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# Comparative yield of crude lipid and fatty acid from *Tetraselmis* sp. using ultrasonic extraction at varying time and solvent systems

[1]Ida G. Pahila, [1]Roland J. C. Calawigan, [2]Nieves A. Toledo, [2]Soledad S. Garibay, [3]Caridad N. Jimenez, [4]Leticia J. Ami, [4]Rose T. Mueda, [1]Mellie J. S. Paulines, [5]Pilar M. A. Retiracion

[1] Department of Chemistry, College of Arts and Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines; [2] Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines; [3] Institute of Fisheries Policy and DevelopmentStudies, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines; [4] Institute of Fish Processing Technology, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines; [5] Division of Professional Education, College of Arts and Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines. Corresponding author: I. G. Pahila, igpahila@gmail.com

**Abstract**. This study was conducted to compare lipid extraction from wet paste-like *Tetraselmis* sp. using isopropanol:hexane, absolute ethanol and chloroform:methanol at different ultrasonic extraction times. The best solvent system and ultrasonic extraction time is chloroform:methanol (1:1) at 50 minutes, respectively. Soaking effect (before and after extraction) gave a higher crude lipid (0.65 % - 28 %) recovery than those that were not. Ultrasonic extraction of *Tetraselmis* sp. gave higher crude lipid yield (15.3 %) than the Folch method (11.78 %). Qualitative analysis of fatty acid profile using different solvents showed no significant difference. Fatty acid present in *Tetraselmis* sp. is predominantly $C_{16}$ (Palmitic acid) with significant amount of $C_{18:0}$ (Stearic acid), $C_{18:1\ n\text{-}9}$ (Oleic acid), $C_{18:2\ n\text{-}6}$ (Linoleic acid) and $C_{18:3\ n\text{-}3}$ (α-Linolenic acid) and $C_{20:5\ n\text{-}3}$ (Eicosapentanoic acid). Fatty acid profile is not dependent on extraction solvent but crude lipid yield varies with the solvent system.
**Key Words**: isopropanol:hexane, absolute ethanol, chloroform:methanol, soaking effect.

**Introduction**. Microalgae are unicellular aquatic plants which utilize the light from the sun, carbon dioxide and water for survival. Many species of microalgae have been found to have a high content of oil (Prescott 1968; Chisti 2007a,b).

There are different methods used to extract lipids. These are the Soxhlet method (Takagi & Yanagita 1985; Manirakiza et al 2001), the Bligh & Dyer method (Bligh & Dyer 1959; Pratoomyot et al 2005; Manirakiza et al 2001) or the modified Folch method (Folch et al 1957; Takagi & Yanagita 1985; Mulbry et al 2009) and Goldfisch method (Shahidi 2001). These methods have been used for conventional extraction to quantitatively estimate the amount of lipid in a particular sample. One disadvantage of these methods is most of them are time consuming and laborious (Shahidi 2001). These methods may not be economically efficient since they consume a lot of time for extraction, hence, higher energy consumption.

To address the disadvantage mentioned above, scientists have looked upon the use of ultrasound energy. During sonication, microscopic bubbles are formed and collapsed releasing tremendous energy as heat, pressure and mechanical sheer (Chemat et al 2004). Sonication has been widely used to disrupt microbial cells (Engler 1985; Lee et al 2010) and some microalgae (Supriya & Ramachandra 2012). It breaks the structure of the cell improving material transfer with the assistance of solvent such as hexane supporting lipid extraction (Dunstan et al 1992; Hielscher 2011).

To approximate the lipid yield for different microalgae species, studies and researches often used the Bligh & Dyer method (Lewis et al 2000; Mulbry et al 2009). However, other methods such as the sonication method and the use of different solvent systems have not yet been thoroughly explored for microalgae (Metherel et al 2009).

In this study, wet *Tetraselmis* sp. microalgae samples were used for evaluating crude lipid content and fatty acids using different solvents and ultrasonic extraction time. To facilitate the extraction through this type of sample, pure polar solvents and a mixture of polar and nonpolar solvents were used. This is to enhance the penetration of solvents to the sample matrix, and maximize the extraction of lipids.

