CHAPTER 6

OPTIMIZING LIPID EXTRACTION USING LOW POWER ULTRASONIC – ASSISTED SOLVENT EXTRACTION

6.1 Introduction

Microalgae can be considered suitable material for biofuel production. However, the productivity of extracted total lipids is still low because lipid extraction from biomass is not effective. Extraction is another process in recovering lipids from microalgal cells before transesterification step. Several methods have been applied for lipid extraction from microalgal biomass. However, some methods require long extraction time and high energy inputs.

In the previous experiment, lipid extraction from *Carteria* sp. AARL G045 has been conducted by traditional solvent extraction according to the method of Bligh and Dyer (1959). However, the main disadvantage of this method is the long extraction time and low extraction yield (Prommuak *et al.*, 2012). Recently, many techniques for lipid extraction have been developed, including the ultrasonic - assisted solvent extraction (UASE). This technique promotes the damage of cell walls through the cavitation from ultrasonic device. Ultrasonic waves will create bubbles in the solvent and these bubbles will collapse and surround the cell walls of microalgae, causing cell disruption and the lipids will be released into the solvent (Cravotto *et al.*, 2008).Therefore, using of ultrasound has been developed to increase the capability of lipid extraction in short time.

However, UASE from many studies were done with high power ultrasound (HPU) which led to higher energy consumption. Lipid extraction from algae in this study was attempted via the UASE with low power ultrasound (LPU). LPU can be performed without high energy requirement and costly HPU equipment (Cravotto *et al*., 2008). Thus, this will support the production of bio oil from microalgae by an economically feasible process.

In this experiment, lipid extraction was conducted by UASE using chloroform and methanol, (2:1v/v). Moreover, the ultrasonic frequencies, ultrasonic power, extraction time and the sample powder on the solvent ratio were optimized for obtaining the best condition for lipid extraction.

6.2 Materials and methods

6.2.1 Culture condition and harvest

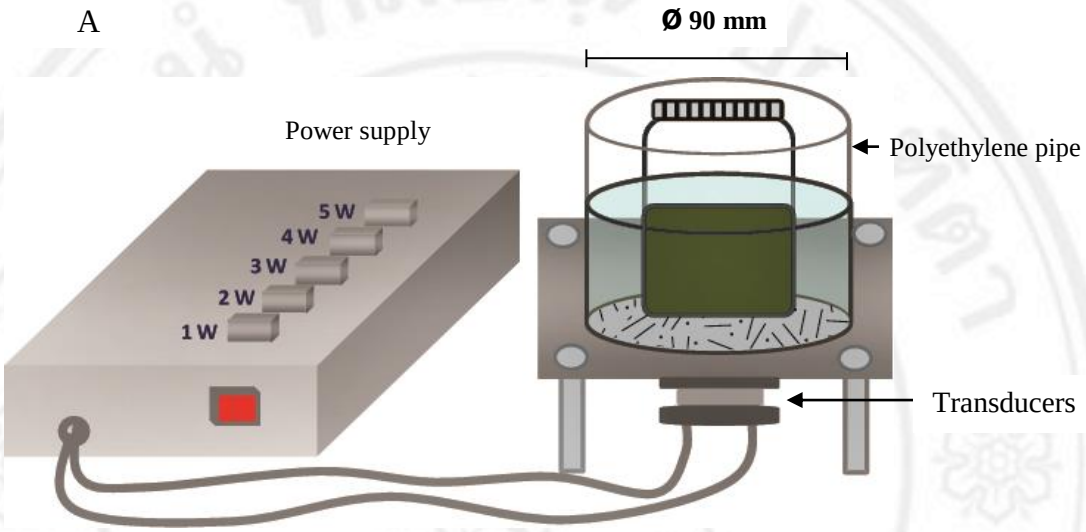
Seed culture of *Carteria* sp. AARLG045 was inoculated in triplicate sets in the open plastic tank (77x108x28 cm) containing 100 L of CMU03 medium to obtain an initial optical density at 665 nm (OD_{665}) of 0.05. The microalga was cultured under natural sunlight without temperature control, and aeration was provided by the bubbling air-line for 14 days. When the growth reached stationary phase, the cells were harvested. The dry cells were pulverized in a mortar for lipid extraction.

6.2.2 Ultrasonic –Assisted Extraction Apparatus setup

An ultrasonic bath was obtained from Honda Electronics Co., Ltd. (Toyohashi, Aichi, Japan) (Figure 23). The bath was made of a cylindrical polyethylene pipe (inner diameter: 90 mm, height: 90 mm) and a stainless steel

bottom plate, which is equipped with ultrasonic transducers (frequencies: 45 - 2,000 KHz, power: 1-5 W).

