracts from Yuzu leaves using liquefied DME, raw material, and extracted residue: (a) extracts from Yuzu leaves, (b) raw Yuzu leaves, (c) extracted residue

Figure 2.1.3 shows the time-dependent change in the color of the extracted solution and that of the extraction residue compared to that of the Yuzu leaves. The color of the initial extract was dark green. The extract gradually changed into colorless by increasing the consumption of DME towards the end of the extraction process. On the other hand, the color of the residue turned to light green from dark green immediately after the extraction. Such change in the color intensity implied that the pigments of the sample were also extracted into the liquefied DME.

2.1.3.2 Comparison of yields of essential oils obtained by liquefied DME and Steam distillation methods

The extraction yields of essential oils from the samples by using the DME method were compared with those obtained by using the SD method, as shown in Figure 2.1.4.

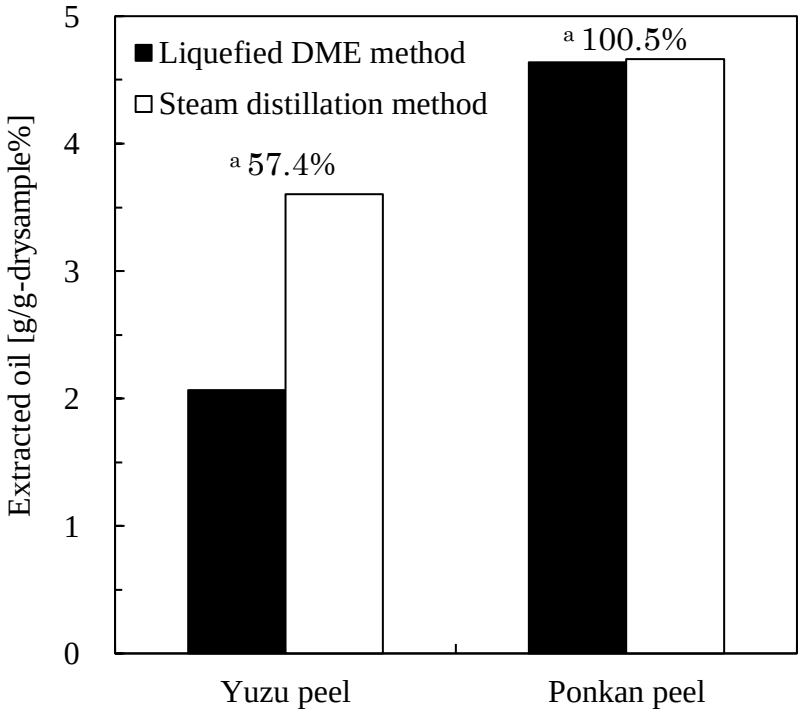

Figure 2.1.4 Amounts of essential oils extracted by liquefied DME and steam distillation methods. [a] Extraction yield by DME method relative to that obtained by steam distillation method

The extraction amounts of the essential oils were small. Figure 2.1.4 shows the final extraction amounts of the essential oils from the citrus peel. The black columns indicate the DME method extraction ratios based on the dry weight of the samples, while the white columns indicate the results by using the SD method. The numbers besides the superscripts “a” indicate the extraction yields obtained using DME relative to those achieved by using the SD method. In the case of extraction by using the SD method, the yields of essential oils from the Yuzu and Ponkan peels were 2.1 and 4.7 g/g-dry sample, respectively, which were typical for citrus peel [30, 31]. Interestingly, in the case of

extraction by using the DME method, the amounts of essential oils obtained were almost equal to those obtained by using the SD method. Furthermore, in the DME method, the extracts were not affected by thermal denaturation; hence, their compositions were close to that of the raw material. However, the Yuzu peel was thicker than the Ponkan peel; hence, the yield of the essential oils from the Yuzu peel was comparatively lower. In order to examine the effect of citrus peel thickness on the efficiency of the extraction of essential oils using the DME method, an additional experiment was carried out using *Citrus grandis* peel as the raw material. The reason for choosing *Citrus grandis* was its similarity to the Yuzu peel, making it possible to easily compare the extraction yield between the *Citrus grandis* and Yuzu peels. For the *Citrus grandis* peels, only the albedo parts were cut, leaving a 2-mm-thick peel. In another test experiment, we used a *Citrus grandis* peel containing the albedo parts, with a total thickness of 5 mm. For simplicity, the peel containing the thick albedo part is considered as untreated and the peel without the albedo parts is considered as pre-treated. The yield of the essential oils from the peel without treatment was 0.3 g/g-dry sample while the yield from the pre-treated sample was 3.4 g/g-dry sample. This result suggests that the albedo parts were preventing the extraction of the essential oils.

2.1.3.3 HPLC analysis of extracts obtained by DME method

Figure 2.1.5 shows the chromatograms of the citrus flavonoids extracted by using the liquefied DME method, which is typical for flavonoids [32]. Table 2.1.1 shows the quantitative results for these flavonoids. In the case of the Yuzu peel, naringin, hesperidin, and neohesperidin were extracted in 2.9 mg, 45.6 mg, and 1.4 mg, respectively. Hesperidin, nobiretin, and tangeretin were extracted from the Ponkan peel in 1.6 mg, 3.8 mg, and 1.2 mg, respectively. Hesperidin, neohesperidin, and phroletin were extracted from the Yuzu leaves in 0.3 mg, 17.9 mg, and 0.2 mg, respectively. Citrus flavonoids are usually extracted from the citrus peel using 1:1 DMSO/methanol (v/v). The DMSO/methanol combination has high extraction ability, and is used for the quantitative analysis of flavonoids. While using DMSO/methanol as the extraction mixture, approximately 2% hesperidin per dry sample weight was extracted from the orange peel [13]. The extracted amounts of citrus flavonoids using liquefied DME were one-tenth of those obtained by using the DMSO/methanol method. These results suggested that the citrus flavonoids remained in the extracted residue. However, the use of DMSO for food processing is prohibited in Japan. In this study, it was possible to selectively extract essential oils from the citrus residues using liquefied DME. Therefore, there remain the

citrus flavonoids in the extraction residue.

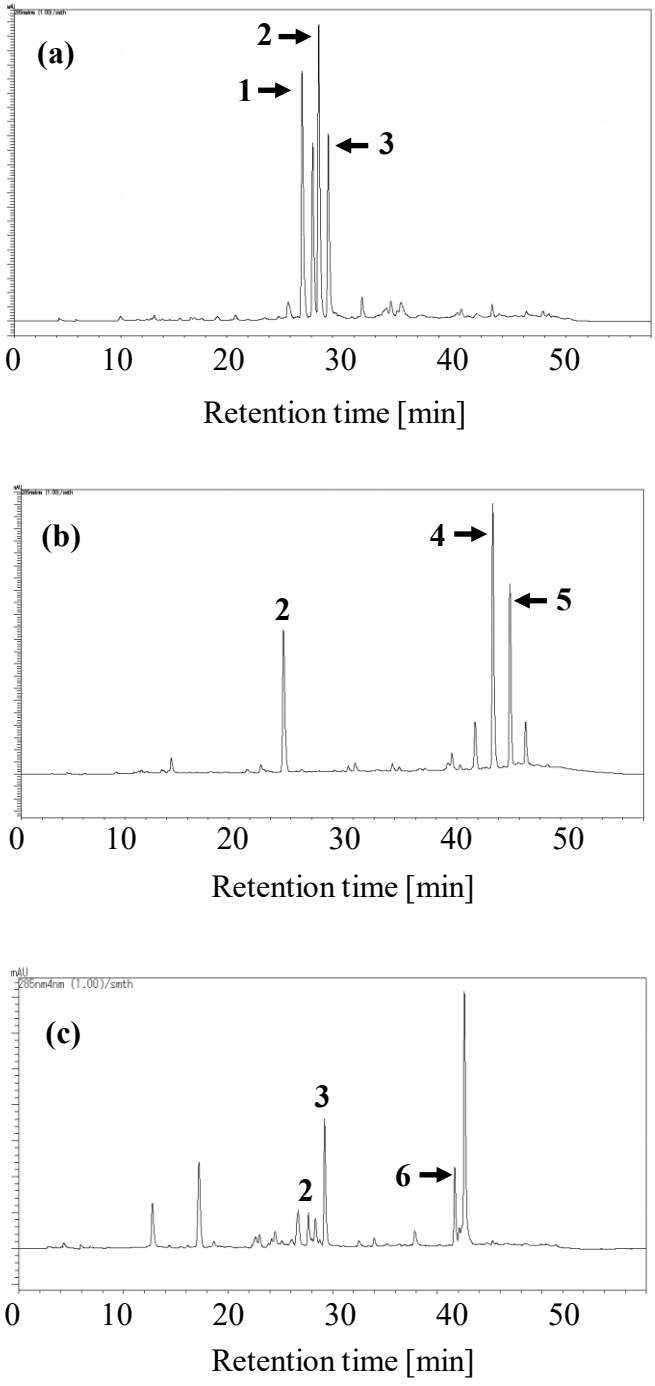

Figure 2.1.5 HPLC chromatograms of citrus flavonoids in the water phase at 285 nm: (a) Yuzu peel, (b) Ponkan peel, (c) Yuzu leaves. (1) Naringin, (2) Hesperidin, (3) Neohesperidin, (4) Nobiretin, (5) Tangeretin, (6) Phloretin

Table 2.1.1 Amount of citrus flavonoids extracted in water phase by DME extraction

Citrus flavonoid	Yield [mg/g-dry sample]		
	Yuzu peel	Ponkan peel	Yuzu leaves
Naringin	2.9	-	-
Hesperidin	45.6	1.6	0.3
Neohesperidin	1.4	-	17.9
Phloretin	- [a]	-	0.2
Nobiretin	-	3.8	-
Tangeretin	-	1.2	-

[a]Not detected.

2.1.4 Conclusions

Water, essential oils, and citrus flavonoids were extracted from the citrus leaves and peels using liquefied DME. More than 70 wt% water was removed from the test samples. The essential oils were also extracted in this process. In particular, almost the same amount of essential oils was extracted from the Ponkan peel. In addition, citrus flavonoids such as hesperidin, neohesperidin, nobiletin, tangeretin, and phloretin were extracted from all the samples. These flavonoids could not be extracted by the SD method. Therefore, DME extraction was highly effective in extracting water and essential oils from the citrus leaves and peels. These results suggested that the liquefied DME extraction method can be used to reduce the volume of citrus pomace, leading to a reduction in the disposal costs.

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2.2 Functional ingredients extraction from *Garcinia mangostana* pericarp by liquefied dimethyl ether

2.2.1 Introduction

Mangosteen (*Garcinia mangostana Linn.*) is one of the tropical fruits belonging to the Guttiferae family which called “queen of the fruit” because of its sweet juicy edible part. It is grow in Thailand, Malaya, India, Vietnam, Singapore, Philippines and Burma [1, 2]. It has a dark red pericarp that has been discarded. However, the valuable compounds such as xanthones, which is a kind of polyphenols are contained in the pericarp. Xanthones are attracting attention as a dietary supplement and a medicament because its powerful functionalities [3-6]. In recent years, the functionality of xanthones has been reported on medical, pharmaceutical and food science fields as follow. Antiproliferative, antioxidation and induction of apoptosis by mangosteen extract on human breast cancer cell line [7-9]. Induction of apoptosis by xanthones occurred in human leukemia cell lines [10]. Garcinone E, one of a xanthone derivatives, has potent cytotoxic effect against liver cancer, gastric cancer, lung cancer and colorectal cancer [11, 12]. Currently, the organic solvent (ethanol, methanol and ethyl acetate, etc.) were used as conventional extraction solvents for obtaining the xanthones [2, 7, 12]. However, the following problems have been concerned; the oxidation and thermal denaturation, the environmental impact, and effects on the human body of residual solvent. As a method of solving these problems, supercritical carbon dioxide (SC-CO_2) and liquefied dimethyl ether (DME) are receiving attention as the extraction solvent because of their useful properties. SC-CO_2 is excellent extraction solvent for the non-polar substance because it has properties similar to hexane. Additionally, the method has shown high selectivity by operating conditions such as temperatures and pressures [13-15]. However, it is necessary to use a special apparatus to establish the high pressure of SC-CO_2 and pretreatment such as sample drying. In the case of DME, the standard boiling point is -24.8°C, and its saturated vapor pressure at 20°C is 0.51 MPa. Because of the weak hydrogen bonds of DME, water dissolves into the liquefied DME to the extent of 7–8 wt% at room temperature. Therefore, those characteristic properties, it can extract functional ingredients from the high moisture content raw materials at the vicinity of room temperature [16-23]. Additionally, DME has been approved as a safe extraction solvent for the production of foodstuffs and food ingredients by the European Food Safety Authority [24]. In this work, the alpha-mangostin which is a kind of xanthone would be extracted from the raw pericarp of

mangosteen using liquefied DME. Experimental conditions were 35°C/0.8 MPa and extracted components were analyzed by using high performance liquid chromatography (HPLC).

2.2.2 Materials and methods

2.2.2.1 Materials and chemicals

For the extraction, pericarp of mangosteen which was cultivated in Thailand was used. Wet sample of pericarps was prepared as powder type (0.5-1.0 mm) and cube type (5 mm). alpha-Mangostin was purchased from Wako Co. Ltd., Japan was used for standard. Other xanthones (3-Isomangostin, Mangostanol, 8-Desoxygartanin, Gartanin, Garcinone E, 9-Hydroxycalabaxanthone, beta-Mangostin) were calculated using the calibration curve of alpha-mangostin. HPLC-grade of methanol and acetic acid used for HPLC analysis. The analytical–grade of ethanol which used for ethanol extraction and dilution solvent were purchased from Wako Co. Ltd., Japan.

2.2.2.2 DME extraction

The apparatus shown in Figure 2.1.1 was used for the DME extraction. Liquefied DME tank (100 ml; TVS-1-100, Taiatsu Techno Corp., Saitama, Japan), the extraction column (10 ml; HPG-10-5, Taiatsu Techno Corp., Saitama, Japan) and collector (96 ml; HPG-96-3, Taiatsu Techno Corp., Saitama, Japan) were employed for the experiment. Raw materials were prepared for powder type (moisture content of 61.6 wt%) and cube type (moisture content of 62.9 wt%). In each experiment, approximately 1, 3, and 6 g of wet samples were loaded into the extraction column and the remaining volume was filled with glass beads (diameter between 1.5 and 2.5 mm; BZ-2, ASONE Co., Inc., Osaka, Japan) and cotton at the bottom and top of the column [18, 25]. The liquefied DME in the tank was controlled at 35 ± 2°C in a water bath, while the saturated vapor pressure of DME in the tank was 0.78 ± 0.03 MPa [16]. The liquefied DME was supplied to the extraction column, and cooled down to room temperature using the long tube, which connected the tank and the extraction column. The temperature of the DME in the tube and extraction column was 20 ± 1°C. DME flowed owing to the pressure difference between tank and the extraction column while the DME flow rate was maintained at 7 mL/min using a pressure-reducing valve in the outlet of the extraction column. After the liquefied DME was passed through the extraction column at different time intervals, the DME was

evaporated by opening the pressure-reducing valve of collector. The amount of the total extracts remaining in collector was equal to the total amount of extract and water extracted from the samples. The total extract was weighed after collection, and the yield of water, extract, and xanthones were determined using the equation shown below (1), (2), and (3). In addition, the amount of alpha-mangostin in the extract was analyzed using high-performance liquid chromatography (HPLC).

$$\text{Yield of water} = \frac{Extracted\ water\ [g]}{Initial\ amount\ of\ water\ in\ sampls\ [g]} \times 100\% \quad (1)$$

$$\text{Yield of extract} = \frac{Extracts\ [g]}{Dry\ sampls\ [g]} \times 100\% \quad (2)$$

$$\text{Yield of xanthone} = \frac{Extracted\ xanthone\ [mg]}{Dry\ sampls\ [g]} \times 100\% \quad (3)$$

2.2.2.3 Conventional method using ethanol as extraction solvent

Ethanol extraction for comparison was performed. Powder type (0.5-1.0 mm) was used as an extraction material in wet sample (moisture content of 61.6 wt%) and dry sample (moisture content of 5.3 wt%). Using a batch extractor, 100 mL ethanol was heated while mixing on a hot stirrer (500 rpm) at 35°C and ambient pressure. A sample of milled mangosteen pericarp (1.0 g) was added when the pre-treatment temperature condition was achieved, then the extraction was started. 1 mL extract liquid was collected at 10, 20, 30, 60, and 90 min.

2.2.2.4 HPLC analysis

The HPLC system consisted of a Tosoh LC-8010 system (Tosoh Co. Japan) which equipped with a UV detector. The extracts including xanthones were separated using a TSK-GEL (Tosoh Co. Japan) ODS-80Ts column (150 x 4.6 mm) at 40°C. The detection wavelength was set at 340 nm. Extracts were dissolved in 4 mL of ethanol and injected

into the HPLC system. The injection volume was 5 or 50 µL, which was selected based on the extract concentration to avoid detector saturation. Two mobile phases were used during the separations: (A) methanol and (B) 0.1% acetic acid in water. The methanol gradient profiles were as follows: 75% over 0-10 min; 90% over 50-60 min; 75% over 60-70 min. The flow rate was set at 0.8 mL/min.

2.2.3 Results and discussion

DME extraction for the wet sample of mangosteen pericarp was successfully carried out. Figure 2.2.1 shows the pictures of raw material, residue, and extracts which dissolved in ethanol after DME extraction. The color of the raw material was dark red-purple, however the color of the residue was bleached to orange-yellow. On the other hand, the color of the initial extract was orange, the extract gradually lost its color with increasing extraction time because of the consumption of solvent towards the end of the extraction process.

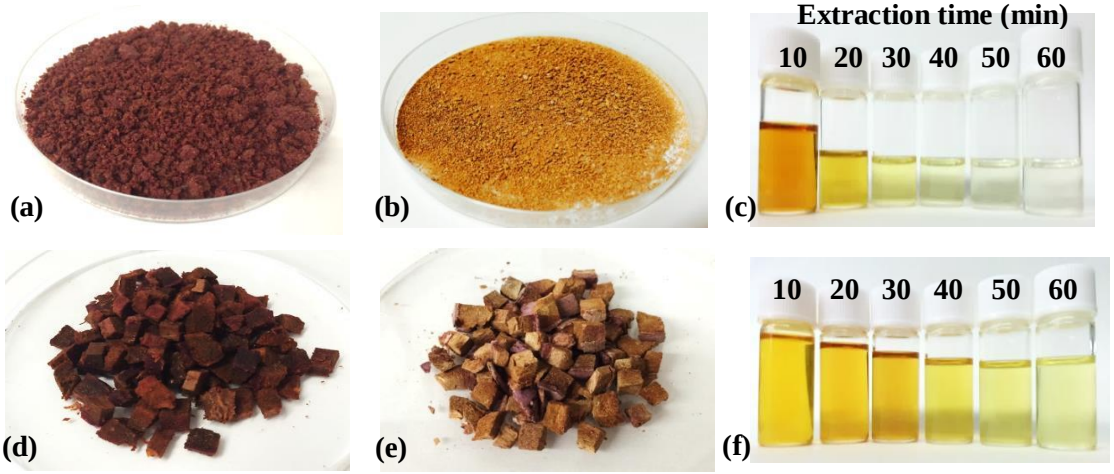

Figure 2.2.1 Appearances of extracts from mangosteen pericarp using liquefied DME, raw material, and extracted residue: (a) raw material of powder type, (b) extracted residue of powder type, (c) extracts from powder type, (d) raw material of cube type, (e) extracted residue of cube type, (f) extracts from cube type

2.2.3.1 Water extraction

Figure 2.2.2 shows the amounts of water removal from the samples using liquefied DME. In order to differing water contents in each sample, the DME consumption (horizontal axis) was expressed as the ratio between the consumption of DME and the total initial amount of water in the tested sample. In the case of shape of the sample, the extracted water from cube sample was slightly lower than the powder type due to its thickness [16]. Moreover, higher amount of DME was required to extract the same amount of water compared to powder type. On the other hand, in the case of powder sample, extraction equilibrium has been nearly reached in the initial stage. Therefore, it is considered that the DME consumption maybe reduced by milling in order to increase the contact area.