## Material and Method

***Sample preparation***. Wet paste-like *Tetraselmis* sp. samples were provided by the University of the Philippines Visayas Biodiesel Project 3 (Manipulation of culture techniques to improve lipid content and establish effective protocols/structures for mass production), a research program on Biodiesel from Philippine Algae funded by the Department of Science and Technology (DOST). These were cultured using combined fertilizer and standard culture methods. Microalgal cells were concentrated by flocculation prior to harvesting.

***Moisture content***. Wet paste-like *Tetraselmis* sp. samples were analysed for moisture content using gravimetric method. Three replicates (2 g each) of *Tetraselmis* sp. in paste-like form was dried at 105 °C in a WiseVen® oven for 5 hours to ensure samples were dry and attained a constant mass.

***Preparation of solvents***. All solvents used in the analyses were prepared using analytical grade chemicals and HPLC/GC grade for methylation. A preliminary run was done to determine the best ratio for solvent mixtures. Test solvents were: 90:10 isopropanol:hexane (v/v), absolute ethanol and 1:1 chloroform:methanol (v/v).

***Solvent and ultrasonic extraction time***. A 2-factor experiment was performed with 3 solvent systems and 5 ultrasonic extraction times as variables. Crude lipid content of the *Tetraselmis* sp. paste-like samples was evaluated under the different solvents and extraction times (Table 1).

Table 1

The 2-factor experiment with 3 solvent systems and 5 extraction times

| *Solvent* | *Extraction time (min.)* | | | | | *Replicates* |
|---|---|---|---|---|---|---|
| 1:1 Chloroform:Methanol | 0 | 5 | 10 | 20 | 50 | 3x |
| 90:10 Isopropanol:Hexane | 0 | 5 | 10 | 20 | 50 | 3x |
| Absolute Ethanol | 0 | 5 | 10 | 20 | 50 | 3x |

For each solvent, three 1-gram wet paste-like samples with approximately 74.02 % moisture (*Tetraselmis* sp.) were weighed in a 10 mL test tubes using (Sartorious®) analytical balance. For each tube, 5 mL of the solvent were added and were covered using an aluminum foil. The samples were soaked in the solvent for ~18 hours. After soaking, the tubes containing the sample with the solvent were placed in a 200 W Ultrasonic Cleaner (Cole-Palmer®) and were sonicated at 0, 5, 10, 20, and 50 minutes. To maintain the temperature of the sonicator, a water pump was connected to an external ice water source to keep the temperature constant at 33 °C all throughout the experiment. After sonication, the samples were soaked in the same solvent for another ~18 hours. After soaking, each tube was centrifuged for 10 minutes at 1,500 rpm and the extracts were carefully pipetted out using Pasteur pipets to prevent inclusion of solid particles. The collected extracts were placed in a pre-weighed glass vials for gravimetric analysis and the crude lipid content were quantified.

***Effect of soaking***. To determine the effect of soaking in the experiment, a separate determination was performed for the samples. In this experiment, the same procedure was done without soaking the samples. For each solvent, three 1-gram wet paste-like samples with approximately 74.02 % moisture content were weighed in a 10 mL test tubes using (Sartorious®) analytical balance. For each tube, 5 mL of the solvent were added and were covered using an aluminum foil. The tubes containing the sample with the solvent were placed in a 200 W Ultrasonic Cleaner (Cole-Palmer®) and were sonicated according at 5, 10, 20, and 50 minutes. To maintain the temperature of the sonicator, a water pump was connected to an external ice water source. The temperature was kept constant at 33 °C all throughout the experiment. After sonication, each tube was centrifuged for 10 minutes at 1,500 rpm and the extracts were carefully pipetted out using Pasteur pipets to prevent inclusion of solid particles. The collected extracts were placed in a pre-weighed glass vials for gravimetric analysis and the crude lipid content were quantified.

***Folch method***. Crude lipid analysis using a standard extraction method (Folch et al 1957) was done to compare the extraction efficiency of the proposed method – the ultrasonic extraction method. The Folch method introduced by Folch et al (1957) utilizes chloroform:methanol as solvent systems.