A



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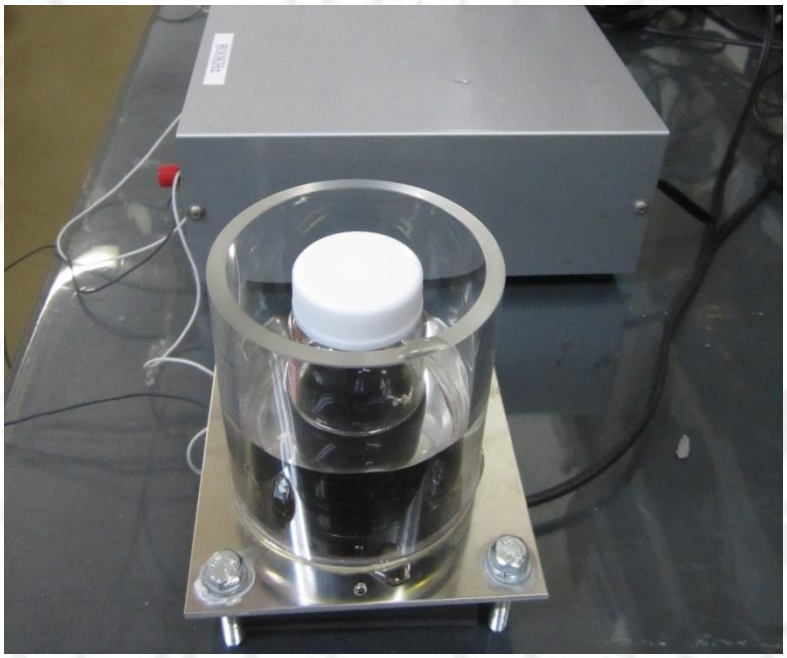

Figure 23 Ultrasonic –assisted extraction apparatus for lipid extraction

(A: diagram, B: real picture)

6.2.3 Lipid extraction

6.2.3.1 Conventional method

Lipids content was determined using a modified procedure according to Bligh and Dyer method (1959). One gram of dried sample was added to 30 mL of the chloroform: methanol (2:1 v/v) in a glass bottle with cover. It was left for 24 hr at room temperature. The extracted lipids were separated from the cell debris by centrifugation at 6000 rpm for 20 min. The lower organic phase was transferred to a new pre-weighed centrifuge tube and dried at 36 – 40 °C to determine balanced dry lipid weight (Figure 24).