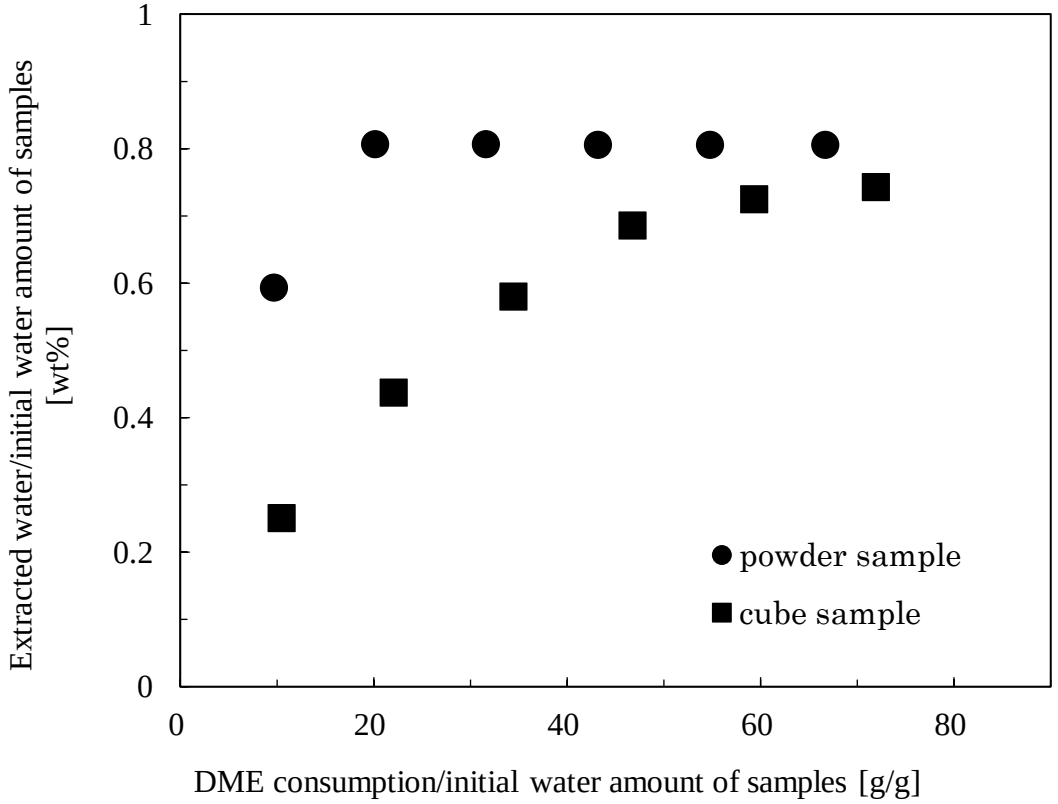

Figure 2.2.2 Dewatering rates of the DME method for mangosteen pericarp of different shape

2.2.3.2 Yield of extract and xanthones

The yield of extracted components and xanthones by DME extraction from powder sample and cube sample with various filling amounts were investigated. The yield of extracted xanthones by DME and ethanol extraction were shown in Table 2.2.1.

Table 2.2.1 Experimental condition with yield of extracted xanthones [mg/g-dry sample]

Extraction solvent	Liquefied DME		Ethanol	
Sample type	cube wet	powder wet	powder wet	powder dry
Filling amount [g]	6	6	1	1
Various xanthones				
3-Isomangostin	0.3	0.5	-	0.7
Mangostanol	0.6	1.1	-	0.7
8-Desoxygartanin	5.6	6.3	5.7	11.5
Gartanin	0.8	2.1	1.1	4.0
alpha-Mangostin	33.3	42.9	41.1	59.7
Garcinone E	1.1	1. 8	2.1	1.9
9-Hydroxycalabaxanthone	0.8	1.1	0.5	1.3
beta-Mangostin	0.5	0.7	0.8	0.9

Figure 2.2.3 and 2.2.4 shows the yields of extracted components and alpha-mangostin as xanthone derived compounds. In the case of cube sample, the yields of extracted components and alpha-mangostin were lower than powder samples even though it had almost the same yield of water. There are possible reasons that the water does not act as a co-solvent because the solubility of xanthone in water is low [26]. Therefore, it can be considered that preferentially water has been extracted. However, the cube type allows to recover alpha-mangostin of 33.3 mg/g-dry sample (about 80% of the powder sample) without milling. Cube type has advantages during the extraction such as to avoid the milling process and it can be easily handling of the sample. Highest yield of alpha-mangostin (42.9 mg/g-dry sample) was obtained from powder sample. It is corresponding to about 104 % of the ethanol extraction with wet mangosteen pericarp, and 72% of the dried sample.

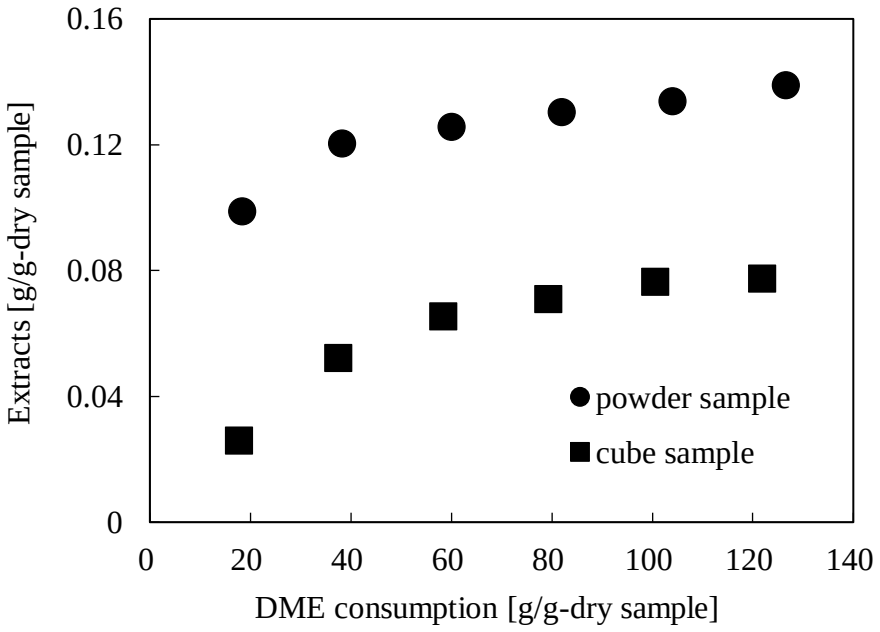

Figure 2.2.3 Extraction rates of the DME method for extracts from mangosteen pericarp of different shape

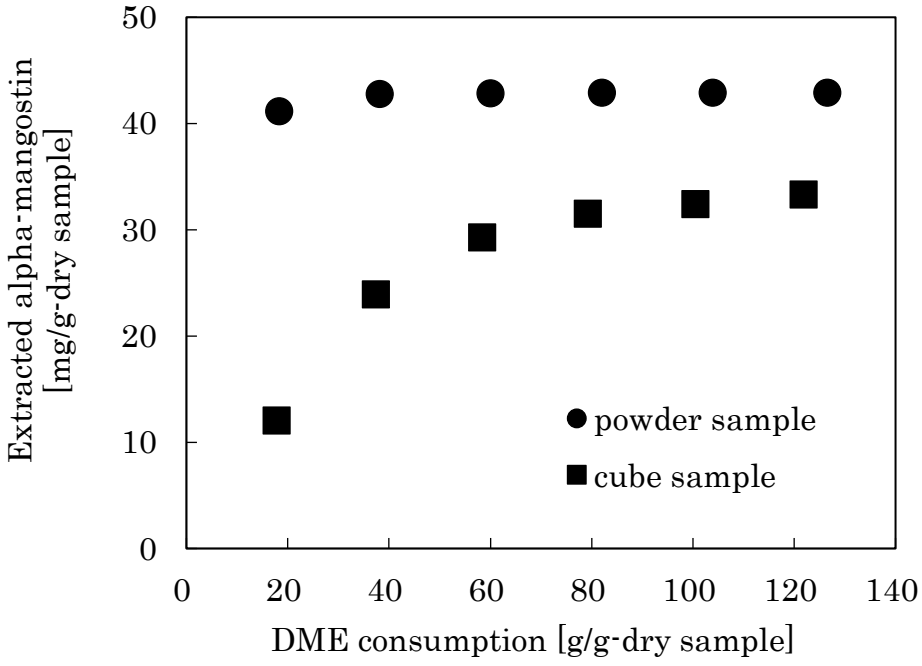

Figure 2.2.4 Extraction rates of the DME method for alpha-mangostin from mangosteen pericarp of different shape

2.2.4 Conclusion

In this work, the liquefied DME extraction method was verified that can be used to extract water, extract and xanthone from the wet mangosteen pericarp. Final extracted yield of water from the cube sample was almost equal compared with the powder type (0.8 g/g_initial water). However, in the case of powder sample, extraction equilibrium has been nearly reached in the initial stage. The highest yield of extract (0.14 g/g-dry sample) and alpha-mangostin (42.9 mg/g-dry sample) were obtained with a powder sample.

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Chapter 3

Utilization of algal biomass as a biofuels resources by liquefied DME

3.1 *Aurantiochytrium limacinum*

3.1.1 Introduction

In recent year, the depletion of fossil fuels such as coal oil are feared, and the technology for making biomass fuel from labyrinthula attracts the world's attention. Labyrinthula *Aurantiochytrium limacinum* (*A. limacinum*) as heterotrophic microorganisms is capable of growing in the presence of organic substances of brackish water region [1]. *A. limacinum* have a potential to rapidly accumulate lipids important for biofuel production [2]. The growth rate of *A. limacinum* is very fast, production efficiency of hydrocarbons production is higher than *Botryococcus braunii* [3]. In general, the lipids content in *A. limacinum* is around 30-60wt% on a dry weight basis [4]. Commonly, chloroform, methanol, ethanol, and n-hexane were used as medium to extract lipids from marine tissue such as microalgae. In particular, solvent mixtures containing a polar and no-polar solvent could extract a total lipids [5]. For example, a combination of chloroform (non-polar), methanol (polar) and water, known as Bligh-Dyer (BD) method, has been used for total

lipid extraction from biological materials [6]. However, they were toxic organic solvents and costly [7–9]. Several step processes were also required when these solvents were employed as extraction media. They are as follows: drying of starting materials, cell disruption as a pre-treatment, evaporation of extraction solvents by heating, and condensation of them by cooling [10–12]. Figure 3.1.1 (A, B) describes the procedures of conventional organic solvent extraction and BD method to extract biofuel from wet microalgae. Therefore, it could be said that these extraction processes consume a lot of energy and is environmentally-unfriendly.

Instead of organic solvents as extractants, liquefied DME would be used as an extractant to solve their drawbacks. Li et al. 2014 [13] proposed the energy-saving extraction of bio-solid fuel and bio-crude from vegetal biomass without pre-treatments such as drying and cell-disruption by using liquefied DME. Figure 3.1.1 (C) describes the procedures of proposed liquefied DME extraction. They used common vegetal biomasses such as spent coffee ground, tea leaf waste, orange peel and gramineous weed as selected materials. They informed that the liquefied DME could efficiently extract lipids, implying its utilization to produce bio-liquid fuel from lipid-rich biomass. Kanda et al. 2014 [14] also extracted carotenoid by using liquefied DME in semi-continuous flow-type system from microalgae. Compared with ethanol Soxhlet and supercritical CO_2 (SC-CO_2) extraction, which includes drying and cell disruption, the yield of carotenoid from Undaria pinnatifida was quite high. Moreover, DME as an extractant is the simplest form of ether with the following characteristics: (i) standard boiling point is -24.8 °C [15], (ii) high affinity to oily compositions and partial miscibility with water [16], (iii) safe extraction solvent for the production of foodstuff and food ingredients [17], (iv) resistance to autoxidation unlike other alkyl ethers [18]. In this work, the efficacy of DME extraction was evaluated using labyrinthula *A. limacinum* at 0.51 MPa and 20°C to verify its versatility. The results would be compared with SC-CO_2 extraction, hexane extraction and BD extraction as other methods. Furthermore, the extracted lipids and the extracted residues would be analyzed by elemental analysis, molecular weight distribution (MWD), fatty acids analysis and functional substances.

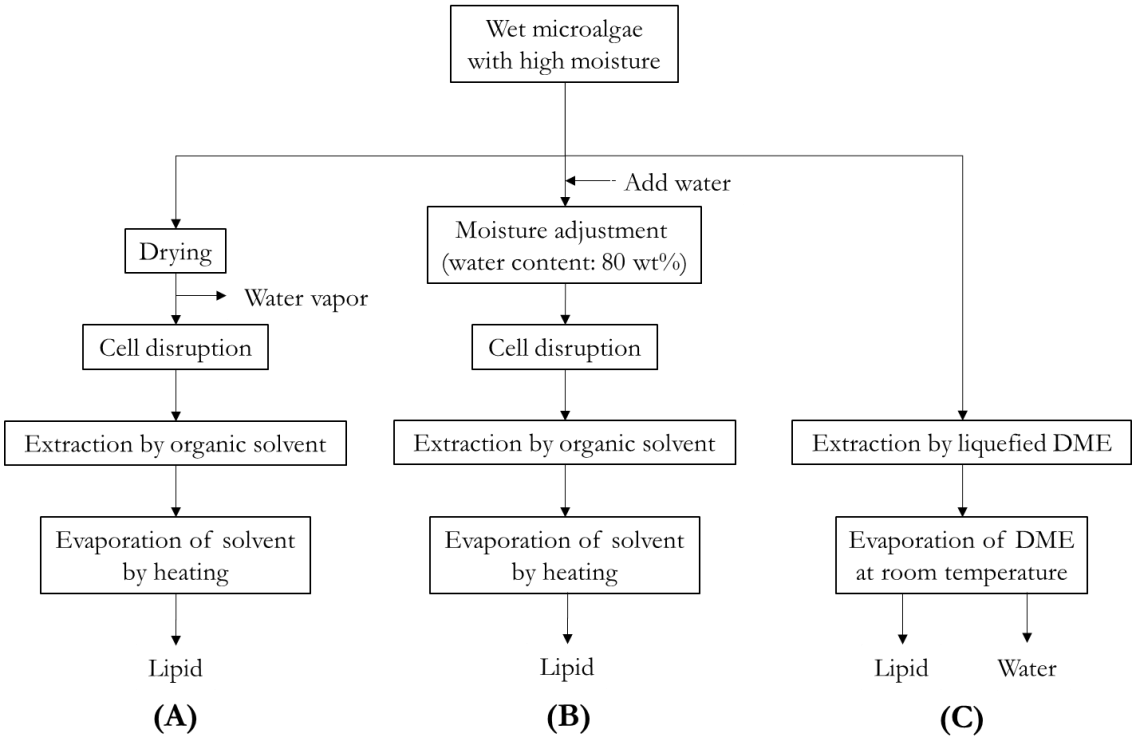

Figure 3.1.1 Comparison of the liquefied DME and conventional organic solvent extraction procedures. (A) Conventional organic solvent extraction. (B) Bligh-Dyer method. (C) Proposed method.

3.1.2 Materials and methods

3.1.2.1 Materials

A. limacinum strain was provided by Nippon Suisan Kaisya, Ltd, Tokyo, Japan and used as a starting material. The moisture content was determined by continual drying at 107 °C until constant weights. It was around 67.9 wt%. HPLC grade tetrahydrofuran (THF), n-hexane, chloroform, and methanol were purchased from Wako Pure Chemical Industries, Ltd., Japan. CO_2 (99.9 wt%) was obtained from Sogo Co., Aichi, Japan. For GPC analysis, the series of polystyrene calibration standards (Tosoh Co., Tokyo, Japan) were used.

3.1.2.2 Lipids extraction

Four methods of lipid extraction were performed: liquefied DME method; SC-CO_2

method; hexane-Soxhlet extraction method; and BD extraction method. The apparatus shown in Figure 2.1.1 was used for the DME extraction. Schematic diagrams of the apparatus used for SC-CO_2 extraction are shown in Figure 3.1.2.

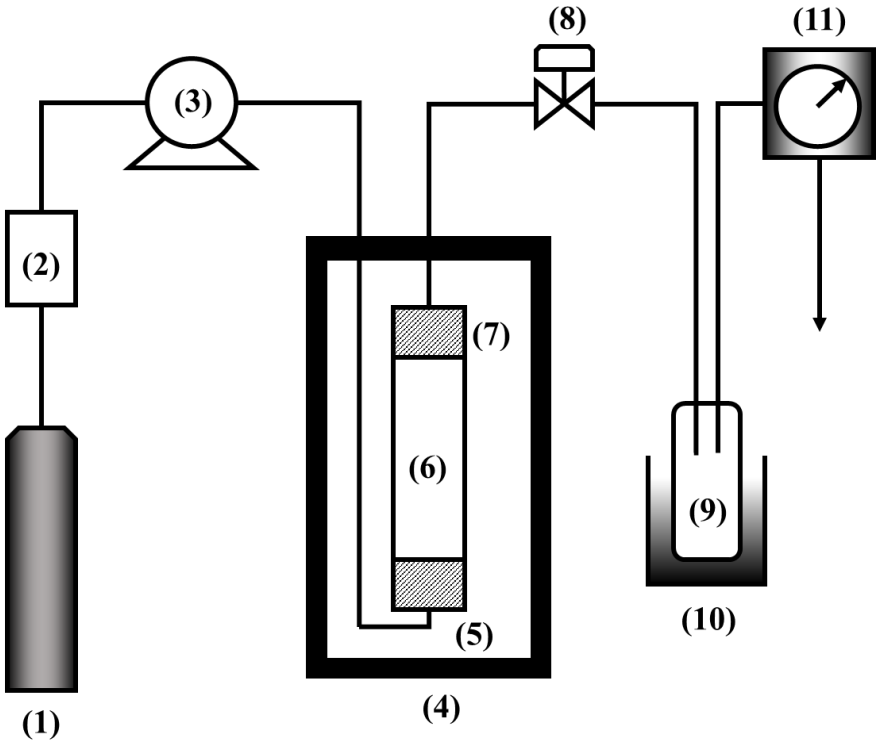

Figure 3.1.2 Schematic diagram of SC-CO_2 extraction apparatus: (1) CO_2 cylinder, (2) Chiller, (3) CO_2 pump, (4) Heating chamber, (5) Extraction column, (6) Dry sample, (7) Cotton, (8) BPR, (9) Recovery vial, (10) Water bath, (11) CO_2 gas meter

In the liquefied DME method, wet *A. limacinum* was fed directly without drying and cell disruption as pre-treatments. However, in the case of SC-CO_2, hexane and BD methods, the *A. limacinum* cells were disrupted using a pestle and mortar for 10 min. The liquefied DME extraction apparatus and procedure has been described in detail in previous studies [14, 19, 20]. A storage tank of liquefied DME (volume: 100 cm^3) an extractor (diameter: 11.6 mm, length: 190 mm, volume: 10 cm^3) and a collector, were connected in series. 2.51 g (dry weight, 0.81 g) of *A. limacinum* slurry was loaded into the lower half of the extractor, and glass beads were placed at upper half of the extractor. A filter paper (DURAPORE MEMBRANE FILTERS, pore size: 0.65 μm, material: hydrophilic

polyvinylidene difluoride, Merck Millipore, Darmstadt, Germany) was placed at the outlet of the extraction column. The flowrate of liquefied DME was 10 mL/min, and the temperature was kept at 20°C. In the SC-CO_2 extraction method, the main apparatus consists of a high-pressure pump for CO_2, a heating chamber, a extractor (volume: 25 cm^3), a CO_2 chiller, back-pressure regulator, and a wet gas meter. In order to determine the effect of temperature and pressure on the yield of extracted components, lipids were extracted from *A. limacinum* at temperatures between 60, 80, 100, 120°C, pressures of 40 MPa and CO_2 flow rate of 5 mL/min. In each experiment, 2.0 g of dried *A. limacinum* was loaded into the extraction column, and the remaining volume was filled with glass beads at the bottom and top of the extraction column. The cell was placed in a heating chamber maintained at the set operating temperature. The extract collection was collected at 15, 45, 75 min. In order to investigate the extracted components using the hexane-Soxhlet extraction method, 1.0 g of pre-treated *A. limacinum* were loaded in a Soxhlet apparatus with 200 cm^3 of n-hexane for 15 h at 70 °C. In this work, the total lipid content in *A. limacinum* was determined by BD method [6]. 1.0 g of pre-treated *A. limacinum* were extracted using a Soxhlet apparatus using 200 cm^3 of mixture of chloroform and methanol (1:1, v/v) for 15 h at 70 °C.