***Fatty acid analysis***. The effect of sonication and solvent on fatty acid extraction was also investigated. For each solvent, the lipid extracted at the best sonication time was trans-esterified to convert fatty acids to Fatty Acid Methyl Ester (FAME). The procedure for the methylation of samples was based from AOAC Hydrolytic Extraction Gas Chromatographic Method. Three replicates of the extracted lipid were dissolved in 2 - 3 mL chloroform and 2 - 3 mL diethyl ether and were transferred into a glass vial and evaporated to dryness in 40 °C under nitrogen stream. Two (2) mL of 7 % $BF_3$ with methanol reagent and 1.0 mL toluene were added and covered with screw cap top coated teflon/silicon septum. The vials were heated in the oven for 45 min at 100 °C. Vials were shaken gently every 10 mins. The vials were allowed to cool at room temperature. Five mL of water, 1.0 mL of hexane and 1.0 g of anhydrous $Na_2SO_4$ were then added to remove traces of water. The layers were allowed to separate and the top layer was transferred to another vial containing 1.0 g of $Na_2SO_4$.

***Statistical analysis***. All data were statistically analyzed by Two-Way ANOVA and One-Way ANOVA using the SigmaStat v3.5 and SPSS v16 program to differentiate the crude lipid yield with respect to sonication time and solvent systems and to check if there is interaction between the two variables.

## Results and Discussion

The physical and chemical properties of the microalgae determined before and after flocculation are presented in table 2.

Table 2

Physical and chemical analysis of sample before and after flocculation

| *Parameter* | *Before flocculation* | *After flocculation* |
|---|---|---|
| pH | 8.95 | 6.16 |
| Dissolved oxygen (DO), mg $L^{-1}$ | 7.02 | 6.36 |
| Salinity, ppt | 34.0 | 34.0 |
| Algal density, cells $mL^{-1}$ | 320,000 | nd* |
| Temperature, °C | 29.2 | 29.9 |
| Light intensity, kLux | 7.5 | 92.3 |

* not detected.

***Preliminary experiment***. The solvents that were used in the extraction are mixtures of Isopropanol:Hexane, Choloroform:Methanol, and Ethanol. To evaluate the best ratio for

the two-component solvents, a preliminary run was done and the optimization results are presented in table 3 & 4.

Table 3

Solvent optimization for Isopropanol/Hexane

| *% Ratio Isopropanol:Hexane* | *Crude lipid* (%)* |
|---|---|
| 10:90 | 1.39±0.60 |
| 20:80 | 1.55±0.30 |
| 30:70 | 1.93±0.07 |
| 40:60 | 2.64±0.50 |
| 50:50 | 3.43±0.76 |
| 60:40 | 4.40±0.18 |
| 70:30 | 5.30±0.32 |
| 80:20 | 8.85±1.41 |
| 90:10 | 8.82±0.35 |

*expressed as dry weight. Mean values of triplicate groups ± SD.

Table 4

Solvent optimization for Chloroform/Metanol

| *Ratio Chloroform:Methanol* | *Crude lipid* (%)* |
|---|---|
| 1:2 | 7.98 ± 0.74 |
| 2:1 | 1.21 ± 0.25 |
| 1:1 | 11.60 ± 1.2 |

*expressed as dry weight. Mean values of triplicate groups ± SD.

The crude lipid yield for Isopropanol/Hexane solvent ratio is not statistically different for the ratio 80:20 and 90:10 while in the Chloroform:Methanol ratio, there is a significant difference in the crude lipid yield between each ratios evaluated. Hence, the Isopropanol:Hexane (90:10) and Chlorofom:Methanol (1:1) were selected. Likewise absolute ethanol was also considered as another solvent system.

***Solvent-extraction*. *Time experiment*.** The extraction of lipid from wet paste-like microalgae samples were evaluated in five different extraction times (0, 5, 10, 20 and 50 min) and three different solvent systems namely: Isopropanol:Hexane (90:10), absolute ethanol and Choloroform:Methanol (1:1). The % crude lipid for each solvent-time extractions determined is presented in table 4. The result presented in figure 1 shows that the Isopropanol/Hexane (90:10) has the highest % crude lipid yield at 20 minutes with a crude lipid yield of 5.71 ± 0.22 % and at 50 minutes for both Ethanol and Chloroform:Methanol (1:1) mixture with a crude lipid yield of 6.19 ± 0.99 % and 15.3 ± 0.04 %, respectively. It reveals that the solvents and sonication times has no statistically significant interaction. This means that the crude lipid content at different extraction time is not dependent on the solvent used.