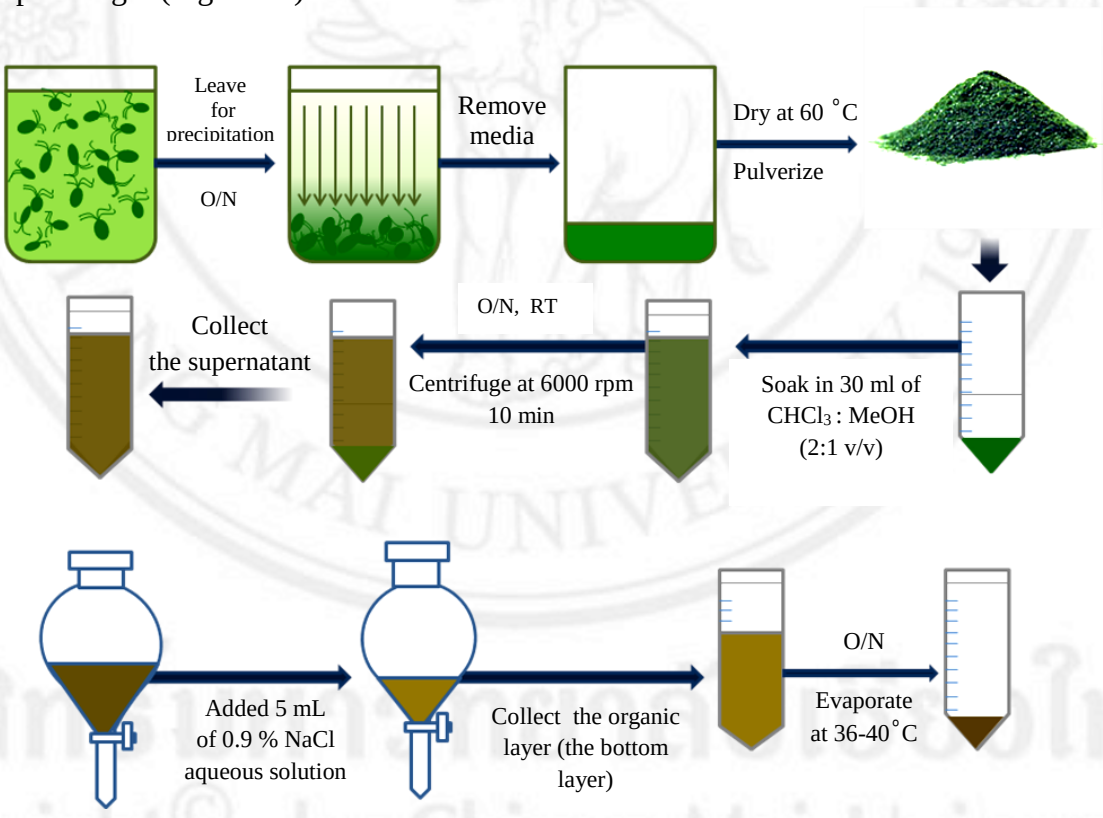

Figure 24 Modified Bligh and Dyer method for lipid extraction

6.2.3.2 Ultrasound assisted solvent extraction method

UASE was applied with a modified Bligh and Dyer method. A glass bottle with a mixture of sample and solvent was immersed in an ultrasonic bath, which contained 100 mL of water. The frequencies, ultrasonic power, time and solid – liquid ratio were optimized in this experiment to obtain the best rate of extraction efficiency.

Frequencies

Different frequencies (45, 200, 350, 500, 600, 800, 1000 KHz) were used to optimize the extraction efficiency. The ultrasonic power was maintained constant at 1 W for 1hr.

Ultrasonic power

The percentage of lipid content was observed from two frequencies (45 KHz and 1 MHz), for which the treatments were carried out at 1-5 W. All the extractions were performed over 1 hr.

Extraction time

The UASE time was considered for extraction efficiency, which was examined at varying times of 10, 20, 30, 60, 90 min, and 120 min. The treatments were carried out at the optimal frequencies (1 MHz) and power input at 4W from the previous experiment.

The solid – liquid ratio

The effect of the sample powder on the solvent ratio was considered another influential factor that was examined in determining the optimum extraction rates. One gram of sample per 30 mL of chloroform:methanol (2:1) or 1:30 (w/v)

was used in the previous experiments. This ratio was the standard value used in this experiment. Dried samples weighing 1 g, 1.2 g, 1.5 g, 2 g, 3 g and 6 g were placed in 30 ml of mixed-solvent in order to prepare mixtures with sample-solvent ratios of 1:30, 1:25, 1:20, 1:15, 1:10 and 1:5 w/v, respectively, to evaluate the effect of the sample powder to the solvent ratio. The treatments were carried out at the optimal frequencies (1 MHz) and power intensity (4 W), time was 30 min from the previous experiment.

6.2.4 Gas chromatography – mass spectrometry (GC-MS) analysis of the fatty acid fraction

Crude lipid which was extracted by conventional method (6.2.3.1), was analyzed by GC-MS to identity the type of fatty acid and compare with UASE method (6.2.3.2). Fatty acid contents from microalgae were analyzed by Agilent 7890 gas chromatography equipped with a flame ionization detector and Agilent HP-5MS capillary column (30 m x 0.25 mm ID x 0.25 μm film thickness). Helium was used as a carrier gas at a flow rate of 0.1 $mL.min^{-1}$. One microliter of the crude extract was injected in the split (25:1) injection mode. The inlet and detector temperatures were 270 °C, and the oven temperature was programmed at an initial temperature of 100 °C and then increased at 10 °C. min^{-1} intervals to 250 °C and held for 3 min. Fatty acid methyl esters were identified by chromatographic comparison with authentic standards (Sigma Chemical Co., St. Louis, MO).

6.2.5 Statistical analysis

The data were expressed as mean ± S.D. Statistical comparison between groups

was analyzed using SPSS for Windows ™ version 15.0., and was examined by one-way ANOVA and Paired - Sample T-Test. Significance was inferred at $p<0.05$.

6.3 Results and discussions

6.3.1 Screening of ultrasonic frequency

To examine the effect of ultrasonic frequency, the experiments were operated at 45, 200, 350, 500, 600, 800, 1000 KHz. Figure 25 shows the lipid recovery from each frequency.