3.1.3 Analytical methods

The extracted lipids and the extracted residues were analyzed by elemental analysis, molecular weight distribution (MWD), fatty acids analysis and functional substances.

3.1.3.1 Elemental analysis

Elemental analysis was performed for original *A. limacinum*, extracted lipids and the extracted residues. The original *A. limacinum* and the extracted residues were conditioned at 107°C until constant weights. The carbon, hydrogen, and nitrogen content were determined by a CHN analyzer (Yanaco, CORDER MT-6) based on flash combustion, which converts all organic substances into combustion gases. The oxygen content was calculated using the differences.

3.1.3.2 Molecular weight distribution

The molecular weight distributions (MWD) of the extracted lipids was determined by gel permeation chromatography (GPC) performed at 40°C by diluting the lipids in THF. The

MWDs were compared with that of a polystyrene standard. A lipid concentration of 1 mg/mL was used for each measurement and the injection amount was 20 µL. The analytical equipment and condition used for GPC were HPLC system controller and UV/Vis detector with chromatographic columns. The wavelength was set at 250 nm [21]. The MWDs of lipids obtained by liquefied DME extraction were compared with those obtained by SC-CO_2, hexane and BD extraction methods.

3.1.3.3 Fatty acid profile

The major lipid components were identified by gas chromatograph mass spectrometer (GC/MS) 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 Technologies Tokyo Ltd., Japan). The temperature program was as a follow: initially at 60°C for 5 min, then ramped to 320°C with 4°C min^{-1}. The injector and detector temperatures were set at 250°C. The split ratio was 1:1; with a helium gas flow rate of 24 mL/min and the injection volume was 1.0 µL. Qualitative analysis of the detected fatty acids methyl esters (FAME) were carried out by comparison of their retention time and mass spectra with FAME standard (Supelco 37 Component. FAME Mix. Sigma–Aldrich St. Louis, MO, USA). The esterification of extracted lipids was carried out by using a fatty acid methylation kit and fatty acid methyl ester purification kit, which purchased from Nakalai Tesque, Inc., Kyoto, Japan.

3.1.4 Results and discussion

3.1.4.1 Extraction yield of water and lipid

Figure 3.1.3 described the yield of water that extracted by liquefied DME. The dewatering rates was 86.7 wt% by using liquefied DME extraction. In addition, Figure 3.1.4 shows the yield of lipids by using DME method. The increasing amount of liquefied DME consumed was followed by increasing the amount of lipid extracted. It seems to increase rapidly when the liquefied DME consumed was around 300 g then remained constant after 335 g. The yield of lipid could approach up to 46 g/g- dry sample. Compared with other methods, such as SC-CO_2, hexane Soxhlet and BD extraction methods, the yield of lipid by liquefied DME was quite high. The maximum yield of lipids was 22.5, 43.6, and 50.7 g/g- dry sample when SC-CO_2, hexane Soxhlet and BD extraction methods were employed as extraction medium, respectively. As informed above, that liquefied DME

could dissolve a wide range of polar and non-polar substances. DME is also good solvents for many hydrogen-bonded substances. To dissolve hydrogen bonded substances, high solvation energy was needed to break the hydrogen bonds. Due to DME has ability to act hydrogen bond acceptors, forming hydrogen bonds with hydrogen-bonding solutes, liquefied DME can penetrate into *A. limacinum* and flow out together with *A. limacinum* components include lipid. In addition, liquefied DME could also produce a lower viscosity of the analytes in the matrix and, accordingly, a better diffusion rate of the solute from the solid phase to the solvent. Consequently, the lipid in *A. limacinum* might be extracted easily.

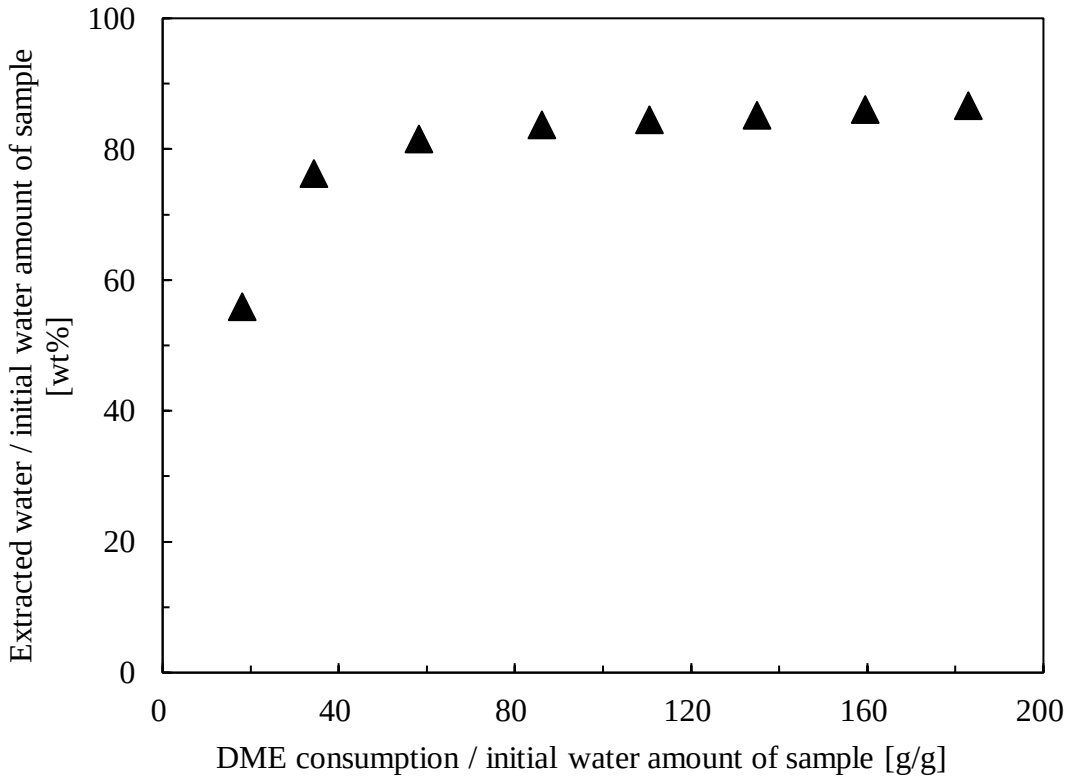

Figure 3.1.3 Extraction behavior of the water from *A. limacinum* by using liquefied DME

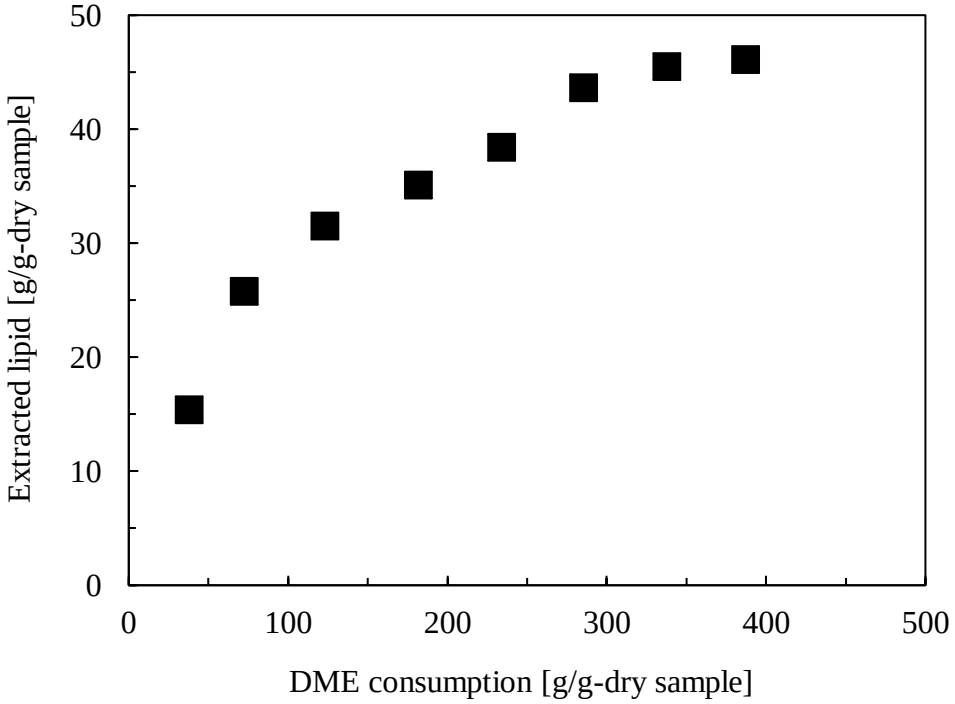

Figure 3.1.4 Extraction behavior of the lipids from *A. limacinum* by using liquefied DME

Figure 3.1.4 shows the yield of lipid extracted from *A. limacinum* by SC-CO_2 at various extraction temperatures. Generally, the yield of extract influenced by the increase of pressure or temperature at constant temperature or pressure when SC-CO_2 was applied as an extraction medium. This phenomenon occurred due to direct change of density and hence solubility of SC-CO_2 [22, 23]. In this work, it should be noted that the lipid extraction by SC-CO_2 was performed at pressure of 40 MPa with various extraction temperatures. As shown in Figure 3.1.5, the yield of lipid increased with increasing extraction temperature. Except at 60°C, the yield of lipid increased rapidly till 520 g CO_2 consumed then remained constant after 1000 g CO_2 consumed in each extraction temperature. The highest extraction rate of lipid was reached when the extraction temperature is 100°C. It could approach to 22 g/g- dry sample. This extraction condition likely had a stronger effect on the solubility, consequently the yield of lipid is lower than at 100°C when the extraction temperature was performed at lower or higher 100°C. As explained before, in the case of hexane Soxhlet extraction, the main drawbacks of this extraction methods are the long time needed and the large amount of solvent wasted, which is costly and cause environmental problems. Like hexane Soxhlet extraction, the

toxic solvents also were used in large amount when using BD extraction technique. Judging the results, it could be said that the lipid extraction by DME is very simple and versatile technique. In addition, the extraction process not required the pre-treatments such as drying and cell-disruption. Therefore, DME method is an economical method that can significantly reduce the energy required for lipid extraction from wet materials.

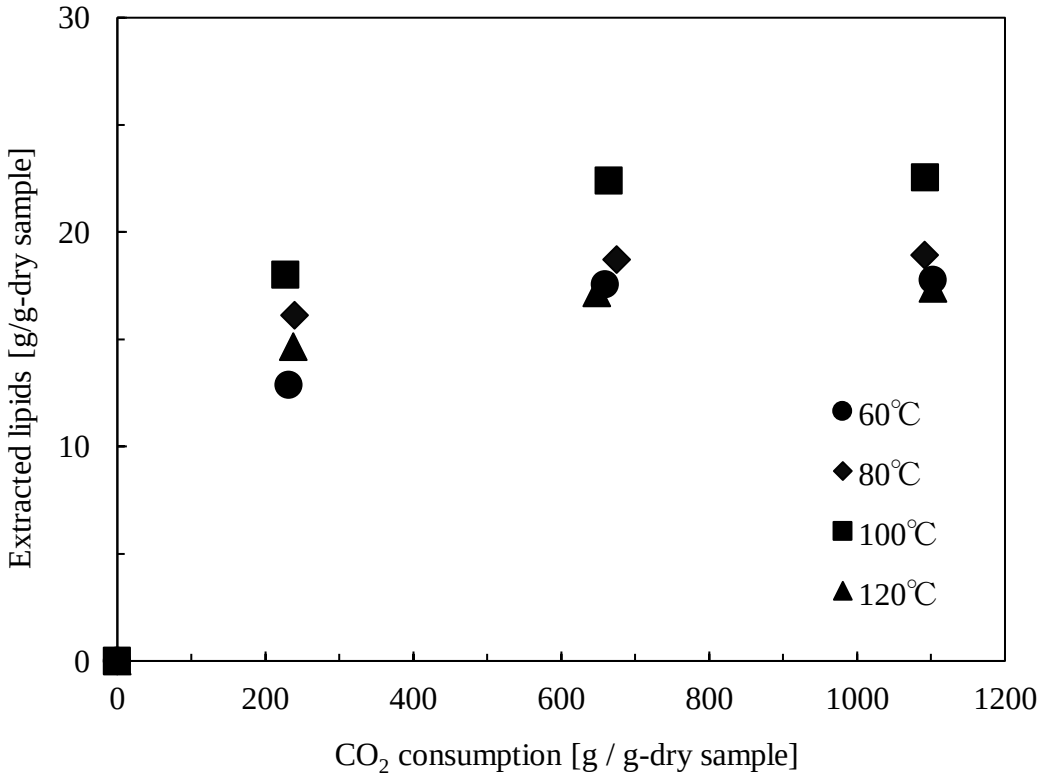

Figure 3.1.4 Extraction behavior of the lipid from *A. limacinum* by using supercritical carbon dioxide

3.1.4.2 Elemental analysis

It was well known that the elemental analysis for carbon (C), hydrogen (H), and nitrogen (N) content is one of the typical ways to express the components in the organic matter as fuel resources. The three concentrations obtained these components were expressed in % daf. The C/N ratio can be referenced as an indicator to identify the most suitable technique for the biomass conversion. When C/N is lower than 30, the conversion processes of biomass appropriate to biochemical processes, while when the C/N ratio is higher than

30, the thermochemical conversion process could be applied. Table 3.1.1 showed the elemental analysis of extracted lipids from *A. limacinum* and their residues when liquefied DME, SC-CO_2, hexane Soxhlet and BD were applied as extraction method. As shown in Table 3.1.1, the elemental content of C, H, N, and O in the *A. limacinum* as a starting material was 55.9, 8.3, 4.4, and 31.5%, respectively. After liquefied DME was applied as an extractant, the content of these elements in the residue of *A. limacinum* material decreased significanty to 47.2, 7.3, 5.4, and 40.1%, respectively. This indicated that liquefied DME could extract *A. limacinum* consituents successfully. The same phenomenon was also occurred when SC-CO_2, hexane Soxhlet and BD were used as extraction media. However, the lipids extracted using liquefied DME composed of C (68.7%), H (10.4%), N (0.2%), and O (20.8%), respectively. Compared with other extraction methods, except hexane Soxhlet, the C and H contents of the extracted lipids are higher than those extracted lipids by other methods. In general, high nitrogen content is one of the drawbacks in the terms of fuel quality [24]. In this work, the extracted lipids that shown in Table 3.1.1 had low nitrogen content. Accordingly, these extracted lipids from *A. limacinum* was suitable as bio-fuel.

Table 3.1.1 Elemental analysis of extracted lipids and the residues

Analysis [dry ash free]	Liquefied dimethyl ether		SC-CO_2		Hexane		Bligh-Dyer		Original
[%]	Lipid	Residue	Lipid	Residue	Lipid	Residue	Lipid	Residue	Feed
C	68.7	47.2	67.2	48.8	75.2	44.6	64.7	51.1	55.9
H	10.4	7.3	10.0	7.4	10.8	6.8	9.4	7.7	8.3
N	0.2	5.4	0.1	5.4	0.7	5.0	0.2	4.7	4.4
O*	20.8	40.1	22.7	38.5	13.4	43.6	25.7	36.5	31.5

*By difference

3.1.4.3 Molecular weight distribution

In principle, the different extraction methods provide the different molecular weight extracted constituents of matrix which may give the different properties in chemically and physically. Hence, the properties of extracted lipids from *A. limacinum* by liquefied DME, SC-CO_2, hexane Soxhlet and BD extraction methods were analyzed by GPC. This technique is simple and has become a powerful method for the determination of the MWD of extracted compounds. Figure 3.1.6 showed the MWD of extracted lipids relative to the polystyrene standards. The MWD of extracted lipids by liquefied DME were compared with those obtained by SC-CO_2, hexane Soxhlet and BD extraction methods using GPC with THF as a solvent. As illustrated in Figure 3.1.5, the MWD of extracted lipids are almost the same. They seemed to have molecular weights ranging from 1 to 100 kDa. This indicated that liquefied DME that employed as a solvent media allowed to extract lipid components from *A. limacinum* matrix. The result also showed that liquefied DME may have high polarity property which is suitable for extracting higher-molecular-weight polar compounds.

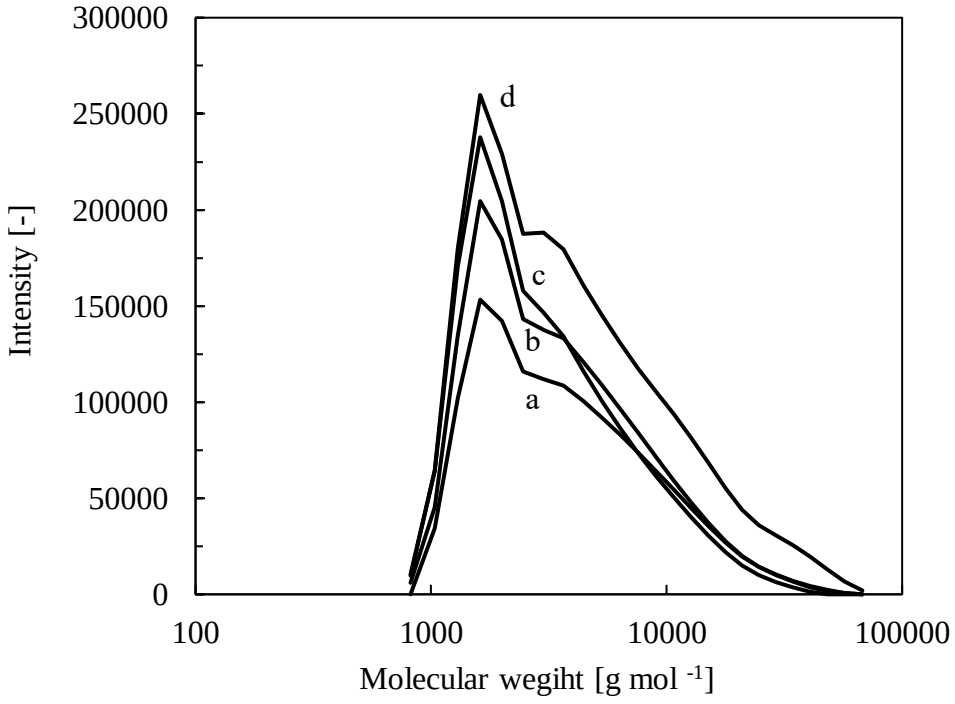

Figure 3.1.6 Molecular weight distribution curve of the extracted lipids: (a) Bligh-Dyer, (b) Hexane, (c) DME, (d) SC-CO_2

3.1.4.4 Fatty acid profile

In order to understand the fuel properties, the systematic analysis of the fatty acid composition is very important for species selection for biodiesel production. The most common components of fatty acid are Palmitic-(hexadecanoic-C16:0), Stearic-(octadecanoic-C18:0), Oleic (octadecenoic-C18:1), Linoleic-(octadecadienoic-C18:2) and Linolenic-(octadecatrienoic-C18:3) acids [25]. However, the small amounts of eicosapentaenoic acid (EPA) (C20:5) and docosahexaenoic acid (DHA) (C22:6) were also found in several biodiesel resources, including microalgae. The fatty acid composition of lipids from *A. limacinum* was shown in Table 3.1.2. Their compositions varied significantly with the different methods. In the liquefied DME extraction, the amount of saturated fatty acid was 95.5 % with palmitic acid (C16:0) as main components of extracted lipids from *A. limacinum*. The amount of palmitic acid was 89.4 %, followed by 3.7 % myristic acid (C14:0) and 2.5 % stearic acid (C18:0). The small amounts of docosapentaenoic acid (DPA; C22:5n-6) and docosahexaenoic acid (DHA; C22:6n-3) were obtained. This is, of course, a beneficial result in terms of biodiesel fuel where the high content of polyunsaturated fatty acids are not suitable for use as biodiesel oil [26]. Compared with other methods, liquefied DME could extract lipids containing more saturated fatty acid than unsaturated fatty acids. Thus, extraction by using liquefied DME appears to be a good method for lipids recovery from *A. limacinum* with improved yields.