To determine the effects of ultrasonic extraction time and solvents, One Way ANOVA was used for each factor. For the ultrasonic extraction time, there is a significant difference in the values of % crude lipid for the different extraction times. 0 minute has a significant difference on 50 minutes, while it has no significant difference between 5 minutes and 10 minutes. The 20 minutes on the other hand, has no significant difference with 50 minutes. On the effect of solvent on crude lipid content, there is a significant difference ($\alpha<0.05$) for each solvent. Isopropanol:Hexane (90:10) is significantly different from Ethanol and Chloroform:Methanol (1:1). The same is true for Ethanol to Chloroform:Methanol (1:1). The result of the analysis showed that the % crude lipid yield of the sample depends on either the time of extraction using sonication method or the solvent used. For a higher % of crude lipid yield, it can be deduced from the data that the

best extraction time and solvent for wet paste-like *Tetraselmis* sp. is 50 minutes using Chloroform:Methanol (1:1).

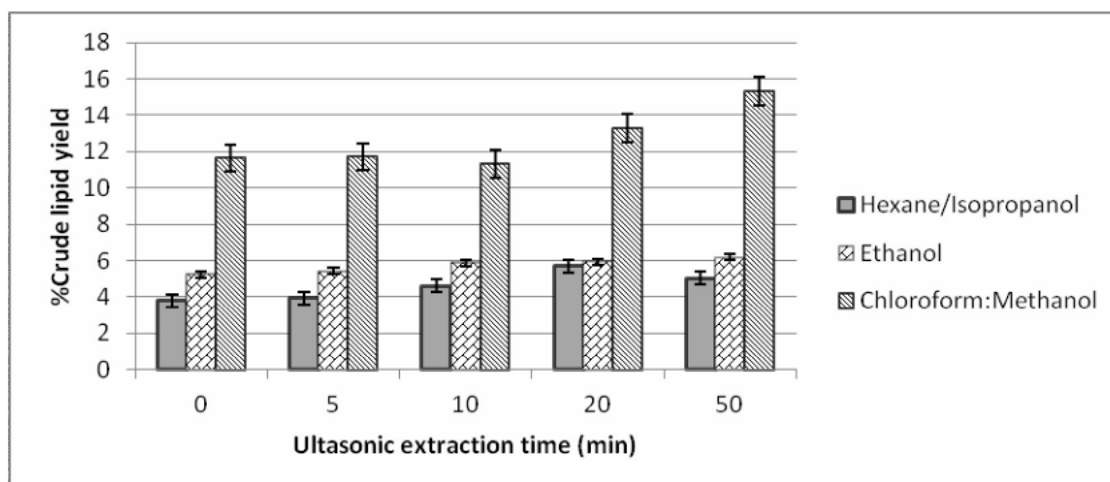


Figure 1. % Crude lipid expressed in dry weight basis extracted using different solvents at different sonication times.

***Soaking effect***. The effect of soaking before and after sonication on the crude lipid yield was also investigated. Table 5 compares the % of crude lipid showing the effect of soaking together with the sonication time and solvent factors. There is a significant difference between the % of crude lipid yield of the samples that were soaked and was extracted directly. It is evident that the lipid yield is lower for samples extracted directly than those that were soaked.