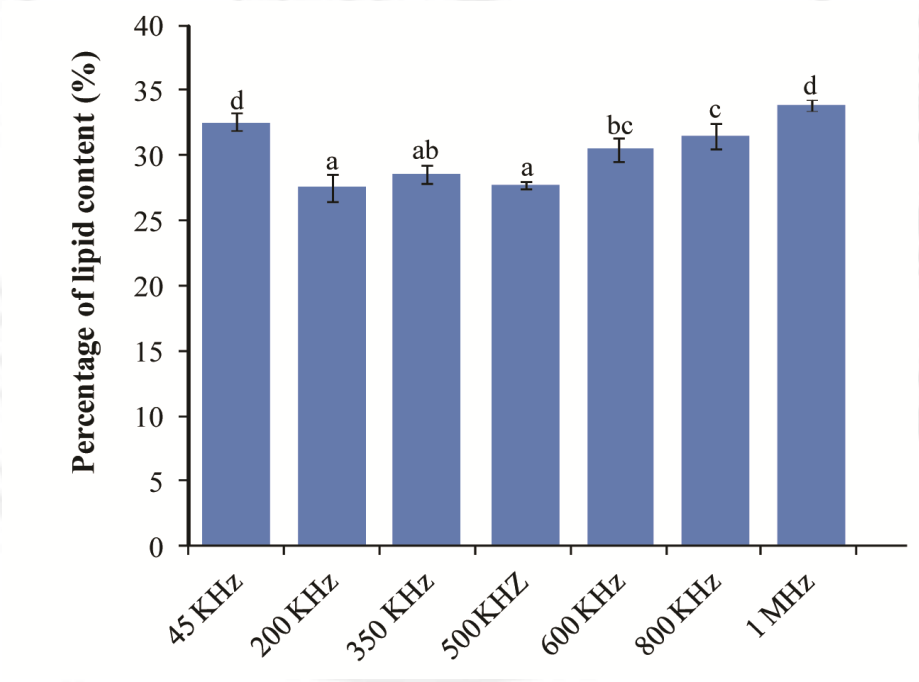

Figure 25 Effects of frequency on lipid yield. [Conditions: ultrasonic power: 1 W; time: 1hr] Letters on the top (a, b, c and d) are a statistical comparison among groups using ANOVA and LSD) test, ($p< 0.05$)

The percentage of lipid content was similar at 45 KHz and 1 MHz, which were not significantly different ($p>0.05$). Therefore, these two frequencies were chosen for further experiments.

6.3.2 Effect of ultrasonic power

The ultrasonic power was also optimized because the driving force of ultrasonic power encourages the dispersal of the solvent to the sample. The device, which powers the ultrasonic generator can be adjusted within a range of 1-5 W and was used. In this experiment, two suitable frequencies (45 KHz and 1 MHz) were considered. It was found that the lipid extracted yield at 1 MHz was significantly higher than that at 45 kHz, except at 2W (Figure 26).

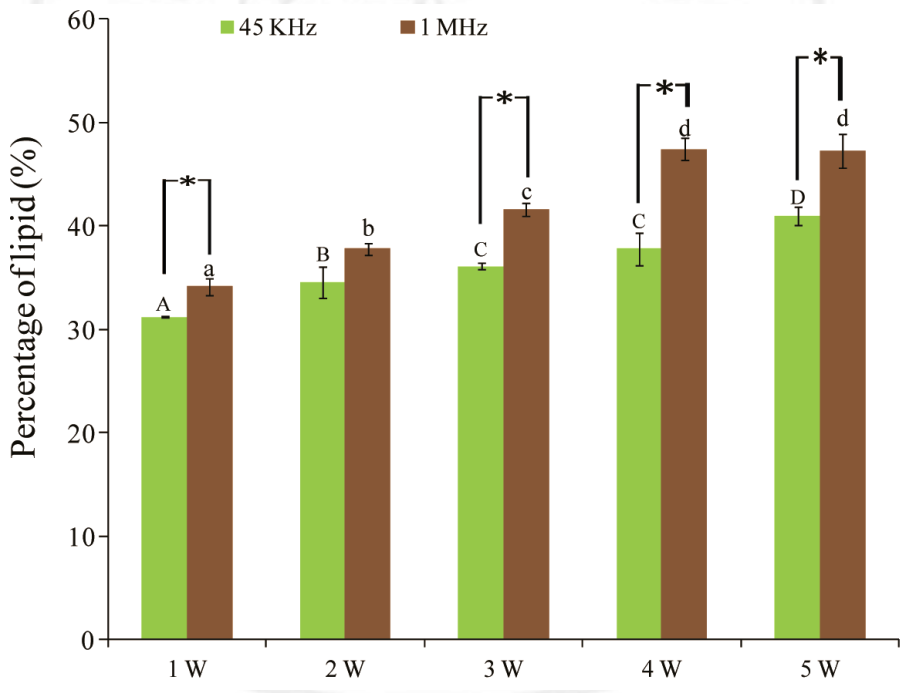

Figure 26 Effects of ultrasonic power on lipid extraction, [Conditions: sample: 1 g; extraction volume: 30 mL; ultrasonic power: 1 - 5 W; time: 1 hr] Letters on the top are a statistical comparison among groups using ANOVA and LSD test, (p<0.05). Whereas, * indicated a significant difference between (p<0.05) 45 KHz and 1MHz

Actually, cavitation may occur in solvents at both low frequency (LF) and high frequency (HF). Normally the bubble from LF is larger in size and the cavitation generated stronger collapse than that from HF (Alliger, 1975). However, in this study, the cavitation at HF has higher efficiency in lipid extraction than LF because HF could enhance mass transfer, the dispersion of solvents and the disaggregation of catalyst particles more than those of LF. Although, the resonant radius of bubble from HF is smaller in size than that from LF, hence the bubble requires only fewer acoustic cycles for creating before it reaches to resonant size and collapse to release the energy. For this reason, HF could generate a greater number of acoustic cycles per unit of time which results in more rapidly diffusion than at LF (Thangavadivel *et al.*, 2012). Moreover, an increase in liquid temperature from HF produces pyrolysis inside the bubble and results in an increase in the amount of the vapour inside the bubble, which can promote the formation of free reactive radicals from vapour dissociation as the chemical effect (Gong and Hart, 1998; Tang *et al.*, 2004). In this study, the temperature of the solvent at 1 MHz was 45 ℃ which was higher than the LF (45 KHz), which was 30 ℃. The smaller bubbles from the HF had a greater influence on the chemical effects from the free radicals (Mason *et al.*, 2011). These free radicals, which were created from cavitation in the chloroform, are strong oxidant (Kim *et al.*, 2003; Oturan *et al.*, 2008). These radicals can be destructive to cells, which could help extracting lipids from algal biomass. Therefore, 1 MHz was chosen for the optimal frequency.