Table 3.1.2 Percentage fatty acid components of the lipids from *A. limacinum*

Fatty acids	Components [Peak area %]			
	Liquefied DME	SC-CO_2	Hexane	Bligh-Dyer
C14:0	3.7	1.5	1.6	2.9
C16:0	**89.4**	**63.7**	**57.9**	**76.9**
C18:0	2.5	0.8	1.0	1.4
C22:5n-6	1.1	7.1	7.8	4.0
C22:6n-3	3.4	27.0	31.7	14.8
Saturated fatty acid	95.5	66.0	60.5	81.2
Unsaturated fatty acid	4.5	34.0	39.5	18.8

3.1.5 Conclusion

This work has clearly verified that the liquefied DME method can be used to directly extract lipids from the wet *A. limacinum* at room temperature. Comparison of the extraction yields, elemental analysis, the MWD curves, and the GC/MS spectra, the liquefied DME extraction technique indicated that this technique performs as well as hexane or BD extraction techniques despite the steps of drying, cell disruption, and heating at high temperature. Moreover, the *A. limacinum* lipids were consisted of saturated fatty acids, implying that *A. limacinum* is suitable as a fine fuel source and easily modified into a biodiesel fuel. Thus, liquefied DME method, which can skip drying and cell disruption, has the advantage of being energy-saving for lipid extraction.

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3.2 *Pleurochrysis carterae*

3.2.1 Introduction

Biofuel is generally produced from oleaginous plants, such as palm, sunflower, soybean, and rapeseed via a transesterification reaction of their oils with short-chain alcohols. Recently, microalgae have been considered to be one of the most promising alternative sources for biofuels due to the several advantages. They are such as higher photosynthetic efficiency, higher biomass productivities, a faster growth rate than higher plants, highest CO_2 fixation and O_2 production, etc [1, 2]. Moreover, biofuel was a renewable resource of energy that might be sustainably supplied and easily available from general plant biomass sources. It seems to have several favorable environmental properties resulting in no net increased release of carbon dioxide and very low sulfur content, therefore biofuel has a considerable environmentally friendly potential [3-5].

Beside microalgae have a number of advantages when they were applied as a raw material for biofuels production, they also have high lipids content. The different species and strains of microalgae resulted in the different of lipid content. Generally, the amount of lipids in microalgae was in the range of 15% to 50% in dry base, even the lipids content in *Botyococcus braunii* could be up to 80 % under certain circumstances [6]. Therefore, microalgae offers many potential advantages over conventional biomass sources. Here, *pleurochrysis carterae* (*P. carterae*), one of the microalgae species, would be subjected as a biofuels resources. Similar to others microalgae, *P. carterae* has high lipids content in the range of 30% to 50 % in dry base. This microalgae also has ability for long-term outdoor culture, and low potential for contamination of protozoans or other algae owing to the high pH of the growth medium during outdoor cultivation [7].

In the present work, the liquefied dimethyl ether (DME) would be employed as a solvent extraction to extract lipids from *P. carterae*. DME is the simplest form of ether and has high affinity to oily substances and partial miscibility with water. The relative permittivity of DME is around 1.08 and 5.34 at 30.5°C, in gaseous and liquid states, respectively. Due to these beneficial properties, it allowed to apply DME as a solvent extraction to extract lipids from *P. carterae* without any process such as drying treatment. In addition, DME also has a low boiling point (-24.8°C), hence, it was not found in the final products at normal temperatures. This liquefied DME extraction method has been successfully implemented to recover the lipids from a series of feedstocks [8-11]. Kanda et al. [9] conducted extraction of hydrocarbons and lipids from wet *B. braunii* showing the same yield as obtained by hexane soxhlet extraction with dried algae biomass. They also

informed that DME-based extraction method may reduce the energy needed for drying and cell disruption of algae biomass. Similar results were also found when the DME-based extraction method was applied to extract lipid from the common vegetal biomasses such as spent coffee ground, tea leaf waste, orange peel, and gramineous weed [10].

3.2.2 Materials and methods

3.2.2.1 Materials and chemicals

Microalgae corporation, gifu city was used as a starting material. The analytical reagents used were n-hexane, chloroform, and methanol (HPLC-grade) from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). The series of polystyrene calibration standards was purchased from Tosoh Co., Tokyo, Japan. All chemicals were used as received. DME was purchased from Tamiya, Inc. Japan and used without further purification.

3.2.2.2 Hexane soxhlet extraction

This technique is one of the solvent extraction techniques that can be applied with relative simple and ease for the extraction of lipids from microalgae in the mass production of biofuels. In this work, the hexane soxhlet extraction was carried out at temperature of 80 ^{o}C with reaction time 8 hours. Initially, the dried *P. carterae* and hexane were loaded in flask. The amounts of them were 8 g and 200 mL, respectively. When the soxhlet extraction time was reached, the mantle heater electric source was unplugged. Then, the flask was removed from the mantle heater, and the liquid extracts were transferred to bottle that covered with aluminium foil and refrigerated until analysis.

3.2.2.3 Bligh-Dyer extraction

Similar with hexane soxhlet extraction technique, the Bligh - Dyer extraction technique is also a simple and a typically used technique to extract lipids from microalgae biomass. This technique uses mixture of chloroform and methanol as solvents to extract lipids. The mixture of chloroform-methanol (1:1 v/v) and dried *P. carterae* were loaded slightly in the flask. The ratio of solvent-to-sample was 1:1. The mixtures of solvent-sample was then transferred into a separatory funnel and shaken for 5 min. The lipid fraction was separated from the separatory funnel and the solvent evaporated using a rotary evaporator.

3.2.2.4 Liquefied DME extraction

The apparatus shown in Figure 2.1.1 was used for the DME extraction. The main apparatus consisted of extractor (HPG-10-5; Taiatsu Techno Corp., Saitama, Japan; volume: 10 cm^3), needle valve to control the DME flow rate, and extract storage tank (HPG-96-3; Taiatsu Techno Corp., Saitama, Japan; volume: 96 cm^3). The extractor and DME storage tank were made of pressure-resistant glass coated with polycarbonate. 1.79 g of the raw *P. carterae* (moisture content: 61.99 wt%) was introduced into the lower half of the extractor, and the upper half was loaded with colorless glass beads. The DME flow rate was 10 mL/min. The extraction was conducted at temperature of 25°C and pressure of 0.59 MPa. After passing liquefied DME through the extractor at different time intervals, the DME was removed by opening the reducing valve of the storage vessel. The total extraction time was less than 45 min.

3.2.3 Analytical methods

The extracted lipid components were identified and quantified by gas chromatograph mass spectrometer (GC/MS; Agilent 7890A GC) which connected to an Agilent 5975C mass spectrometer. The installed column was a phenyl arylene capillary column (HP-5MS; Length: 30 m, I.D: 0.25 mm, Film Thickness: 0.25 μm). The temperature program was as a follow: initially at 60°C for 5 min, then ramped to 320°C with 4°C min^{-1}. The injector and detector temperatures were set at 250°C. The flow rate of helium gas as a carrier gas was 24 mL/min and the injection volume was 1.0 μL. The fatty acids methyl esters standard (FAME; Supelco 37 Component. FAME Mix. Sigma–Aldrich St. Louis, MO, USA) was used to determine the amount of extracted fatty acids methyl esters from *P. carterae*. The molecular weight distributions (MWD) of extracted lipids was determined by using gel permeation chromatography (GPC) at 40°C with tetrahydrofuran (THF) as a solvent. Polystyrene was employed as a standard determination. The elemental analysis was conducted for original *P. carterae*, extracted lipids and the *P. carterae* residues by a CHN analyzer (Yanaco, CORDER MT-6). The thermo-gravimetric analysis (TG/DTA; Thermoplus; TG8120) was also conducted to investigate the behaviour of *P. carterae* residues.

3.2.4 Results and discussion

Figure 3.2.1 described the yield of water that extracted by liquefied DME. The dewatering rates was 85.2 wt% by using liquefied DME extraction.

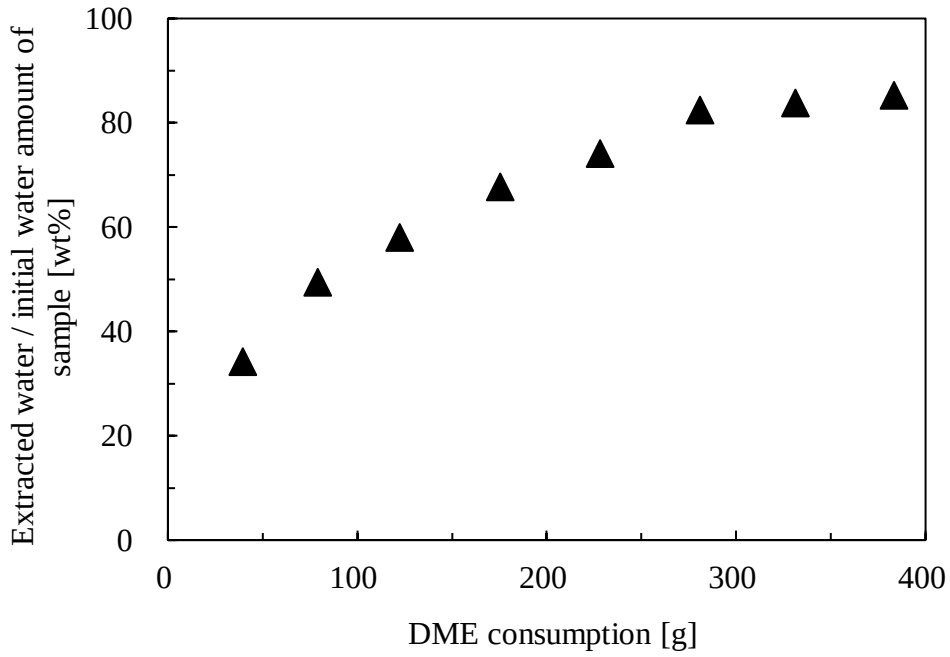

Figure 3.2.1 Yield of water from *P. carterae* by liquefied DME extraction method

On the other hands, in hexane soxhlet extraction process, beside simple and easy, this process allows to use a cycles series of hexane solvent evaporation and condensation to provide a continuous supply the fresh hexane solvent to the *P. carterae* in a thimble filter. Hence, the hexane solvent used could be reduced and the extracted lipids from *P. carterae* are kept in a concentrated form within a limited volume of hexane solvent. Halim et al. [12] informed that the hexane soxhlet extraction to be significantly more (280%) efficient than a batch system when applied for extracting lipids from *Chlorococcum sp*. In this work, the maximum amount of extracted lipids from *P. carterae* by using hexane soxhlet extraction was 7.5 g/g- dry sample. At these conditions, hexane as a single extractant has ability to destroy the *P. carterae* cell walls, and to extract the existence of lipids in its cells. It was well known that the temperature acts a significant role for extraction, elevated temperatures can disrupt the strong solute-matrix interactions caused by van der Waals

forces, hydrogen bonding, and dipole attractions of the solute molecules and active sites on the matrix [13]. It was expected the use of hexane as an extract solvent at elevated temperature is enhanced solubility and mass transfer effects, and disruption of surface equilibria. However, since the lipids content in *P. carterae* was around 30% to 50% [6], the hexane soxhlet extraction technique seems not adequate for the *P. carterae* lipids extraction.

In order to complete lipids extraction from *P. carterae*, all the bonds between the lipids and other cell components must be disrupted and, at the same conditions, the disruption process must not affect any degradation of the extracted lipids. Consequently, the usage of any solvent or two-solvents based extraction process must cover a choice of solvent that results a set of chemical interactions between the analyte and solvent molecules that is more favorable than the chemical interactions between the solvent molecules themselves and the analyte with the matrix. Next, the Bligh-Dyer extraction method was applied to extract lipids from *P. carterae*. The water molecules will partition into the methanol solvent, and the lipid molecules in *P. carterae* will partition into the chloroform solvent. The interactions between methanol sovent and water molecules are stronger than they are between the methanol and chloroform, while the interactions between chloroform solvent and lipids molecules are stronger than the interactions between water molecules or methanol solvent and lipids molecules. As results, the amount of extracted lipids from *P. carterae* by using Bligh-Dyer method was 9.0 g/g- dry sample. Compared with hexane soxhlet extraction, the Bligh-Dyer extraction was more effective extraction method. It indicated that methanol as a polar solvent may disrupt the hydrogen bonding and electrostatic forces between the lipids and proteins in *P. carterae*. Next, chloroform as a non-polar solvent could extract lipids easily [14]. In addition, Halim et al. [15] also informed that the elevated temperatures during hexane soxhlet extraction potentially caused lipids degradation.

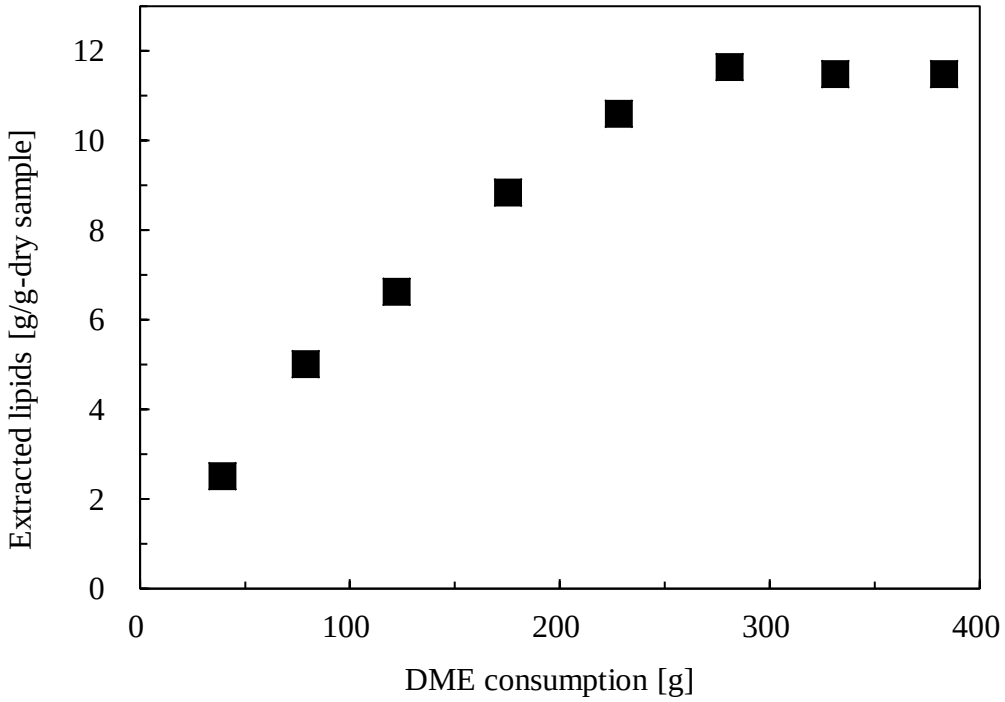

Figure 3.2.2 Yield of lipids from *P. carterae* by liquefied DME extraction method

Figure 3.2.2 showed the yield of extracted lipids from *P. carterae* by using liquefied DME. Proportionally, the amount of liquefied DME consumed was followed by increasing the increasing amount of extracted lipids. As shown in this figure, the amount of extracted lipids increased rapidly until 280 g of liquefied DME consumed. At this point, the amount of extracted lipids was around 11.6 g/g- dry sample. When the consumption of liquefied DME was added to 383 g, the amount of extracted lipids seemed constant at 11.6 g/g- dry sample. It indicated the maximum of extracted lipids from *P. carterae* was reached. Compared with hexane soxhlet extraction and Bligh-Dyer extraction methods, the yield of lipids by this method was higher. As explained before, despite liquefied DME had high dissolving ability, liquefied DME may also generate a lower viscosity of the analytes in the matrix and, accordingly, a better diffusion rate of the solute from the solid phase to the solvent [11]. Therefore, liquefied DME can enter into *P. carterae* cells and flows out together with *P. carterae* components. As results, the substances including lipids in *P. carterae* might be extracted easily. It should be noted that the others component in the extract was not determined.

(a) (b)

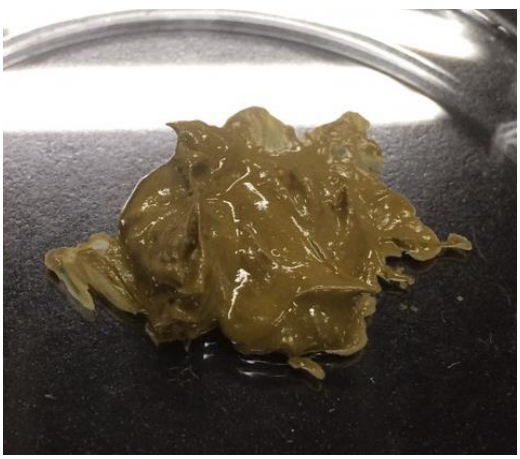

Figure 3.2.3 *P. carterae* before (a) and after (b) liquefied DME extraction

Figure 3.2.3 showed the residues of the *P. carterae* after extraction by using liquefied DME. The residue of the *P. carterae* was almost perfectly dry as like as such kind of dry paper. The color of *P. carterae* was changed from dark green-yellow to light green-white color. It indicated that the water content in the *P. carterae* matrix was also removed simultaneously when the liquefied DME as an extractant was applied to extract lipids as a target compound. This is, of course, a positive effect in terms of extracting the substances from the plant matters. This confirmed that the extraction by using liquefied DME was very different from the conventional methods. It could be directly applied to extract the organic substances from the natural feedstock at room temperature that the heating of the extractant and the downstream hot-drying are both avoidable.

Table 3.2.1 Elemental analysis of starting material, extracted lipids and residues

Elements	DME		Hexane		Bligh-Dyer		Original
[%, dry ash free]	Lipids	Residue	Lipids	Residue	Lipids	Residue	Feed
C	71.0	27.0	71.9	29.8	57.0	25.9	31.3
H	10.0	3.7	9.7	4.1	7.7	3.4	4.2
N	1.3	4.0	1.5	4.3	1.7	3.6	3.8
O*	17.7	65.3	16.9	61.9	33.6	67.1	60.7

*calculated by difference

Table 3.2.1 shows the elemental composition of extracted lipids from *P. caterae* obtained after hexane soxhlet, Bligh-Dyer, and liquefied DME extraction methods. This analysis is one of the typical ways to state the components in the organic matter as fuel resources. The three concentrations of carbon (C), hydrogen (H), and nitrogen (N) obtained these components were showed in % daf. The C/N ratio can be used as a reference to know the most suitable technique for the biomass conversion [16]. When C/N is lower than 30, the conversion processes of biomass suitable to biochemical processes, while when the C/N ratio is higher than 30, the thermochemical conversion process could be employed. Hence, the used methods in this work seems suitable method to extract lipids from *P. caterae*. From this table, it could be observed that the composition of the extracted lipids was almost similarly for both hexane soxhlet and liquefied DME extraction methods. The results exhibited that liquefied DME extraction method was appropriate method to extract lipids from *P. carterae*. The low nitrogen content in the extracted lipids was also indicated that the extracted lipids from *P. carterae* was suitable as a biofuel. In case of Bligh–Dyer extraction method, the lower carbon content in the extracted lipids might be affected by a wide range of oily components which may isolated from *P. carterae* matrix.

When the concept of utilizing microalgae to produce lipids as biofuel resources, the development and commercialization of lipids extraction method become major focus for research. It was already known that the efficient extraction of lipids from microalgae is highly affected by the polarity of the organic solvent or solvent mixture used, although an appropriate technique of cell disruption was applied. Therefore, the different extraction methods gave the different molecular weight extracted lipids of matrix which may provide the different properties in chemically and physically.