Table 5

% of crude lipid at different sonication times, solvents and soaking factor

| *Solvent* | *Time (min.)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *5* | | *10* | | *20* | | *50* | |
| | *Without soaking* | *With soaking* | *Without soaking* | *With soaking* | *Without soaking* | *With soaking* | *Without soaking* | *With soaking* |
| $C_3H_8O:C_6H_{14}$ (9:1) | 3.58±0.37 | 3.92±0.20 | 4.58±0.47 | 4.61±0.43 | 4.74±0.11 | 5.71±0.22 | 4.72±0.36 | 5.03±0.61 |
| $C_2H_6O$ | 5.10±0.09 | 5.42±0.37 | 5.47±0.17 | 5.85±0.15 | 5.45±0.08 | 5.96±0.12 | 5.74±0.51 | 6.19±0.99 |
| $CHCl_3:CH_4O$ (1:1) | 9.50±0.82 | 11.7±0.62 | 9.90±0.69 | 11.3±0.86 | 10.4±0.49 | 13.3±3.23 | 13.8±0.92 | 15.3±0.04 |

$C_3H_8O$ – Isopropanol, $C_6H_{14}$ – Hexane, $C_2H_6O$ – Ethanol, $CHCl_3$ – Chloroform, $CH_4O$ – Methanol.

***Sonication versus standard Folch extraction method***. The result of the crude lipid yield using ultrasonic extraction method and the standard Folch method is presented in table 6.

Table 6

Comparison between sonication method and standard Folch method

| | *P value* | *Crude lipid yield (%)* |
|---|---|---|
| *Sonication** | 0.011 | 15.30 ± $0.04^a$ |
| Folch Standard Method | | 11.78 ± $0.53^b$ |

Values are means of triplicate group ± SD. Mean values in a column bearing similar superscripts are not significantly different (P>0.05), *Data used was taken from the highest crude lipid yield of sonication experiment.

The crude lipid yield using sonication method is statistically higher than the Folch method. Both methods used Chloroform:Methanol solvent mixtures but the difference is the ratio of the chloroform to methanol. For Folch Method, the ratio of Chloroform:Methanol is 2:1 while for the sonication method, the used ratio is 1:1.

***Fatty acid analysis***. Table 7 shows the fatty acid profile of *Tetraselmis* sp. extracted using different solvents. The fatty acids are composed of $C_{16:0}$ (Palmitic acid), $C_{18:0}$ (Stearic acid), $C_{18:1\ n-9}$ (Oleic acid), $C_{18:2\ n-6}$ (Linoleic acid) and $C_{18:3\ n-3}$ (α-Linolenic acid) although $C_{14}$ (Myristic acid), $C_{16:1n-7}$ (Palmitoleic acid), $C_{20}$ (Arachidic acid), $C_{20:5n-3}$ (Eicosapentanoic acid). Palmitic acid is the major fatty acid with the highest % molar concentration in all extraction solvents. The amount fatty acid in the samples extracted using different solvents were found to be not statistically different ($p>0.05$).

Table 7

Fatty acid profile of *Tetraselmis* sp. lipid extracted using different solvents

| *Fatty acids/ Extraction solvents* | *% mole fatty acid* | | |
|---|---|---|---|
| | *Isopropanol:Hexane (9:1)* | *Ethanol* | *Chloroform:Methanol (1:1)* |
| $C_{16:0}$ | 83.1 | 85.1 | 82.9 |
| $C_{18:0}$ | 1.43 | 1.44 | 1.56 |
| $C_{18:1\ n-9}$ | 5.17 | 4.49 | 5.25 |
| $C_{18:2\ n-6}$ | 8.87 | 7.61 | 8.68 |
| $C_{18:3\ n-3}$ | 0.177 | 0.098 | 0.16 |

Extraction of wet paste-like *Tetraselmis* sp. samples showed higher % of crude lipid recovery using Chloroform:Methanol (1:1, v/v) with sonication at 50 minutes together with the aid of soaking. Soaking the samples before and after the extraction improved the lipid yield by 0.65 % - 28 % compared to extraction without soaking. In a study conducted by Coats & Karnosky (1949) on oil-bearing substance, soaking the sample in the solvent for half the extraction time is just as effective as extraction for the entire time with fresh solvent. An increase in lipid content was also observed in extraction from soy milk with soaking (Wilkens & Hackler 1969). When compared to the standard Folch method, the lipid recovery using sonication also gave a significantly higher value. Studies showed that ultrasound assistance in extraction can increase lipid recovery (Metherel et al 2009) by breaking the structure of the cell improving material transfer with the assistance of solvent supporting lipid extraction (Dunstan et al 1992; Hielscher 2011). Conventional extraction techniques employ dry samples and use hexane for lipid recovery. Although hexane is widely used in industries, it was reported that it has the lowest lipid recovery in extraction of lipids for flaxseed samples (Metherel et al 2009). Nonpolar solvents can extract neutral lipids such as triacylglycerols, however, polar lipids such as phospholipids can be lost during the extraction (Christie 2003). Employing both qualities of solvents as what has been done in this study, can increase the lipid yield together with the aid of sonication those results to an emulsification-extraction leading to an efficient and rapid extraction of total lipids in solid matrices (Perez-Serdilla et al 2007). Drying process is an energy intensive technique, thus, employing wet samples for extraction can minimize the cost of lipid extraction. In the study of Carabias-Martinez et al (2005) and Focant et al (2004) the use of polar of solvent or polar solvent mixtures also improves the extraction efficiency of wet samples.