In addition, the lipid extraction yield increased with the increase in power at the same frequency (1MHz), but it was constant after 4W-5W. The highest lipid content was 47.43%, which was obtained from 1 MHz at 4 W of input power (0.04

$W.cm^{-3}$). The high power ultrasound (HPU), 4W is more effective than the low power ultrasound (LPU) because the sonication requires less time to generate the bubbles when the frequency increases. However, the cavitation needs greater power to achieve the desired extraction yields in the solvent (Mason and Lorimer, 2002). It also increases the dispersion of solvent to the sample.

Zu *et al*. (2012) studied the effects of the ultrasound power on the extraction efficiency of phenolcarboxylic acids, carnosic acid and rosmarinic acid from *Rosmarinus officinalis*. The results showed that the increased ultrasound power could enhance the compound yields because the HPU could destroy the cell walls via ultrasound energy and promotes the theory that the solvent could enter cells and target compounds. Therefore, the ultrasonic power was set at 4W in all further experiments. However, the energy used in this study was lower than other UASE techniques. Soybean-germ oils were successfully extracted by HPU (60-85W) (Cravotto *et al.*, 2008), while fresh microalga, *Chlorella* lipid was extracted by the application of UASE with HPU (1000W) (Broekman *et al.*, 2010). Thus, using LPU requires less energy and resulted in the significant energy saving.

6.3.3 Effect of extraction time

The effect of time was investigated by varying the time at 10, 20, 30, 60, 90 and 120 min. The results are shown in Figure 27. It was shown that the percent yield of lipids increased during the first 20 min and remained constant after 30 min.

The conventional extraction method requires at least 24 hrs for lipid extraction (Metherel *et al.*, 2009) while UASE only need less time as 30 min; hence this implies that the UASE technique can reduce the time needed for extraction about 48 times and

it also reduced energy consumption during longer time. Thus, the contact time at 30 min was chosen as the optimal time.

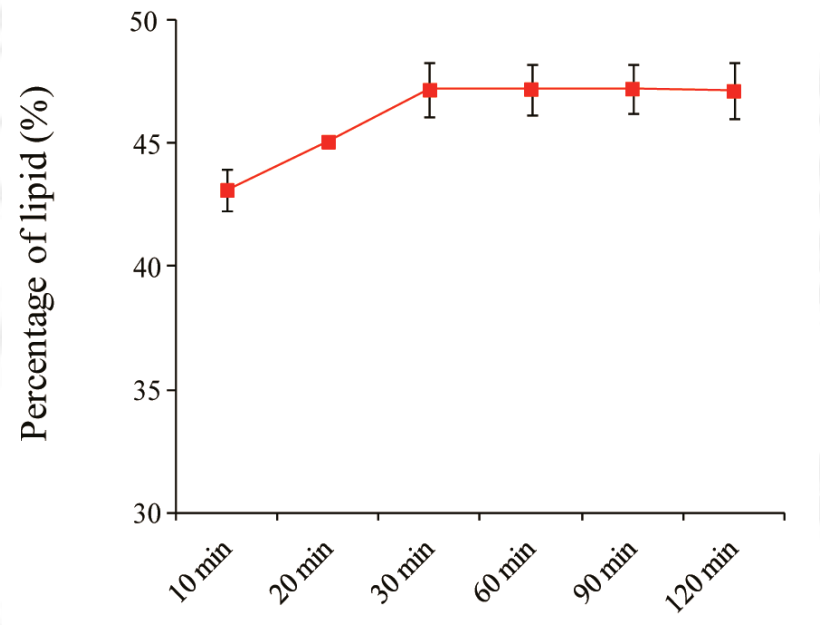

Figure 27 Effects of extraction time on lipid extraction. [Conditions: sample: 1 g.; extraction volume: 30 mL; ultrasonic power: 4 W at 1 MHz]

6.3.4 Influence of the sample powder to solvent ratio

The solid-liquid ratio was found to have a significant role in the extraction efficiency. In this extraction, the ratios of 1:30, 1:25, 1:20, 1:15, 1:10 and 1:5 w/v sample powder to solvent were used. The results are shown in Table 18, the solid-liquid ratio increased from 1:5 to 1:25. The sample ratio of 1:25 (w/v), or 1.2 g in 30 mL of solvent gave the highest lipid productivity of 0.4776±0.0304 g. g DW^{-1}. An increase of the sample from 1 g to 1.2 g reduced solvent consumption and solvent waste disposal costs. Thus, it indicates that a ratio of 1:25 was suitable for extraction to obtain higher lipid productivity. Conversely, other ratios show lower lipid recovery because of the greater samples while the volume of solvent was constant at 30 mL.