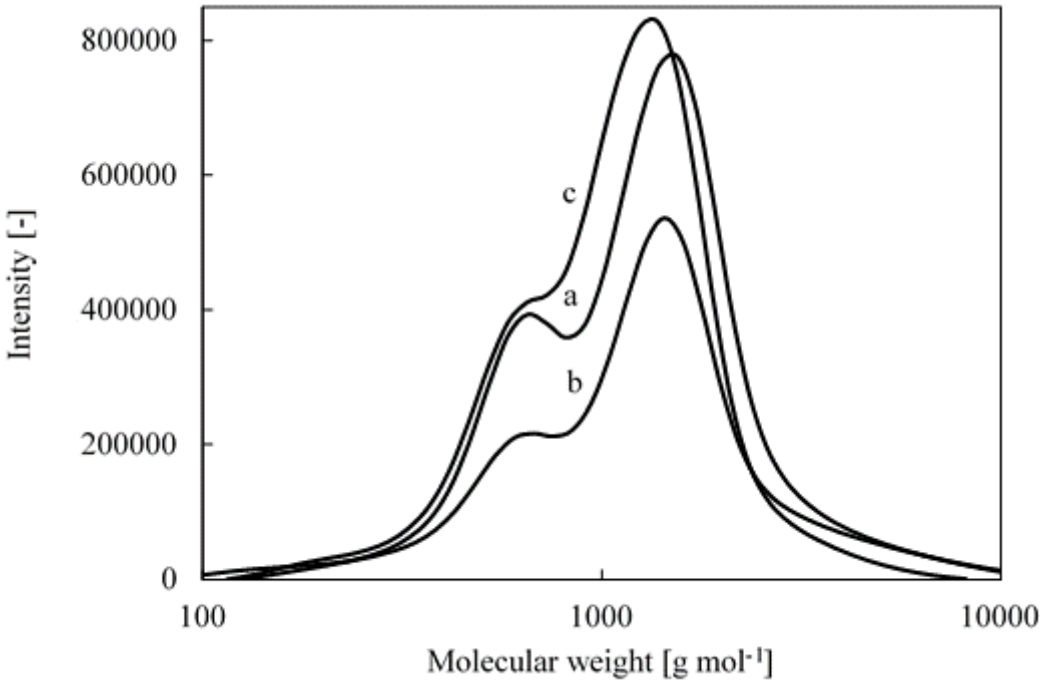

Figure 3.2.4 Molecular weight distribution of extracted lipids: (a) hexane soxhlet, (b) Bligh-Dyer, and (c) liquefied DME extraction methods

Figure 3.2.4 showed the MWD of extracted lipids from *P. carterae* by hexane soxhlet, Bligh-Dyer, and liquefied DME extraction methods by using GPC. This analysis is simple technique and has become a powerful technique for the determination of the MWD of extracted compounds from plant matters. From this figure, it could be known that the MWD of extracted lipids were around 100 Da to 10 kDa. Regarding to the large molecular weight of extracted lipids from *P. carterae*, the crude oils obtained may has high in viscosity, thus requiring conversion to lower molecular weight constituents in the form of fatty acid alkyl esters as a main components in biofuels. This figure also gives information that the liquefied DME allows to extract lipids from *P. carterae* matrix and gives results similar with hexane soxhlet and Bligh-Dyer extraction methods. Due to the unique properties of DME [11], the intensity of extracted lipids MWD was higher than that extracted using the hexane soxhlet and Bligh-Dyer extraction methods.

For further analysis, GC-MS was performed to identify and quantify the extracted lipids profile in *P. carterae*. According to the lipids polarity, lipids can be roughly classified into two groups, neutral lipids and polar lipids. Polar lipids where fatty acid carboxylic group head is attached to a charged head group and neutral lipids where the carboxylic group

end of the fatty acid molecule bonded to an uncharged head group [15]. In this work, the extracted lipids class content was not investigated. Prior to GC-MS analysis, the extracted lipids were esterified by using acid-catalyzed esterification method. The identified compounds of the main peaks were carried out by using a NIST mass spectral database and are compared with the FAME standard. Next, the amount of extracted lipid components in the extract can be quantified.

Table 3.2.2 presents the major components of fatty acid which extracted from *P. carterae*. The typical fatty acid components of microalgal species are C16 and C18 groups, which are similar to those of vegetable oils, and thus suitable for producing biofuels, such as biodiesel. As shown in this table, the prominent fatty acid components were palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and stearidonic acid (C18:4) for each method. Other fatty acids, such as (C16:4(n-3)) hexadecatetraenoic acid, (C18:0) stearic acid), (C18:2) linoleic acid, and (C20:1) gondoic acid, were also exist in smaller quantities. According to Knothe [17], palmitic acid, oleic acid, linoleic acid, and stearic acid were recognized as the most common fatty acids obtained in biodiesel. Judging the result, it could be said that the extraction of lipids which contained the ideal biodiesel components by using liquefied DME, without using any pretreatment like cell-drying or cell-disruption, has been demonstrated.

Table 3.2.2 Compounds identified in extracted lipids from *P. carterae*

Fatty acids (FA)	DME	Hexane	Bligh-Dyer
C12:0	0.4	n.d	n.d
C14:0	0.5	0.4	0.3
C15:0	0.1	n.d	0.1
C16:0	**26.2**	**30.1**	**25.3**
C16:1	0.2	0.2	n.d
C16:3 (n-3), HTA	0.4	n.d	0.3
C16:4 (n-3)	3.2	2.9	1.5
C17:0	0.5	0.4	0.4
C17:1	1.4	1.4	1.4
C17:2	1.7	1.5	1.9
C18:0	5.1	4.0	3.7
C18:1	**26.5**	**30.5**	**27.1**
C18:2	7.2	8.0	6.9
C18:3 (n-6)	n.d	n.d	n.d
C18:4 (n-3), SDA	**15.7**	**13.5**	**14.5**
C20:0	0.5	n.d	n.d
C20:1	2.8	3.1	2.8
C20:2	0.1	0.1	0.2
C20:3 (n−6)	n.d	n.d	0.1
C20:4 (n-3), ETA	0.1	n.d	0.2
C20:4 (n-6)	2.0	n.d	5.2
C20:5 (n-3), EPA	1.1	0.1	1.7
C21:5 (n-3)	0.1	0.2	0.2
C22:0	0.1	n.d	n.d
C22:5 (n-3), DPA	n.d	n.d	n.d
C22:5 (n-6)	n.d	n.d	n.d
C22:6 (n-3), DHA	1.6	3.2	6.3
other	2.6	0.2	0.0
Saturated FA	33.2	34.9	29.7
Unsaturated FA	64.2	64.9	70.3

*HTA: hexadecatrienoic acid; SDA: stearidonic acid; ETA: eicosatetraenoic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid: DHA: docosahexaenoic acid, n.d.: not detected.

Figure 3.2.5 shows TG analysis of *P. carterae* before and after extraction process. Under oxidative (air) atmosphere, an approximately 10 mg of sample was placed in aluminum pan. The samples were heated at 5°C min^{-1} from 30 to 500°C with 50 mL/min gas flow rate. This analysis records the change of weight of samples at given temperatures while the temperature is increased over time. By the comparison between the *P. carterae* before and after extraction process, the temperature range of TG applied may correlate to the existence of substances in microalgae. Generally, the thermal degradation processes of microalgae are similar to each other and can be divided into several different zones [18]. The first and second stages were from the initial temperature to 200°C and from 200°C to 350°C. At these zones, the dehydration of microalgae and the decomposition of protein and carbohydrate occurred. The decomposition of lipids took place at 350 to 550°C. In the next zone (> 550°C), the decomposition of other substances occurred. Accordingly, the characterization of *P. caterae* before and after extraction processes by TG analysis were carried out at temperatures of 30 to 500°C. As depicted in Figure 3.2.5, the slow drop of the TG curves happened when the elevating temperature was lower than 200°C. Conversely, the rapid drop of the TG curves took place at 200 to 450°C due to the reactions of depolymerization, decarboxylation, cracking, carbohydrate, protein, and lipids [18, 19]. These phenomena could be found on the TG curves of all samples except the residue of *P. carterae* after treatment by liquefied DME. Again, it indicated that the most of *P. carterae* substances including lipids has been extracted by liquefied DME.

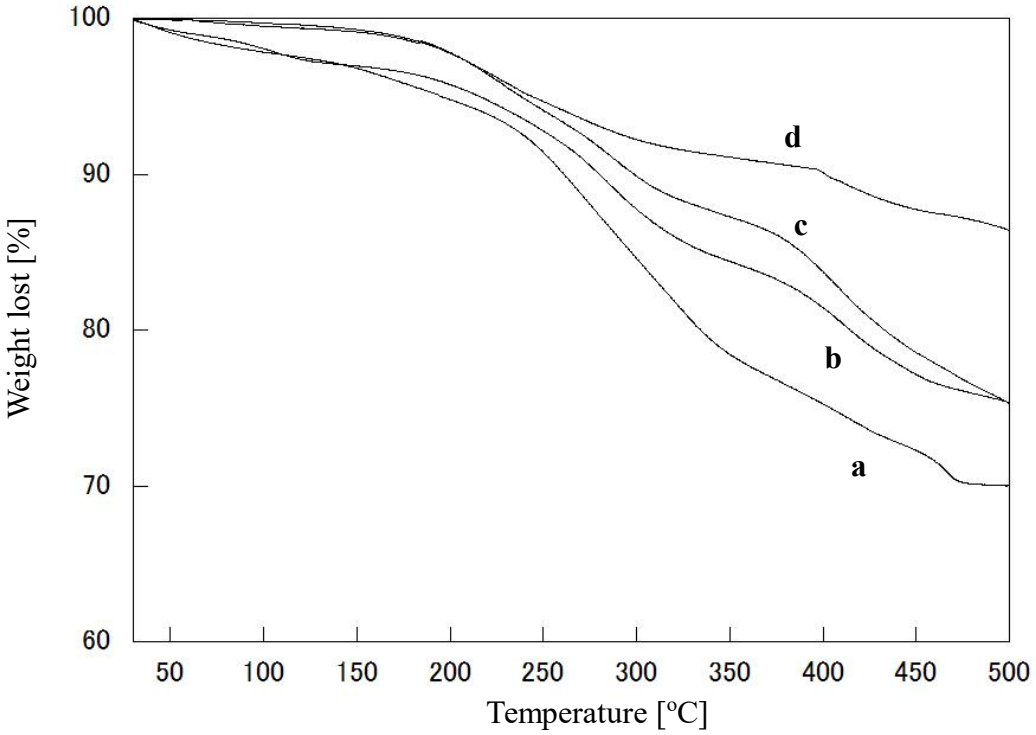

Figure 3.2.5 TG curves of (a) starting material and residues of (b) hexane soxhlet, (c) Bligh-Dyer, and (d) liquefied DME extraction methods

3.2.5 Conclusion

Extraction of lipids from *P. carterae* has been performed by different extraction methods. As a simple and environmentally friendly extraction method, liquefied DME could be applied directly to extract lipids from *P. carterae* matrix without using any pretreatment like cell-drying or cell-disruption. Liquefied DME extraction was carried at temperature of 25°C and pressure of 0.59 MPa using a semi-continuous flow-type system. The maximum of extracted lipids was 11.6 % when the amount of DME consumed was 280 g. Compared with hexane soxhlet extraction (7.5 %) and Bligh-Dyer extraction (9.0 %) methods, the result was quite high. The GC-MS analysis showed that the extracted lipids by using liquefied DME contained the ideal biodiesel components, such as palmitic acid, oleic acid, linoleic acid, and stearic acid as main components. Judging the result, liquefied DME extraction method seems to be an apt method for lipids extraction from *P. carterae*.

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3.3 *Arthrospira platensis*

3.3.1 Introduction

In recent years, the microalgae have attracted attention as potential biofuels, because their photosynthetic capacity is higher than that of other plants, and microalgae do not compete with food or feed stocks [1]. Generally, the recovery of lipids and other high value products from microalgae is performed via hexane extraction. The procedure for hexane extraction involves the following steps: drying, cell disruption, hexane evaporation by heating, and hexane condensation by cooling. The high temperature is required for heating in the drying and hexane evaporation steps. This is energy-consumptive and represents a drawback in hexane extraction [2–4]. Recently, many life cycle assessments of algal biodiesel production from microalgae were conducted, and indicated that drying and extraction accounted for a large part of the total energy requirement of the algal biodiesel production [5, 6]. Thus, they suggested direct lipid extraction from wet microalgae as a viable alternative option that helps decrease energy consumption in the drying process. For example, 1,2-dimethoxyethane was proposed as extraction solvent for wet *Botryococcus braunii*. Nonetheless, this method still requires significant energy for the recovery of 1,2-dimethoxyethane that has to be extracted at high temperature because the boiling point of 1,2-dimethoxyethane is 82–83°C [7].

In previous studies, we proposed a simple, energy-saving extraction method for the recovery of lipids from wet blue-green microalgae [8, 9]. The proposed method employed liquefied dimethyl ether (DME) as solvent for lipids. Dimethyl ether is the simplest ether, having the following characteristics: (i) low standard boiling point (–24.8°C) [10], (ii) high affinity for oily compositions and partial miscibility with water [11], (iii) is a safe extraction solvent for the production of foodstuff and food ingredients [12, 13], and (iv) resistance to autoxidation, unlike other alkyl ethers [14]. The DME extraction was derived from combination of our previously proposed dewatering [15] and oil removal from solids [16]. DME can be easily removed from lipids and residues by decompression because the standard boiling point of DME is very low. The advantages of DME extraction are that it eliminates the need for drying, cell disruption, and solvent evaporation at high temperature, and is, thus, a simpler method that requires low amounts of energy [17]. In the previous study, DME extraction method was examined for natural blue-green microalgae which was not customized for production.

In this study, we applied the proposed method to cyanobacterium microalgae *Arthrospira platensis* (vernacular term is Spirulina), which is resource of lipids and food and feedstock

supplement in the aquaculture and poultry industries. *A. platensis* can be easily separated from the culture using a screen filter [18] because the size of *A. platensis* is 0.3–0.5 mm, larger than other microalgae. In general, *A. platensis* cells contain 7–8 wt% lipids of dry cell weight [19]. *A. platensis* contains functional substances as co-production for cost reduction of lipids processing for fuels. *A. platensis* contains blue chromoprotein phycocyanin [20], which is used as a pigment for food additives, and has antioxidant properties and an anti-inflammatory effect [21, 22]. In addition, *A. platensis* contains β-carotene and zeaxanthin as carotenoid pigments; these have anti-cancer effects, and help prevent macular degeneration and cataract [23, 24]. Furthermore, it also contains the green pigment chlorophyll [25], which is used as a natural colorant in food, cosmetic, and pharmaceutical products.

Previously, extraction of these lipids and functional substances from *A. platensis* using supercritical carbon dioxide (SC-CO_2) and organic solvents such as hexane, acetone, and dimethyl sulfoxide have been conducted [26–29]. SC-CO_2 enters a supercritical state above the critical point (Tc: 31.1°C, Pc: 7.3 MPa), where it exhibits liquid-like density and gas-like viscosity [30]. SC-CO_2 is the most used supercritical fluid, because it is non-flammable, harmless, and inexpensive compared with other chemicals. SC-CO_2 has been mainly utilized in the extraction of lipids, carotenoids and other non-polar substances, because its properties are similar to those of hexane [31–33]. The effects of temperature and pressure on the extraction of lipids from *A. platensis* using SC-CO_2 were investigated by Andrich et al. [27]. However, in the SC-CO_2 extraction, drying and cell disruption steps are needed for the pre-treatment of the test sample [34]. Moreover, SC-CO_2 extraction requires high pressure and temperature for operation.

In this study, we examined the extraction of lipids and functional substances from *A. platensis* using the liquefied DME method. Extraction amounts by liquefied DME were compared with the results by SC-CO_2 and hexane Soxhlet extraction methods where a conventional drying and cell disruption are necessary.

3.3.2 Materials and methods

3.3.2.1 Materials

A. platensis were provided by DIC Corporation (Tokyo, Japan). The moisture content of *A. platensis* was 80.1 wt% that was determined by continual drying at 107°C until constant weight. Three methods of lipid extraction were performed: liquefied DME extraction method, SC-CO_2 extraction method, and hexane extraction method. In

liquefied DME extraction, wet *A. platensis* was used without drying and cell disruption. For SC-CO_2 and hexane Soxhlet extractions, *A. platensis* was dried at 107°C and disrupted by a cutter mill (MF-10 basic coupled with a cutter: MF-10.1, holes diameter of the filter mesh: 0.25 mm, IKA Inc., Osaka, Japan) for 10 min at 6,500 rpm.
Phycocyanin (C-Phycocyanin from Spirulina sp.; purity 40.0%, Sigma-Aldrich Co., LLC, Japan), β-carotene (purity >90.0%, Wako Pure Chemical Industries, Ltd., Osaka, Japan), zeaxathin (purity >98.0%, Funakoshi Co., Tokyo, Japan), and chlorophyll-a (DHI LAB Products., Horsholm, Denmark) were used as standards to determine the extraction amounts of these functional substances. The HPLC grade methanol, HPLC grade tetrahydrofuran (THF) and n-hexane used for analysis were purchased from Wako Pure Chemical Industries, Ltd., Japan. For GPC analysis, a series of polystyrene calibration standards (Tosoh Co., Tokyo, Japan) were used. CO_2 (99.9%) was obtained from Sogo Co., Aichi, Japan.

3.3.2.2 Lipids extraction

The apparatus shown in Figure 2.1.1 was used for the DME extraction. A storage tank of liquefied DME (volume: 100 cm^3; TVS-1-100, Taiatsu Techno Corp., Saitama, Japan), an extraction column (diameter: 11.6 mm, length: 190 mm; HPG-10-5, Taiatsu Techno Corp., Saitama, Japan), and a recovery tank for extract (HPG-96-3, Taiatsu Techno Corp., Saitama, Japan), were connected in series. 2.88 g (dry weight, 0.57 g) of wet *A. platensis* was loaded into the lower half of the extraction column. Glass beads (diameter between 1.5 and 2.5 mm; BZ-2, ASONE Co., Inc., Osaka, Japan) were loaded into the upper half of the extraction column. A filter paper (Durapore membrane filters 0.65μm DV, Merck Millipore, Darmstadt, Germany) was placed at the outlet of the extraction column. The flow rate of liquefied DME was 10 mL/min, and the temperature and pressure were 20°C and 0.51 MPa. In this way, lipids, water, and functional substances were extracted from wet *A. platensis*. After the liquefied DME was passed through the extraction column at different time intervals, used DME was evaporated by opening the reducing valve of the recovery tank containing the mixture of liquefied DME, water, and extracted materials. Extracts including water in the recovery tank were dried at 40°C until constant weight to determine extracted water weight.
As one of previous extraction methods, SC-CO_2 extractions were carried out by the experimental apparatus shown in Figure 3.1.2. The apparatus consists of a high-pressure pump for CO_2 (PU-2080 100 MPa, Jasco Co., Ltd., Tokyo, Japan), a CO_2 chiller (Cooling Unit CLU-33, Iwaki Asahi Techno Glass Co., Ltd., Shizuoka, Japan), a 50 cm^3 extraction

column (Thar Tech, Inc., Pittsburgh, USA), a heating chamber (EYELA/NDO-401, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to control the temperature of extraction column, a back-pressure regulator (BP-2080, Jasco Co., Ltd., Tokyo, Japan), collection vials, and a gas meter (Sinagawa Co., Inagi, Japan). In each experiment, 7.5 g of dried and disrupted *A. platensis* was loaded into the extraction column, and the remaining space was filled with cotton at the bottom and top of the extraction column. The extract was collected in vials every 60, 120, 200, and 280 minutes. CO_2 pressure was 40 MPa, which was the permissible highest pressure of the apparatus. Temperature dependence on extraction yield of lipids, functional substances were examined at 70, 90, and 110°C. The CO_2 flow rate was 7.3 mL/min.

For hexane Soxhlet extraction, the dried and disrupted *A. platensis* was employed. The time and temperature for hexane Soxhlet extraction were 15 h and 70°C, respectively.

Figure 3.3.1 Schematic diagram of extraction apparatus. (a) DME extraction; (1) Water bath, (2) Liquefied DME storage tank, (3) Extraction column, (4) Test samples, (5) Glass beads, (6) Filter paper, (7) DME flow control valve, (8) Recovery tank, (9) Pressure reducing valve. (b) SC-CO_2 extraction.; (1) CO_2 cylinder, (2) Chiller, (3) CO_2 pump, (4) Heating chamber, (5) Cotton, (6) Test samples, (7) Extraction vessel, (8) Back-pressure regulator, (9) Collection vial, (10) Water bath, (11) CO_2 gas meter.

3.3.2.3 Property analysis

Elemental analysis was performed on the dried *A. platensis*, the extracted lipids, and the residues. The original *A. platensis* microalgae and the residue were pretreated by continual drying at 107°C until constant weights were obtained. The carbon, hydrogen, and nitrogen contents were determined by a CHN corder (MT-6 Elemental analyzer, Yanaco New Science Inc., Kyoto, Japan) based on flash combustion, which converts all organic substances into combustion gases. The oxygen content was calculated from the difference.

Proximate analysis and higher heating value (HHV) measurements were performed for the extracted lipids and the residues according to the Japanese Industrial Standard (JIS) M 8812:2004 7, and have been described in a previous study [35].

Molecular weight distribution (MWD) of the lipids was determined by gel permeation chromatography (GPC) performed at 40°C by diluting the lipids in tetrahydrofuran, which is the solvent typically used for GPC measurements. The analytical equipment and conditions used for GPC consisted of an HPLC system controller (LC-NetII/ADC, Jasco

Co., Ltd., Tokyo, Japan) and a UV/Vis detector (UV-2075 Plus, Jasco Co., Ltd., Tokyo, Japan) with chromatographic columns (Shodex KF-805L, Showa Denko K.K., Tokyo, Japan). The detection wavelength was set at 250 nm. The MWDs were compared with that of a polystyrene standard. Lipid concentration was 0.1 mg/mL for each measurement, and the injection amount was 100 μL. The MWDs of the lipids by liquefied DME were compared with those obtained by SC-CO_2 and hexane.

The fatty acid components in the lipids were identified by gas chromatography-mass spectrometry (GC/MS). The GC/MS analyses were performed using an Agilent 7890A GC system connected to an Agilent 5975C mass spectrometer, on a phenyl arylene capillary column (DB-5MS; 30 m × 0.25 mm (i.d.) Agilent Technologies Tokyo Ltd., Japan). The GC conditions were as follows: the oven temperature was initially set at 100°C for 5 min, and then allowed to ramp up to 320°C with a rate of 4°C min^{-1}. The injector and detector temperatures were set at 250°C. The split ratio was 10:1, with a total helium carrier gas flow rate of 24 mL/min. The injection volume was 1.0 μL. Qualitative analyses of the detected fatty acids methyl esters (FAME) were carried out by comparing their retention time and mass spectra with the standards (Supelco 37 Component. FAME Mix. Sigma–Aldrich St. Louis, MO, USA). Methyl esterification of extracted lipids was carried out using a fatty acid methylation kit and a fatty acid methyl ester purification kit (Nakalai Tesque, Inc., Kyoto, Japan). The ratios of fatty acid components contained in lipids were estimated by comparing their peak area with the standard peak area [35].

The initial amount of phycocyanin in the original *A. platensis* was determined based on the extract obtained using a method disclosed in a patent [36]. The detail of the phycocyanin extraction method was as mentioned below. 500g of dried and cell disrupted *A. platensis* was mixed in 10L of 1 wt% $CaCl_2$ solution by stirring, which was then left at rest for 15 hours at 20°C to extract phycocyanin. 250 g of KH_2PO_4 was added to the extract, which was then stirred for 30 minutes. It was then left at rest for 2.5 hours at 20°C to generate $Ca_3(PO_4)_2$, in which phycocyanin was adsorbed. Extraction residue and phycocyanin adsorbed in $Ca_3(PO_4)_2$ was removed by 10000G of centrifuge for 15 minutes. Low-molecular-weight component in the phycocyanin-containing supernatant liquid was removed by ultrafiltration. Molecular weight cut off was 10000. Afterwards, it was dried by freeze dehydration to obtain phycocyanin solid. The amount of phycocyanin was analyzed by a HPLC gradient system (CBM-20, Shimadzu Corp., Kyoto, Japan) equipped with a diode array detector (SDP-M10A, Shimadzu Corp., Kyoto, Japan). 20 μL of extract, dissolved in water, were injected using the autosampler (SIL-10AF, Shimadzu Corp., Kyoto, Japan) and separated on a C-18 column (5C18-MS-II column; 150 mm × 4.6 mm (i.d.), Nacalai Tesque, Inc., Kyoto, Japan) at 40°C. The column was pre-equilibrated with

a 20% (v/v) aqueous acetonitrile (ACN) solution containing 0.1% (v/v) trifluoroacetic acid (TFA). The samples were eluted using a linear gradient from 20 to 100% (v/v) ACN for 1 hour, followed by a 10 min isocratic elution with an eluent containing 100% ACN at a flow rate of 1.0 mL/min, using an intelligent HPLC pump (LC20-AD, Shimadzu Corp., Kyoto, Japan). The detection wavelength was 620 nm. The peak identification of phycocyanin was based on the comparison with the retention times and scanned spectra of standards. The weights of the phycocyanin extracts were calculated by comparing their peak areas with the standards. [37]

The analysis of β-carotene in the extracts was carried out using an HPLC equipped with an intelligent controller (LC-Net II/ADC, Jasco Co., Tokyo, Japan), and a UV/visible detector (UV-970, Jasco Co., Tokyo, Japan). 10 μL of the extract, dissolved in chloroform, were injected and separated on a C-18 column (Pursuit XRs 3 C18; 150 mm × 4.6 mm (i.d.), Agilent Technologies Tokyo Ltd., Japan) at 40°C. The isocratic elution was conducted with THF:methanol (1:9, v/v) as the mobile phase at a flow rate of 1.0 mL/min using an intelligent HPLC pump (PU-980, Jasco Co., Tokyo, Japan) for 25 minutes [38]. The detection wavelength was 477 nm. The identification of β-carotene peaks was based on the comparison between actual retention times and the scanned spectra of standards. The amount of β-carotene in the extract was calculated by comparing peak areas with the peak areas of standards.

The analysis of zeaxanthin in the extracts was carried out using an HPLC equipped with an intelligent controller (LC-Net II/ADC, Jasco Co., Tokyo, Japan), and a UV/visible detector (UV-970, Jasco Co., Tokyo, Japan). 10 μL of extract, dissolved in ethanol, were injected and separated on a C-18 column (Pursuit XRs 3 C18; 150 mm × 4.6 mm (i.d.), Agilent Technologies Tokyo Ltd., Japan) at 40°C. The isocratic elution was conducted with THF:methanol (1:9, v/v) as the mobile phase, at a flow rate of 1.0 mL/min using an intelligent HPLC pump (PU-980, Jasco Co., Tokyo, Japan) for 25 min [39]. The detection wavelength was 477 nm. The identification of zeaxanthin peaks was based on the comparison between actual retention times and the scanned spectra of standards. The amount of zeaxanthin in the extract was calculated by comparing peak areas with the peak areas of standards.

The analysis of chlorophyll-a in the extracts was carried out using an HPLC equipped with an intelligent controller (LC-Net II/ADC, Jasco Co., Tokyo, Japan), and a UV/visible detector (UV-2075, Jasco Co., Tokyo, Japan). 20μL of extract, dissolved in acetone:chloroform (2:1) were injected and separated on a C-18 column (5C18-MS-II column; 150 mm × 4.6 mm (i.d.), Nacalai Tesque, Inc., Kyoto, Japan) at 30°C. The isocratic elution was conducted using THF:methanol (1:9, v/v) as the mobile phase, at a

flow rate of 1.5 mL/min, using an intelligent HPLC pump (PU-2080 Plus, Jasco Co., Tokyo, Japan) for 30 minutes. The detection wavelength was 428 nm. The identification of chlorophyll-a peaks was based on the comparison between actual retention times and the scanned spectra of standards. The amount of chlorophyll-a in the extract was calculated by comparing peak areas with the peak areas of standards. [39]

3.3.3 Results and discussion

3.3.3.1 Extraction yields of lipids and water

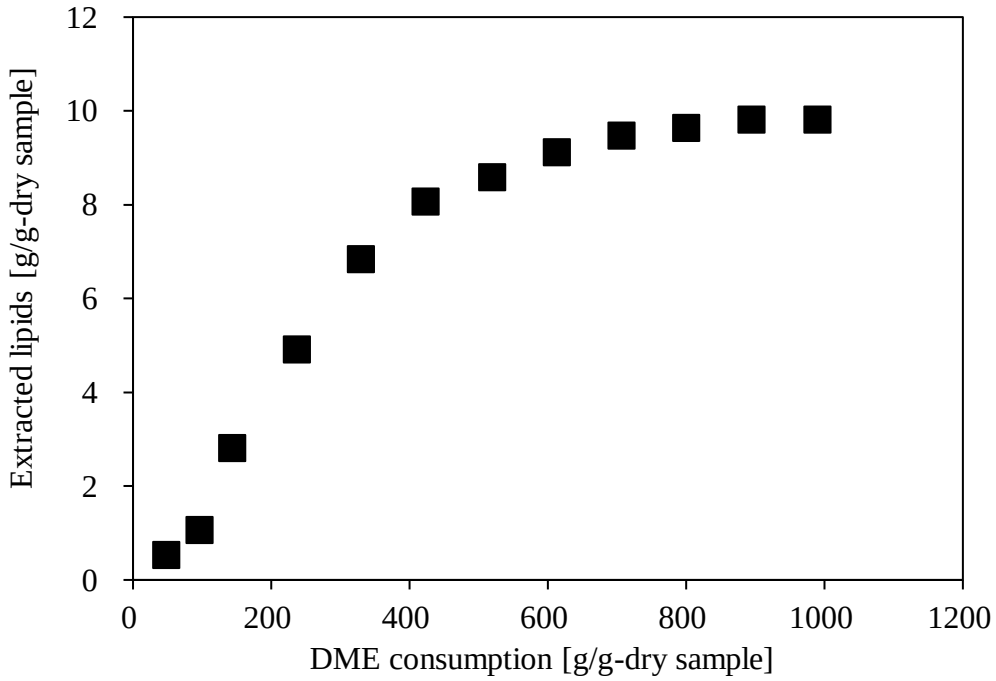

Figure 3.3.2 Extraction of lipids from wet *A. platensis* by liquefied DME

The yield of lipids extracted from wet *A. platensis* using liquefied DME is presented in Figure 3.3.2. In this figure, the liquefied DME consumption (abscissa) is expressed as the ratio between the consumption of liquefied DME and the dry *A. platensis* as tested sample. The final yield for lipids extracted using the liquefied DME method was 9.8 g/g-dry sample. On the other hand, in hexane extraction the final yield for lipids was 7.2 g/g-dry sample. Furthermore, in the liquefied DME method, more than 80 wt% of the water present was simultaneously removed from the wet samples. The residue was completely dewatered as shown in Figure 3.3.3. The temperature effect on the extraction yields of

lipids using the SC-CO_2 extraction is shown in Figure 3.3.4. The extraction yields slightly increased with increasing temperature; however, the highest extraction yield was 2.2 g/g-dry sample, obtained at 90°C at 40 MPa. As we will show hereinafter, the main component of the lipid fraction in *A. platensis* is C16:0, and its melting point is 63°C [40]. Therefore, the yield of lipids at 90°C is higher than the yield obtained at 70°C because at the higher temperature the compounds melted and the viscosity of the lipid fraction was lower [41, 42]. However, the yield of lipids decreased again at 110°C. This decrease is caused by the lowering in SC-CO_2 density at 110°C [43]. As a result, the extraction rate of the liquefied DME extraction method compared with hexane extraction is 136.1%. This proves that an accurate extraction ratio, superior to that of hexane extraction, can be achieved using liquefied DME extraction.

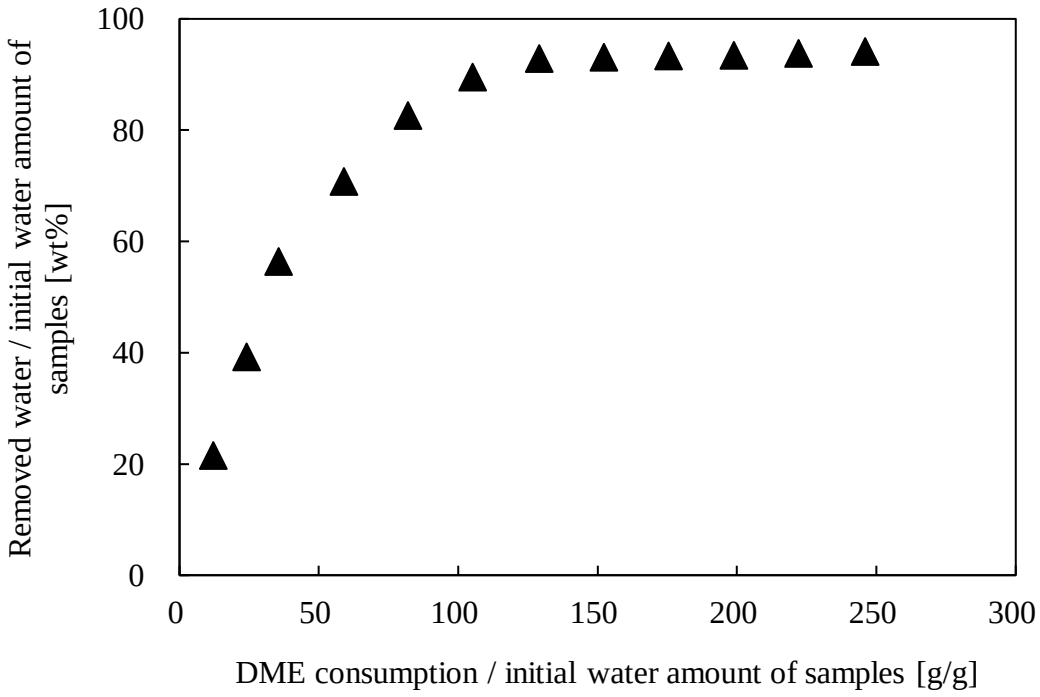

Figure 3.3.3 Extraction of water from wet *A. platensis* by liquefied DM

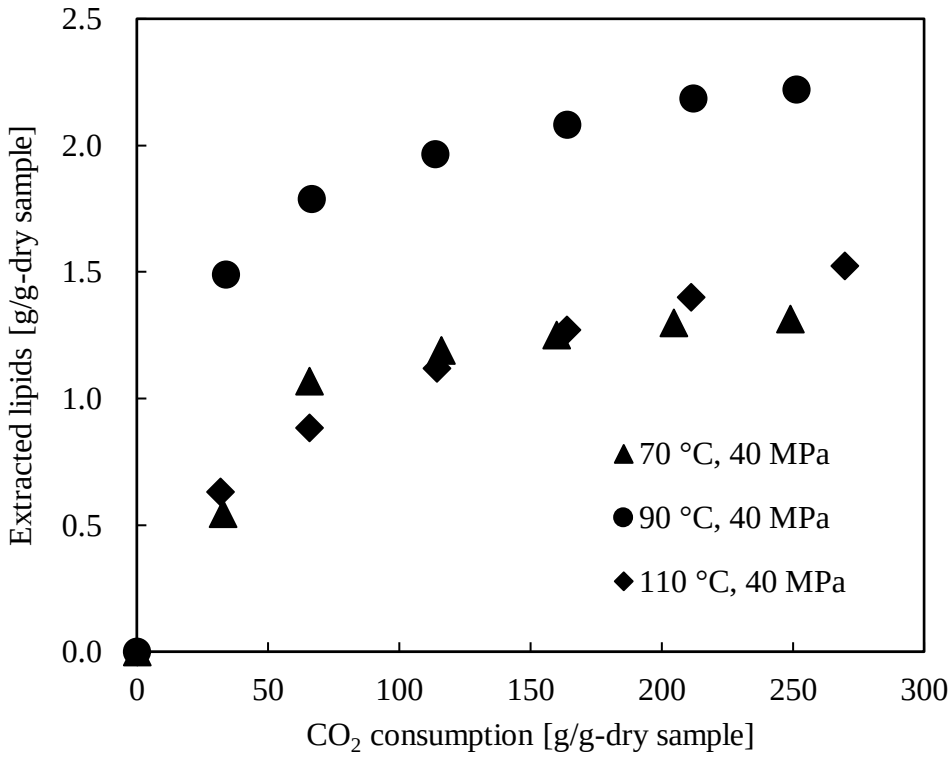

Figure 3.3.4 Extraction of lipids from *A. platensis* by SC-CO_2

3.3.3.2 Elemental, proximate analysis and higher heating value measurement

The results of the proximate analysis, elemental analysis, and calorific value measurements are shown in Table 3.3.1 and 3.3.2. The composition of the lipids fraction extracted from *A. platensis* using the liquefied DME extraction method consisted mainly of C, H, N, and O, in concentrations of approximately 71.6%, 10.7%, 1.5%, and 16.2%, respectively. On the other hands, in the SC-CO_2 extraction method, the contents of which were 74.8%, 10.9%, 0.6%, and 13.7%, respectively. Furthermore, in the hexane extraction method, the contents of which were 63.2%, 9.7%, 0.7%, and 26.4%, respectively. The differences between the lipids and residues obtained using liquefied DME, SC-CO_2 and hexane extraction methods were smaller than the measurement error margin. In particular, the results from liquefied DME and SC-CO_2 extraction were similar to those obtained using the Bligh and Dyer method, considered as the conventional method [44]. In addition, negligible differences were noted between the liquefied DME, SC-CO_2 and hexane extraction in proximate analysis and HHV measurement. The lipids obtained from *A.*

platensis were of high quality compared to those obtained from the wild microalgae studied previously [16]. The HHV of lipids using the liquefied DME method was 35.0 MJ/kg, which is equivalent to that of first-generation biodiesel and is essentially the same as the traditional fossil oil [45]. Moreover, although the HHV of the residue obtained after liquefied DME extraction was 21.3 kJ/kg, proving that it still retained sufficient caloric density to make it a potential carbon-neutral fuel.

Table 3.3.1 Main elemental compositions of extracted lipids and the residues

Analysis [dry ash free %]	Liquefied DME		SC-CO_2		Hexane		Original
	Lipids	Residue	Lipids	Residue	Lipids	Residue	Feed
C	71.6	44.7	74.8	48.4	63.2	47.4	46.5
H	10.7	6.8	10.9	7.1	9.7	7.1	7.1
N	1.5	11.3	0.6	11.3	0.7	0.5	10.4
O*	16.2	37.3	13.7	33.2	26.4	45.0	36.0

*By difference

Table 3.3.2 Proximate analysis of extracted lipids and residues using the liquefied dimethyl ether method

Analysis [wt% dry basis]	Liquefied DME		SC-CO_2		Hexane		Original
Proximate analysis	Lipids	Residue	Lipids	Residue	Lipids	Residue	Feed
Ash yields	-	10.4	-	6.1	-	6.0	6.0
Volatile matter	-	81.9	-	82.2	-	82.6	82.1
Fixed carbon	-	7.7	-	11.7	-	11.4	11.9
HHV (MJ kg^{-1})	35.0	21.3	39.6	22.7	35.7	22.5	22.9

3.3.3.3 Molecular weight distribution

The MWDs of the lipids compared to polystyrene standards are shown in Figure 3.3.5. The MWD curves of the extracts obtained after liquefied DME, SC-CO_2 and hexane extraction were quite similar in GPC analyses. In the extraction using liquefied DME, the weight average molecular weight (Mw) and the number average molecular weight (Mn) were 788 and 618 g/mol^{-1}, respectively. In the extraction using SC-CO_2 and hexane, the Mw and Mn were 777 and 547, 820 and 604 g/mol^{-1}, respectively. The Mw and Mn obtained using the liquefied DME method were smaller than those obtained using SC-CO_2 and hexane, but the ratios were similar.