The excess of sample resulted in less dispersion of solvent into the cells and this might limit the diffusion of ultrasonic wave to a sample.

Table 18 Effect of sample powder on the solvent ratio in lipid recovery

Ratios	Sample (g dry weight per 30 mL)	Lipid productivities (g.g dry weight^{-1})
1:30	1.0	0.4727±0.0184^{c}
1:25	1.2	0.4776±0.0304^{c}
1:20	1.5	0.3910±0.0793^{b}
1:15	2.0	0.3078±0.0197^{ab}
1:10	3.0	0.2300±0.0189^{a}
1:5	6.0	0.1999±0.0089^{a}

Data are expressed as the mean ± standard deviation (SD) of three replicates. Different letters represent the statistical comparisons between groups by using ANOVA and LSD test, (p<0.05)

Thus, the optimal conditions for UASE involved ultrasonic power at 4 W, 1 MHz frequency and a contact time of 30 min with a sample:solvent ratio of 1:25. This condition could extract lipid up to 47%, which was about two times higher than that from a modified Bligh and Dyer method over 24 hrs (Figure 28). UASE can increase lipid yield from cavitation effect and the formation of free radicals (chemical effects) by sonolysis (Chemat *et al.*, 2004). These effects could promote disruption of the cell wall, result in extraction of high lipid yields. In addition, UASE technique can reduce the time needed for extraction around 48 times of the conventional method.

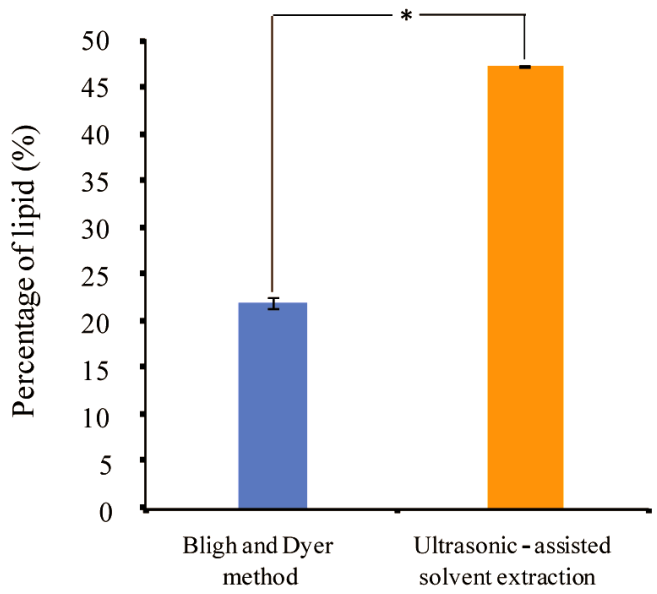

Figure 28 Lipid contents between standard method using chloroform : methanol (2:1) solvent for 24 hrs and using ultrasonic assisted solvent extraction with4 w of ultrasonic power at 1 MHz for 30 min. * indicates significant difference (p<0.05) between standard method and UASE

6.3.5 GC-MS analysis

The relative concentrations of fatty acids analyzed by GC-MS are shown in Figures 29 and 30 and the area percentages of the chemical components of the fatty acids produced by the traditional method and UASE are shown in Table 19. It shows the different compounds of fatty acid content as 37.31%, including fourth unsaturated fatty acids (12.54%) and fourth saturated fatty acids (24.77 %). Palmitic acid (13.81%), myristic acid (6.97 %), 1, 2 - bezene dicarboxylic acid (6.8 %) were the main components in the traditional method, while various compounds in the crude fat from UASE represented at about 66.84% of the total fatty acid composition, including six unsaturated fatty acids (65.12 %) and one saturated fatty acid (1.72 %). The major components of the fatty acids were cis,cis - 7, 10, - hexadecadienal (43.88

%) , adipic acid, bis (2 - ethylhexyl) ester (10.93%), ethyl 9,12,15 -octadecatrienoate (3.67 %).

The relative concentrations of fatty acids by GC-MS indicated that the chemical composition in the two lipid profiles were different, both in terms of quantity and quality, although some of the findings were similar. Some chemical structures were changed due to oxidation from ultrasound. The radical reactions are affected by breaking the C-C bonds and the change in structure. (Bernstein *et al.*, 1996; Li *et al.*, 2008). Moreover, the effect of cavitation which results in the cell disruption encourages more effective extraction to obtain higher fatty acids content than the standard method. Over 50% of the fatty acids in *Carteria* sp. AARL G045 extracted by UASE, were C_{16}-$C_{18.}$ These fatty acids are suitable for biodiesel production. However, these fatty acids should be investigate the suitability as biodiesel. A good biodiesel is considered according to the EN14214 and ASTM D6751-02 biodiesel standards. It should meet the cetane number (CN) standard, which indicates good ignition quality, a suitable cold filter plugging point, low pollutants content, correct density, and viscosity (Islam *et al.*, 2013).

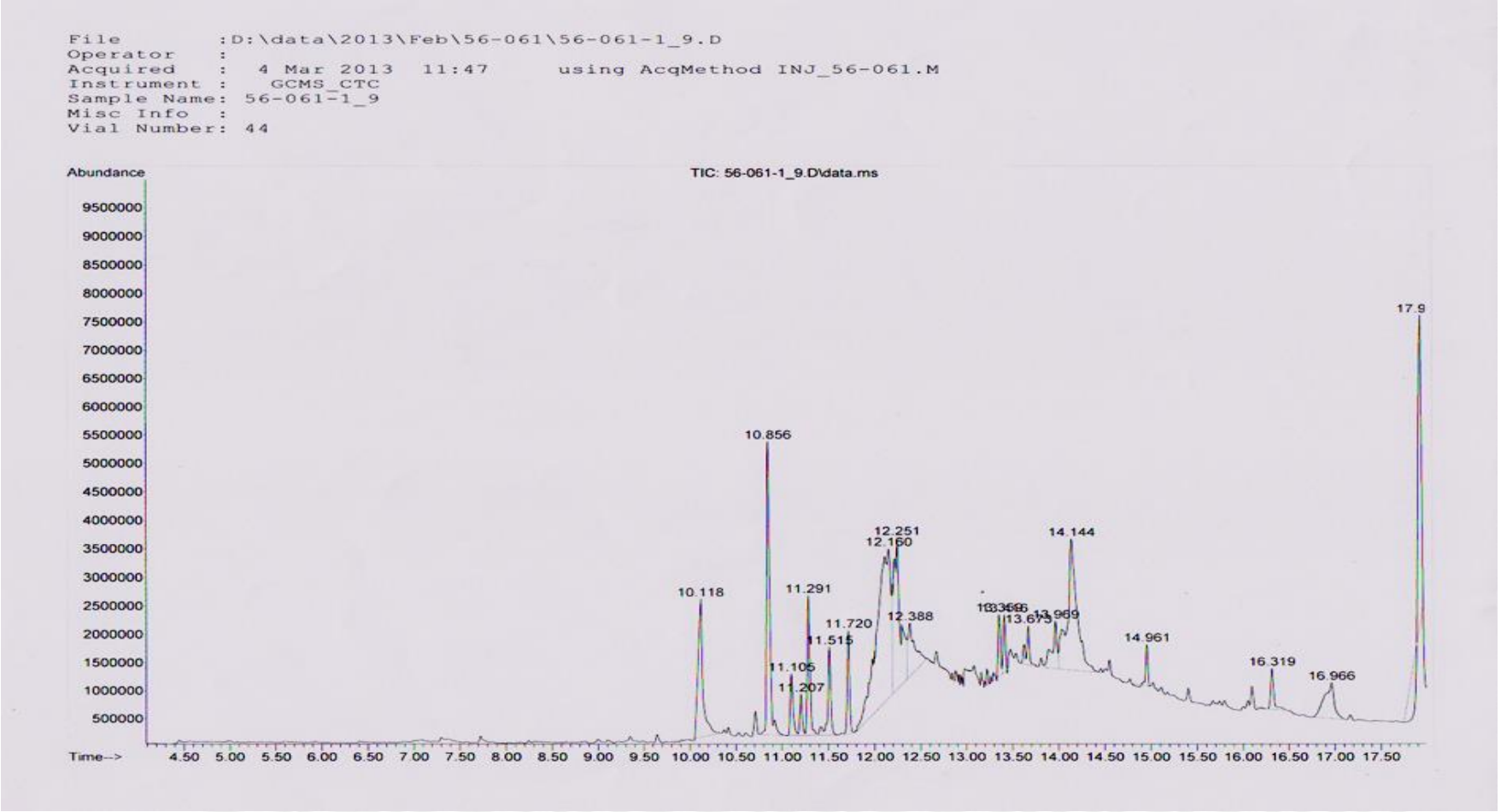

Figure 29 Gas chromatograph of fatty acid concentrations obtained via the standard lipid extraction method