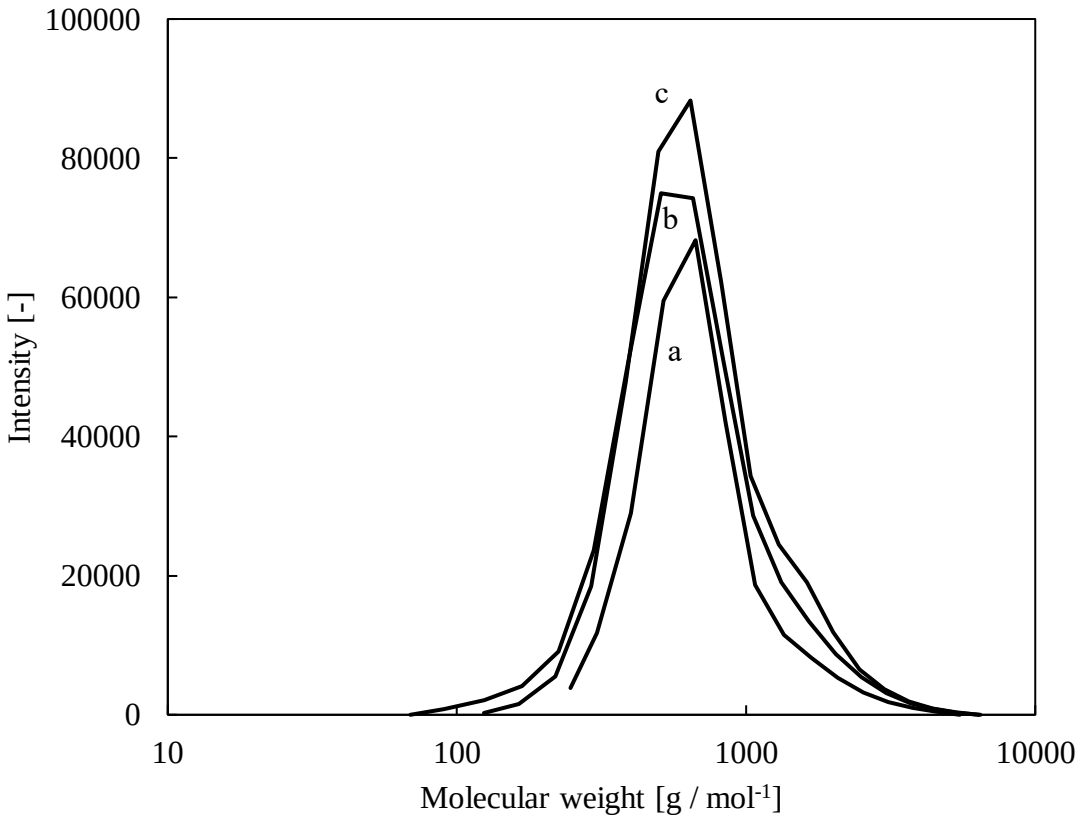

Figure 3.3.5 Molecular weight distributions of the extracted lipids: (a) DME, (b) SC-CO_2, (c) Hexane

3.3.3.4 Fatty acid profile

The fatty acid composition of lipids from *A. platensis* is presented in Table 3.3.3. In the liquefied DME extraction, the percentage of saturated fatty acids was 57.7 %, with palmitic acid (C16:0) as main component of the lipid fraction in *A. platensis* (40). Oleic acid (C18:1), linoleic acid (C18:2) and γ-linolenic acid (C18:3) were extracted in amounts of 0.8 %, 22.8 % and 13.6 %, respectively. However, the other fatty acids such as lauric acid (C12:0), myristic acid (C14:0), pentadecylic acid (C15:0) were not extracted. The ratio of fatty acids extracted using the SC-CO_2 method, was similar to that obtained using the hexane extraction. These results suggest that the lipid fraction obtained using our method has similar properties to the fractions obtained using the more conventional methods, like SC-CO_2 and hexane extraction.

Table 3.3.3 Fatty acid components in the lipids extracted from *A. platensis*

Fatty acids	DME	SC-CO_2	Hexane
C12:0	n.d.*	n.d.	0.1
C14:0	n.d.	0.1	0.1
C15:0	n.d.	0.1	0.2
C16:1(TRANS-9)	n.d.	1.5	1.0
C16:1(CIS-9)	1.9	3.8	3.0
C16:0	**57.7**	**31.1**	**44.4**
C17:2 (ALL CIS-9.12)	n.d.	0.2	0.2
C17:1 (CIS-10)	n.d.	0.1	0.2
C17:0	n.d.	0.1	0.2
C18:3 (ALL CIS-6, 9, 12)	**13.6**	**34.3**	**20.8**
C18:2(ALL CIS-9, 12)	**22.8**	**24.2**	**22.7**
C18:1(TRANS-9)	3.2	1.8	2.7
C18:1(CIS-9)	0.8	0.7	1.1
C18:0	n.d.	1.2	2.8
C20:4(ALL SIS-5, 8, 11, 14)	n.d.	0.2	0.1
C20:5(ALL SIS-5, 8, 11, 14, 17)	n.d.	0.2	0.1
C20:3(ALL TRANS-7,10,13)	n.d.	0.2	0.1
C20:2(ALL CIS-11,14)	n.d.	0.1	0.1
Saturated FA (area %)	57.7	32.5	47.7
Unsaturated FA (area %)	39.1	65.6	49.5

*n.d.: not detected.

3.3.3.5 Extraction of phytochemicals

The yields of functional substances extracted from *A. platensis* using each method are shown in Table 3.3.4. The yields are expressed as milligrams of pigment per gram of dry weight of *A. platensis* as test sample. Using the liquefied DME method, the amounts of carotenoid pigments β-carotene and zeaxanthin extracted were 0.15 and 0.10 mg/g-dry sample, respectively. On the other hand, the yields of β-carotene and zeaxanthin obtained using SC-CO_2 and the hexane extractions were 2.12, 0.10 and 1.66, 0.34 mg/g-dry sample, respectively. As carotenoid pigments are substances soluble in non-polar solvents, SC-CO_2 and hexane are better at solvating them. Furthermore, the chlorophyll yield obtained using liquefied DME, SC-CO_2 and hexane extraction were 0.53, 0.26 and 0.72 mg/g-dry sample, respectively. The quantitative analysis of extracted phycocyanin could not be performed, because the peak was not visible after HPLC. Therefore, extraction of the phycocyanin from residue by the liquefied DME was performed by the DIC method. These results are shown in Figure 3.3.6. The extract was light blue, showing that phycocyanin existed in residue of *A. platensis* obtained by the liquefied DME extraction. The differences in the yields of phytochemicals are caused by the differences in polarity and solvation power between liquefied DME, SC-CO_2 and hexane.

Table 3.3.4 Yields of functional substances in extracts

Extraction method	Functional substances [Yield (mg/g-dry sample)]		
	β-carotene	Zeaxanthin	Chlorophyll a
Liquefied DME	0.15	0.10	0.53
SC-CO_2	2.12	0.10	0.26
Hexane	1.66	0.34	0.72

Figure 3.3.6 Phycocyanin extracted by DIC method from residue obtained by liquefied DME extraction.

3.3.3.6 Structural changes after liquefied DME extraction

To observe what effect had been done to the *A. platensis* cells after liquefied DME extraction, the cells were examined by using an optical microscope. Figure 3.3.7 is a photomicrograph of the untreated cells, which can be compared with the cells were obtained after liquefied DME extraction were shown in Figure 3.3.8. After DME extraction, the almost parts of the initial cell form were maintained, however a gap has generated between the cell. In addition, the bleaching of the cells was confirmed. *A. platensis* as gram-negative bacterium has a cell wall and cell membrane, which consists of the complex lipid, protein, lipopolysaccharide, peptidoglycan and phospholipids, respectively. In general, their lipids are dissolve well in liquefied DME. On the other hands, a protein, lipopolysaccharide and peptidoglycan with a high molecular weight are not dissolve in liquefied DME. Therefore, this observation suggests that the lipid parts of the cell wall and membrane were dissolved in liquefied DME, and then, the lipids, water, and functional substances of were extracted through the gap from the cell without destroying an initial cell form of *A. platenis*.

Figure 3.3.7 Untreated cell of *A. platensis*.

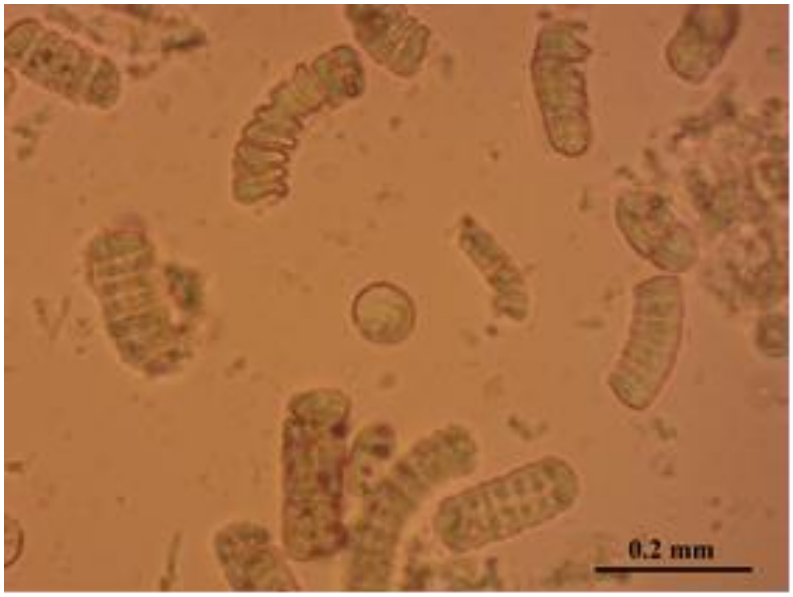

Figure 3.3.8 *A. platensis* residue by liquefied DME extraction

3.3.4 Conclusions

This study has clearly demonstrated that the liquefied DME method can be used to directly extract lipids from the *A. platensis* water slurry. Comparison of the extraction yields, elemental analysis, the MWD curves, and the GC/MS spectra verifying the liquefied DME extraction method, indicate that this method performs as well as the hexane extraction method, despite the fact that some steps have been omitted, such as drying, cell disruption, and heating at high temperature. Moreover, the lipid fraction obtained from *A. platensis* was composed of long-chain fatty acids, implying that *A. platensis* could be suitable as a fine fuel source that could be easily esterified into a biodiesel fuel. Liquefied DME extracted also the phytochemicals present in the algae, which represents an effective method to reduce the price of algal fuel.

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Chapter 4

Antisolvent crystallization of glycine by liquefied DME

4.1 Introduction

Crystallization is one of the most important processes in industrial processes. It is widely used in pharmaceutical, food and fine chemicals industries for separation and/or purification of the production from the solution. In crystallization process, the physical properties of the final product such as its chemical purity, crystal size distribution, and morphology is changed by several factors such as temperature decreasing and ratio of antisolvent per solution. In pharmaceutical industries, these properties effect on the bioavailability of materials [1, 2]. This process can be conducted at the ambient temperature and it was quite convenient for heat-sensitive compounds. Glycine is one of typical products obtained by crystallization. Because glycine is non-toxic and highly soluble in water, generally, it is used as a bulking agent for freeze dried protein formulation. Glycine has been exhibited to inhibit aggregation of certain proteins upon freezing [3, 4]. By using spray-drying, the dry powder of glycine has also been investigated for pulmonary delivery [5]. In generally, energy consumption by antisolvent crystallization is smaller than that due to spray-drying. In a case of spray-drying of glycine,

latent heat of water per unit weight is increased in connection with decreasing of water content of glycine solution. However, even antisolvent crystallization consumes much energy for separation of alcohols antisolvents from water after the crystallization, because of high boiling point of alcohol and azeotropic phenomenon of ethanol as shown in Figure 4.1 (a). Therefore, new antisolvents have been required to decrease the energy consumption. Herein, we propose liquid dimethyl ether (DME) as an antisolvent for glycine crystallization to clear the problem of the alcohols.

The normal boiling point of DME is -24.8°C; therefore it is gaseous at the standard condition. The saturated vapor pressure of DME is 0.5 MPa at 20°C [6]. Liquefied DME is partially miscible with water. The saturated water concentration in liquefied DME is around 7.3 wt% at 20°C [7–9].

Based on the characteristics of DME mentioned above, several researchers conducted dewatering and extraction of several organic components from lignite, soil, plant, and biomass by using DME as a solvent medium [10–24]. By this technique, organic components and water in wet materials were extracted simultaneously. In the case of lipid extraction from wet cyanobacteria, the lipid extracted by liquefied DME consisted mainly of C, H, and O. Conversely, the N content was lower than the starting material [21]. This implied that solubility of amino acids and/or proteins into liquefied DME are lower than those of the lipid.

Generally, DME has been used as a synthesized fuel cheaper alternative to imported LPG in China [25–27]; therefore it has been not so examined as an antisolvent for crystallization of water-soluble materials. Catchpole et al. [16] utilized the DME as an effective antisolvent for proteins in an aqueous solution and for water-soluble coating compounds. Lipids were removed from protein/lipid mixtures solution by liquefied DME, and this technique was applied on fresh and reconstituted egg yolks and on selected dairy by-product streams [19]. After the extractions, DME could be easily removed from extracted organic materials, water and residues at room temperature. These results imply that amino acid is not solved in liquefied DME, and then DME can be used as the antisolvent. From a view point of energy-saving, DME is effective antisolvent because DME can be easily removed from water due to its low boiling point. The procedure of crystallization by liquefied DME and its effect are shown in Figure 4.1 (b).

In this study, efficacy of crystallization process using liquefied DME was evaluated for glycine solution to verify its versatility. The morphologies, sizes, and crystal structures of the glycine crystals obtained under the various conditions were examined.

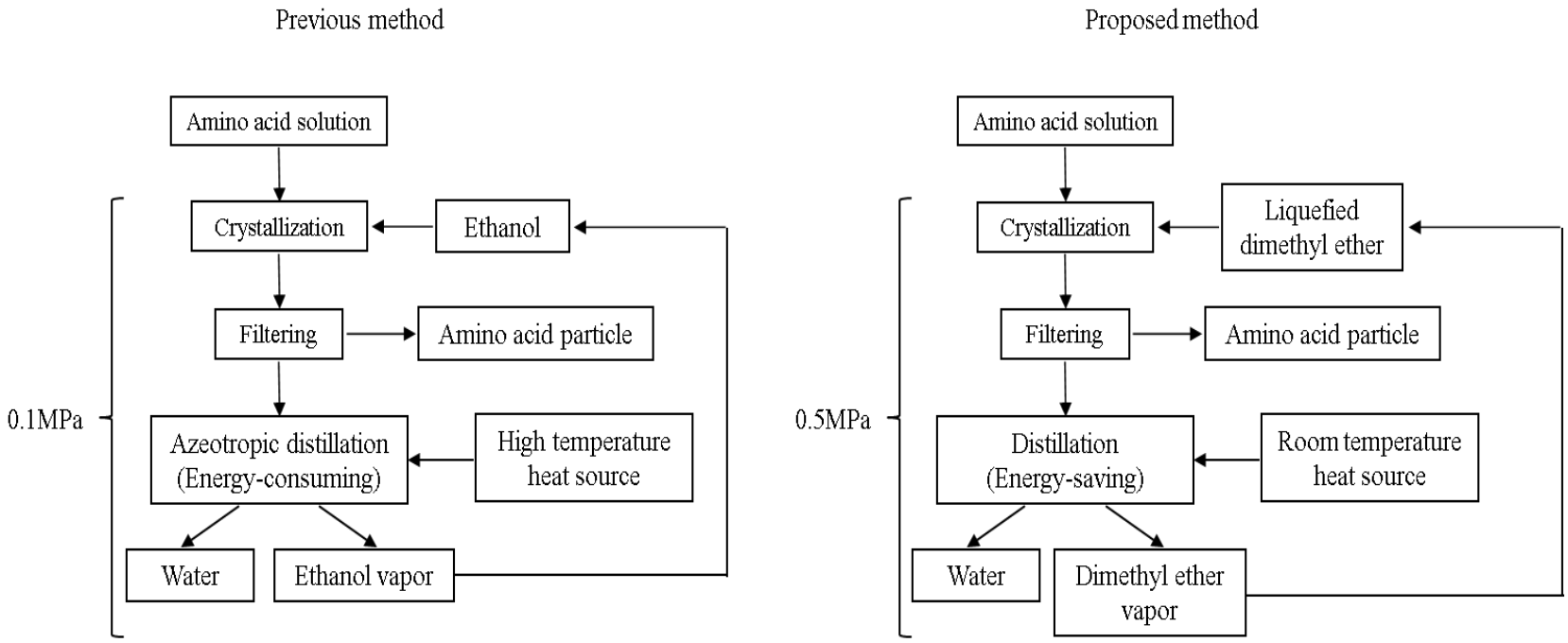

Figure 4.1 Processes of antisolvent crystallization. (a) Previous antisolvent process; (b) The proposed process.

4.2 Materials and methods

4.2.1 Materials

Glycine (99.0%, Wako Pure Chemicals Industries Ltd, Osaka, Japan) was used as the solute. The crystal structure we determined by X-ray diffraction (XRD), and the XRD pattern of γ-glycine was observed. Distilled water obtained from a water distillation apparatus (Auto still WS200, Yamato Scientific Co. LTD., Tokyo, Japan) was used as the solvent. The concentration of glycine solution was 10.3 wt% The concentration was sufficiently lower than the saturated one (20.2 wt.%), then the glycine was completely dissolved. Purified DME (supplied as propellant filled in spray-work air can 420D, Tamiya, Inc., Shizuoka, Japan) was used as the antisolvent.

4.2.2 Experimental setup and procedure

Figure 4.2 showed the schematic diagram of glycine crystallization by liquefied DME apparatus. The main apparatus consisted of a HPLC pump (880-PU; Jasco, Tokyo, Japan) for glycine solution, a crystallization reactor made of pressure resistance glass covered by polycarbonate (upper half of HPG-96-3 and lower half of HPG-96-5 were cut and combined; volume: 96 ml; inner diameter 27 mm; Taiatsu Techno Corp., Saitama, Japan), a DME tank made of SUS 316 (TVS-1-100; volume: 100 ml; Taiatsu Techno Corp., Saitama, Japan) which equipped with needle valve, and a collector made of pressure resistance glass covered by polycarbonate (HPG-96-3; volume: 96 ml; Taiatsu Techno Corp., Saitama, Japan). Flow rate of liquefied DME was controlled by a flow meter (P-100 customized for liquefied DME, Tokyo Keiso Co., Ltd, Tokyo, Japan) and a hand-operated needle valve. These were connected via 1/16 inch SUS 316 tubes as shown in Figure 4.2.

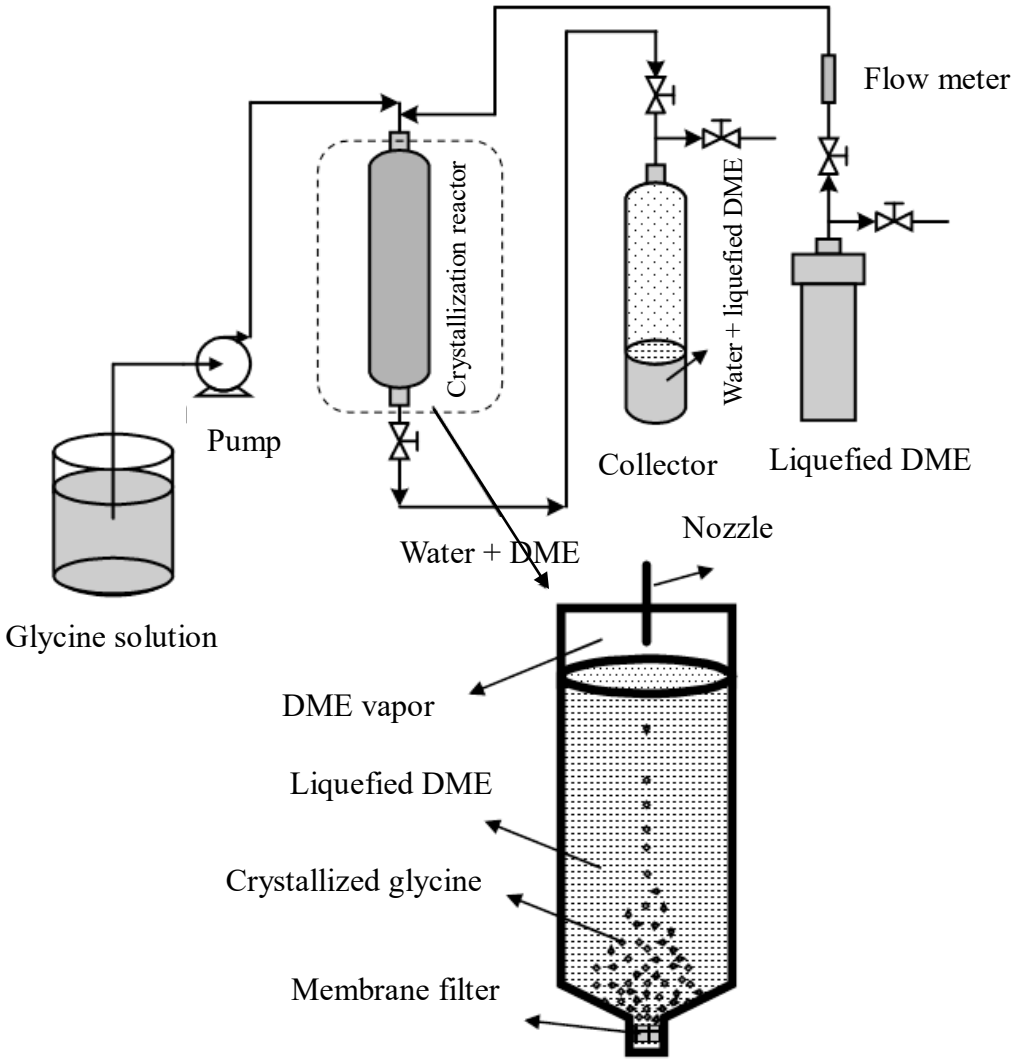

Figure 4.2 Schematic diagram of glycine crystallization.