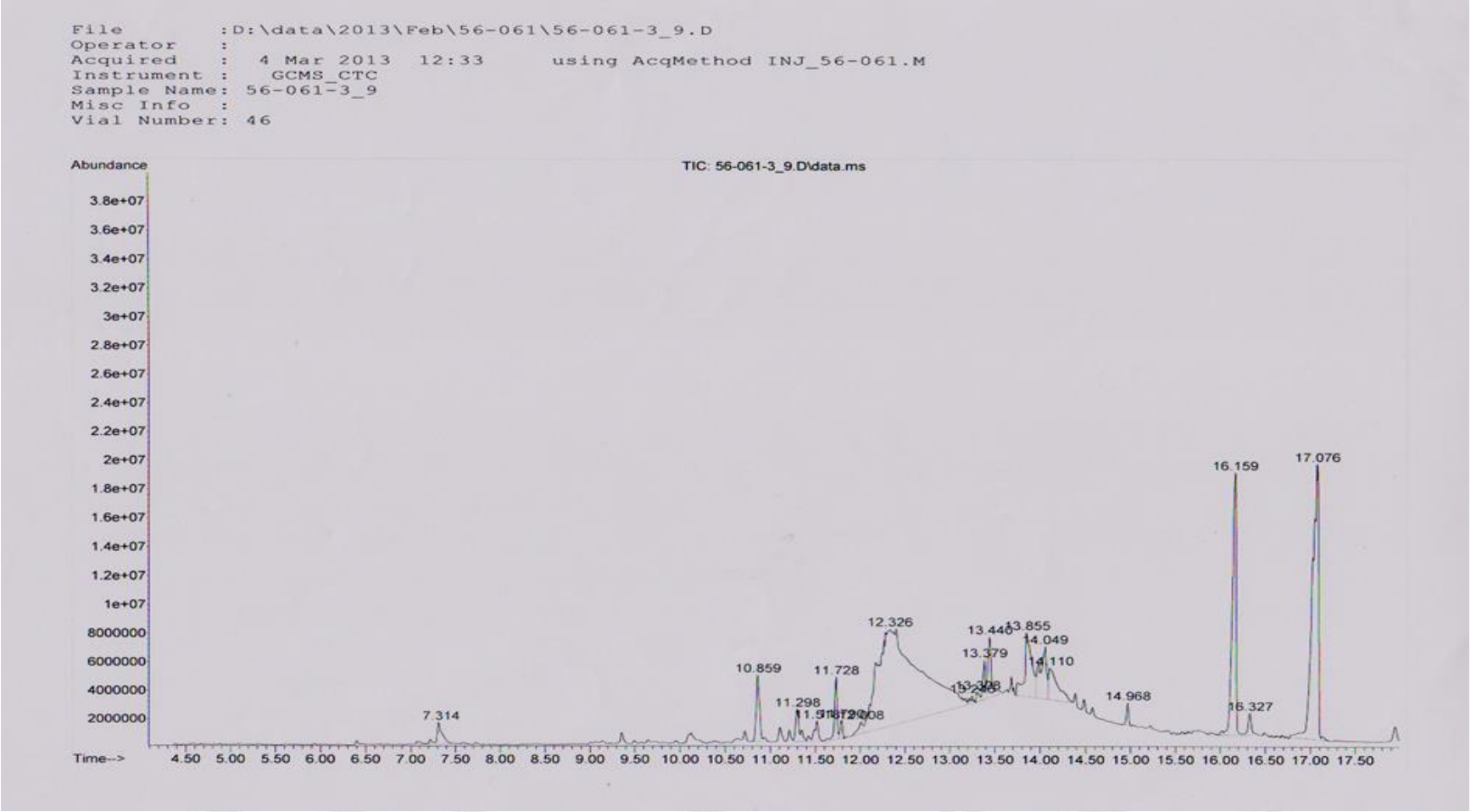

Figure 30 Gas chromatograph of fatty acid concentrations obtained using ultrasound assistance with 4 w at 1 MHz for 30 min.

Table 19 Percentages of components of fatty acids by GC – MS, between the standard extraction method and UASE

Standard extraction method		**UASE with 4W at 1 MHz for 30 min**	
Name of the compound	**% Fatty acids**	**Name of the compound**	**% Fatty acids**
Myristic acid (C 14:0)	6.97	14-Methyl Pentadecanoic Acid Methyl Ester (C17:0)	1.72
Palmitic acid (C16:0)	13.81	Cis,cis -7, 10, - Hexadecadienal (C16:2)	43.88
Tridecanoic acid, methyl ester (C13:0)	3.99	Palmitic acid (C16:0)	0.63
Linoleic acid, methyl ester (C 18:2n6c)	1.98	10,13 - Octadecadienoic acid (C 18:1n13c)	0.98
Oleic acid, methyl ester (C18:1n9c)	1.35	Alpha-linolenic acid methyl ester (C18:3n3)	2.49
9,12- Octadecadienoic acid (C18:2n9c)	2.41	Ethyl 9, 12,15- octadecatrienoate (C20:3)	3.67
1, 2 - Bezenedicarboxylic acid (C8:0)	6.80	Cis, cis - Linoleic acid (C18:2)	3.17
		Adipic acid, bis (2 - ethylhexyl) (C22:0)	10.93