As an antisolvent, liquefied DME was flowed into the crystallization reactor from the DME tank until 83 ml after removing air from the crystallization reactor. The operating temperature and pressure were kept around 25°C and 0.5 MPa in a water bath.

After the desired conditions were achieved, the glycine solution was injected from a nozzle to surface of liquefied DME in the crystallization reactor for 1minute. The flow rate of the glycine solution was controlled by the HPLC pump with 5 ml/min. The inside diameter of the nozzle was 0.25 mm and beginning distance between the nozzle tip and the surface of liquefied DME was 23 mm. During the injection of glycine solution, surface level of liquefied DME was elevated. After 1 minute of glycine solution injection, the

final distances between the nozzle tip and the surface of liquefied DME were 14 mm. Water concentration in liquefied DME was 8.2wt% after 1 minute of glycine solution injection, because the volume of liquefied DME was 83ml, the density of liquefied DME at 25°C is 0.661 g/cm^3, and glycine concentration in water was 10.3 wt %. The saturated water concentration in liquefied DME at 25°C is around 7.5 wt% (8); therefore, after the injection of glycine solution, liquefied DME were saturated by water of glycine solution and apart of water was phase-separated from liquefied DME.

Glycine solution was diluted by liquefied DME, and then glycine was crystalized. Glycine powders were trapped by a filter (DVPP1300; pore diameter < 0.65 μm; Merck Millipore MA, USA) at bottom of the crystallization reactor. However, water content of the trapped glycine powder may be very high because the liquefied DME in the crystallization reactor was saturated by water. In order to remove water from the trapped glycine powders, liquefied DME was supplied again into the crystallization reactor. The bottom of the crystallization reactor was equipped with a hand-operated pressure relief valve. After the injection of glycine solution, the hand-operated pressure relief valve was opened. Afterwards, the liquefied DME saturated by water was flowed from the crystallization reactor into the collector tank. At the same time, pure liquefied DME was supplied again into the crystallization reactor. The flow rate of liquefied DME was 20 ± 3 ml/min. Redundant liquefied DME in the crystallization reactor was pulled out from the bottom of the crystallization reactor with 20 ± 3 ml/min by the hand-operated pressure relief valve; hence, the volume of liquefied DME in the crystallization reactor was maintained constant. Liquefied DME in the collector tank was decompressed and evaporated at room temperature by opening a valve connected with the collector tank.

After several amounts of supplying liquefied DME, the remaining liquefied DME in the crystallization reactor was removed to the collectors. Glycine powders were obtained from the filter in the bottom of the crystallization reactor and then were analyzed.

4.2.3 Crystal characterization

The water contents of glycine powders were measured by using a moisture meter (Frontlab, AS ONE Corporation, Tokyo, Japan). The morphologies and sizes of the glycine powders were observed by a scanning electron microscope (SEM; JSM-6390LV, JEOL Ltd., Tokyo, Japan). The acceleration voltage was 1.0 kV. X-ray diffraction (XRD) (RINT 2100/PC; 40 kV and 200 mA; Rigaku, Akishima, Japan) was used to confirm the crystals structure of glycine. It was equipped with a θ–θ wide-angle goniometer and scintillation detector, using Cu Kα radiation (λ = 1.5406 Å), with a scan rate of 2 /min,

step size of 0.02, and a 2θ range of 10–50.

4.3 Results and discussion

Glycine was successfully crystallized by injection of glycine solution into liquefied DME. In the glycine solution, water obstructs the growth of glycine crystal nascent due to the strong interactions between OHwater–Oglycine and NHglycine–Owater hydrogen-bonds [28]. The crystallization by liquefied DME implies that interactions of hydrogen-bonds between water and liquefied DME is stronger than those between water and glycine, and liquefied DME broke the interactions between water and glycine molecule. Moreover, there was no smell of ethereal odor of DME in the obtained glycine powders and water in the collector. This shows DME was easily removed from glycine powders without heating of them.

Wet glycine powder was obtained and its water content reached 24.1 ± 3 wt. %, before liquefied DME was flowed to remove water from glycine powder. By increasing DME flowing amount to 126 ml, water contents were rapidly reduced to less than 0.1 wt. %.

Figure 4.3 (a-c) shows typical SEM images of glycine powders by several flowing amount of liquefied DME. As shown in Figure 4.3 (a), needle like glycine powders was generated before liquefied DME flowed to remove water. As shown in Figure 4.3 (b and c), during removing of water by flowing of liquefied DME, the shape of glycine powders was not changed. The needle like shape implied α-glycine crystals were observed. [29–31].

To understand whether the glycine powders were amorphous or crystalline, crystal structures were analyzed by powder XRD. In pretreatment of XRD analysis, the powders were hermetically sealed in desiccator at room temperature around one day to be dried completely. Figure 4.4 (a-c) show the XRD patterns of the glycine particles obtained with several flowing amount of liquefied DME. As shown in Figure 4.4 (a), before flowing of liquefied DME, strong peaks of XRD patterns confirmed good crystallinity of α-glycine. As shown in Figure 4.4 (b and c), during flowing of liquefied DME to remove water, the XRD patterns implied that α-glycine was not transformed to other crystal structure [31–34]. The results imply that rapid and local supersaturation of glycine solution injected into liquefied DME initially affected metastable α-glycine thermodynamically.

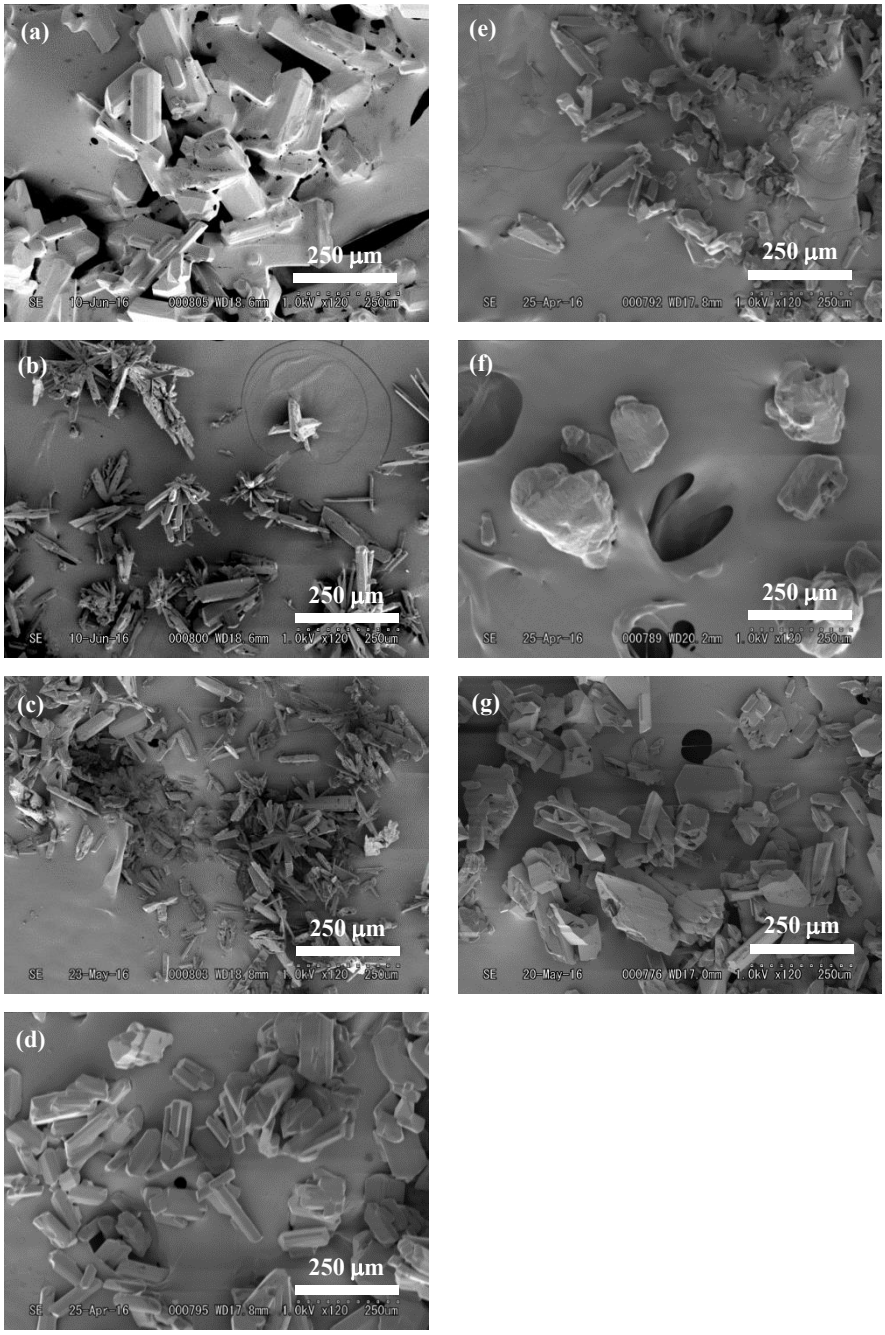

Figure 4.3　SEM images of glycine powders. (a) Before liquefied DME flowed. (b) 126 ml of liquefied DME flowed. (c) 229 ml of liquefied DME flowed. (d) Glycine solution was saturated. Before liquefied DME flowed. (e) Glycine solution was saturated. 106 ml of liquefied DME flowed. (f) Glycine solution was saturated. 222 ml of liquefied DME flowed. (g) Glycine solution was saturated. Liquefied DME was replaced. 215 ml of liquefied DME flowed.

In general, a kind of antisolvent and its concentration affects to polymorphs of crystal [35]. In order to examine influence of concentration of injected glycine solution, crystallization was performed by using saturated glycine solution. The concentration of the injected saturated glycine solution was 20.2 wt.%. Also in a case of saturated glycine solution, wet glycine powder was obtained and its water content reached 31.1 ± 3 wt.%, before liquefied DME was flowed to remove water from glycine powder. By increasing DME flowing amount to 106 ml, water contents were rapidly reduced to less than 0.1 wt. %. Also in a case of the saturated glycine solution, as shown in Figure 4.3 (d), needle like glycine powders was generated before liquefied DME flowed to remove water. The strong peaks of XRD patterns confirmed good crystallinity of α-glycine as shown in Figure 4.4 (d). However, by flowing of liquefied DME, form of glycine powders was changed to cubic like as shown in Figure 4.3 (f). As shown in Figure 4.4 (e and f), the XRD patterns shows that γ-glycine was transformed to γ-glycine, which is the stabilized phase [31–34]. It is a good guess that the transformation was occurred by tiny γ-glycine result from the saturated solution of γ-glycine.

In order to examine the mechanism of the crystal transition, we took particular note of solution-mediated transformation. After the injection of glycine solution, the liquefied DME in the crystallization reactor was nearly saturated by water. Generally, saturated concentration of glycine depends on solvent. We thought that there is a possibility of dependence of water concentration in the liquefied DME on crystal transformation. As an additional examination, after the injection of saturated glycine solution into liquefied DME in the crystallization reactor, all water-saturated liquefied DME was pulled out from the crystallization reactor and the crystallization reactor was refilled with pure liquefied DME. Next, liquefied DME was supplied again into the crystallization reactor with the flow rate of 20 ± 3 ml/min. Redundant liquefied DME was pulled out from the bottom of the crystallization reactor with 20 ± 3 ml/min and the surface position of liquefied DME was maintained constant by adjusting position of the hand-operated pressure relief valve. In this procedure, α-glycine was not transformed to γ-glycine during 215ml of flowing of liquefied DME, as shown in Figure 4.3 (g) and Figure 4.4 (g). The results shows that crystal transformation was not occurred in pure liquefied DME but in water-saturated liquefied DME. In other words, by flowing of liquefied DME, moderate removing of water molecules from α-glycine crystals assisted crystal transformation from α- to stable γ-glycine.

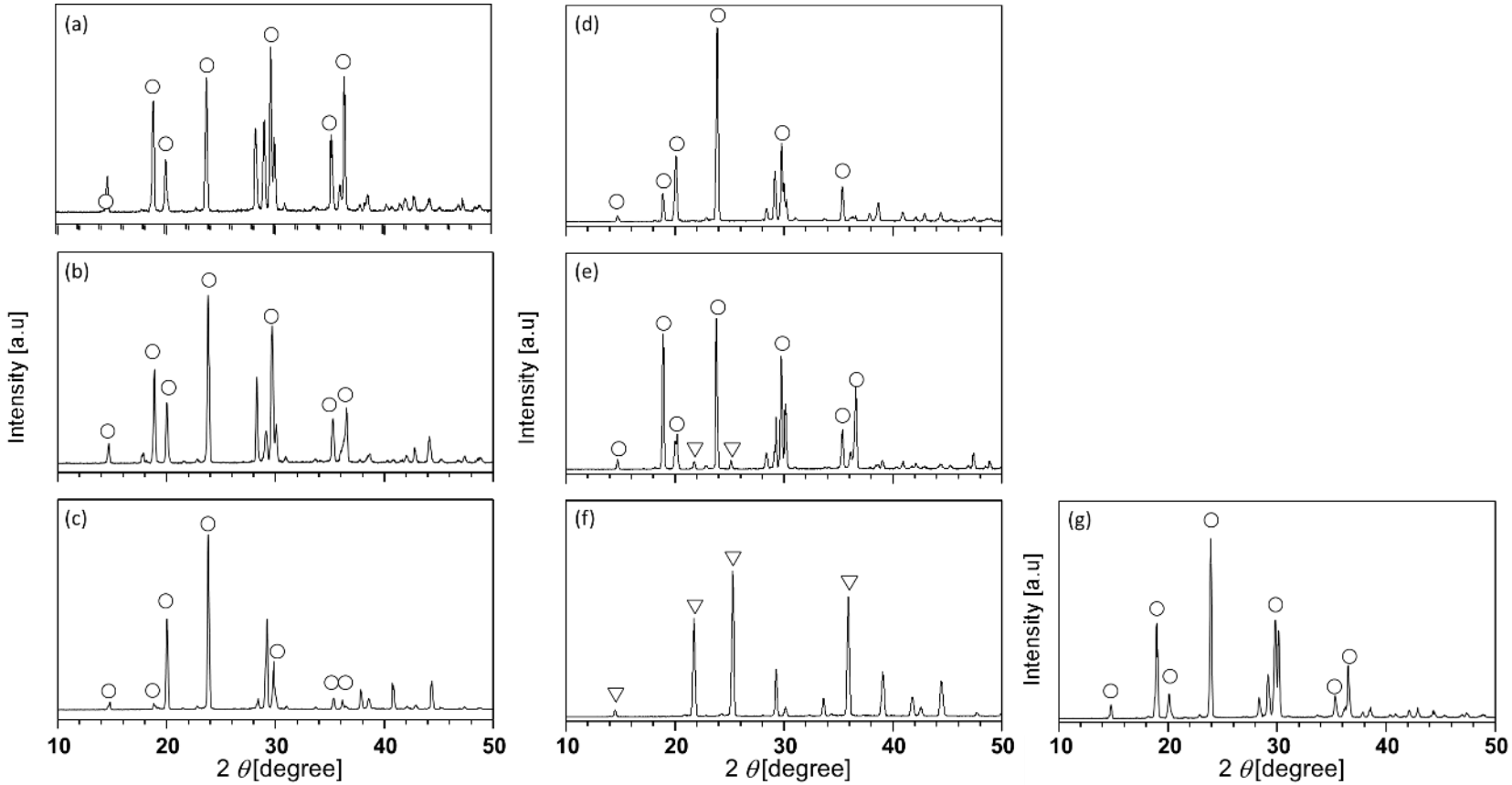

Figure 4.4 XRD patterns of glycine crystals obtained in several conditions. Open circles show peaks of α-glycine, Open triangles show peaks of γ-glycine. (a) Before liquefied DME flowed. (b) 126 ml of liquefied DME flowed. (c) 229 ml of liquefied DME flowed. (d) Glycine solution was saturated. Before liquefied DME flowed. (e) Glycine solution was saturated. 106 ml of liquefied DME flowed. (f) Glycine solution was saturated. 222 ml of liquefied DME flowed. (g) Glycine solution was saturated. Liquefied DME was replaced. 215 ml of liquefied DME flowed.

4.4 Conclusions

We proposed liquefied DME as a new antisolvent for crystallization of glycine from solution. The glycine crystals were successfully obtained by liquefied DME. DME was easily removed from glycine crystals and water without having to be heated. The proposed process using low-boiling liquefied DME has enough potential for energy-saving crystallization. The SEM images and XRD patterns showed that α-glycine was initially obtained by rapid and local supersaturation of the solution in the liquefied DME. By removing of water from glycine crystal, crystal transformation from α- to γ- glycine was observed via solution-mediated transformation only in water-saturated liquefied DME. In the future, the proposed process should be applied to other water-soluble materials and solubility of the materials in liquefied DME with should be examined.

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Chapter 5

Summary and future direction

Liquefied DME method can extract the lipids, water and functional substances from high moisture contents biomass without drying, cell-disruption and solvent removing by heat evaporation. It has been actively researched for development of next-generation biomass energy and environmental application recently. This work focused on the extraction behavior of valuable substances from plants and algal biomass by using liquefied DME.

Utilization of plant biomass as a bioactive compounds resources by using liquefied DME

Application of liquefied DME for oil, water and functional substances extraction from citrus peel, leaves and mangosteen peel as plants biomass were investigated in order to effective utilization of food processing residue. Water, essential oil, citrus flavonoids and xanthone were extracted from wet sample by using liquefied DME. Especially, in the case of citrus essential oil extraction by DME method, the extract amounts were almost equal to those obtained from Ponkan peel by steam distillation method. In addition, the extract amounts were revealed that depend on thickness of peel. The essential oil obtained by DME method was fresh flavor compared with those of SD method, because of the essential oil not to be affected by heat.

Xanthones of mangosteen pericarp were extracted by liquefied DME and the extracted components were evaluated. As a result, it was confirmed that eight kinds of xanthones

such as α-mangostin, 3-Isomangostin, Mangostanol, 8-Desoxygartanin, Gartanin, Garcinone E, 9-Hydroxycalabaxanthone, β-Mangostin were extracted. Therefore, in the extraction of xanthones from the mangosteen, liquefied DME extraction is valid.

Utilization of algal biomass as a biofuels resources by using liquefied DME

The lipid and water were extracted from wet algal biomass without drying and cell-disruption process by using liquefied DME. Lipid extraction amounts by DME method is almost equal to that of the organic solvent extraction method. In addition, the components of fatty acids in lipid is simple, and the purification is easy. Therefore, the lipid is high quality, and its suggested to be possible to use as a biofuel. The ratio of C and H in the extraction residue is high, thus it has a potential of utilization as a fertilizer. Furthermore, the lipid contains a functional substance such as β-carotene and zeaxanthin, it has been suggested that there is a possibility of cost reduction of biofuels. These results showed the superiority for effective utilization of algal biomass by using liquefied DME method. On the other hands, high-molecular compounds such as protein remaining in the extraction residue cannot be extracted by DME method. Moreover, by SEM images, DME is dissolved lipid as structural components of the algal cell, it has been suggested that DME penetrate into the cells through the lipid parts on the cell.

Antisolvent crystallization of glycine by liquefied DME

Application of liquefied DME as anti-solvent for amino acid crystallization was investigated. The glycine crystals were successfully obtained by using liquefied DME. Liquefied DME was easily removed from glycine crystals and water without having to be heated. The proposed process using low-boiling liquefied DME has enough potential for energy-saving crystallization. The SEM images and XRD patterns showed that α-glycine was initially obtained by rapid and local supersaturation of the solution in the liquefied DME. By removing of water from glycine crystal, crystal transformation from α- to γ-glycine was observed via solution-mediated transformation only in water-saturated liquefied DME.

Future direction

Now days, the demand for natural phytochemical compounds is on the increase due to their application in the functional food and pharmaceutical industry. To respond this demand, the development of a suitable extraction technology that is able to produce high quality extracts is required. This problem was solved by applying liquefied DME as an extraction medium. Finally, it could be said that liquefied DME is a very simple, feasible and versatile method for extraction of phytochemical compounds from plants and algal biomass. Moreover, liquefied DME can also be applied for crystallization of amino acid.

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