Fatty acid determination revealed that the dominant fatty acid for *Tetselmis* sp. of the family Prasinophyceae is $C_{16:0}$ (Palmitic acid). The presence of other important fatty acids such as $C_{18:3\ n-3}$ (α-Linolenic acid) and $C_{20:5\ n-3}$ (Eicosapentanoic acid) was likewise reported by some authors (Basova 2005; Cobelas & Lechado 1989). It was observed that

the fatty acid profile of *Tetraselmis* sp. is not dependent on what solvent was used although their crude lipid recovery varies.

Moreover, the high amount of saturated fatty acid in *Tetraselmis* sp. makes it a good candidate for biodiesel production. According Sharma et al (2008), a feedstock with a larger proportion of saturated fatty acid is a good a candidate for biodiesel production. However, higher amounts of saturated fatty acids can increase the low temperature properties such as cloud and pour points (Knothe 2005). On the other hand, saturated fatty acids have higher melting points and a better oxidative stability with fewer NOx emissions (Bowen 2011). In a tropical country like the Philippines, this property for biodiesel is an advantage.

**Conclusions**. Crude lipid extraction from wet paste-like *Tetraselmis* sp. samples showed higher % recovery using Chloroform:Methanol (1:1, v/v) with sonication at 50 minutes together with the aid of soaking. Soaking the samples before and after the extraction improved the lipid yield by 0.65 % - 28 % compared to extraction without soaking. Fatty acid determination revealed that the dominant fatty acid for *Tetselmis* sp. is $C_{16}$ (Palmitic acid). The presence of other important fatty acids such as $C_{18:3\ n\text{-}3}$ (α-Linolenic acid) and $C_{20:5\ n\text{-}3}$ (Eicosapentanoic acid) was also observed. Fatty acid profile is not dependent on extraction solvent but crude lipid recovery varies with the solvent system.

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Authors:
Ida Go Pahila, University of the Philippines Visayas, College of Arts and Sciences, Department of Chemistry, Philippines, 5023, Iloilo, Miagao, e-mail: igpahila@gmail.com
Roland Joseph Cachopero Calawigan, University of the Philippines Visayas, College of Arts and Sciences, Department of Chemistry, Philippines, 5023, Iloilo, Miagao, e-mail: rjcalawigan@hotmail.com
Nieves Aquino Toledo, University of the Philippines Visayas, College of Fisheries and Ocean Sciences, Institute of Aquaculture, Philippines, 5023, Iloilo, Miagao, e-mail: ncatph@yahoo.com
Soledad Santos Garibay, University of the Philippines Visayas, College of Fisheries and Ocean Sciences, Institute of Aquaculture, Philippines, 5023, Iloilo, Miagao, e-mail: sgaribay005@yahoo.com
Caridad Novio Jimenez, University of the Philippines Visayas, College of Fisheries and Ocean Sciences, Institute of Fishery Policy and Development Studies, Philippines, 5023, Iloilo, Miagao, e-mail: cnjimz@yahoo.com
Leticia Jamelo Ami, University of the Philippines Visayas, College of Fisheries and Ocean Sciences, Institute of Fish Processing Technology, Philippines, 5023, Iloilo, Miagao, e-mail: letty_ami@yahoo.com
Rose Toledo Mueda, University of the Philippines Visayas, College of Fisheries and Ocean Sciences, Institute of Fish Processing Technology, Philippines, 5023, Iloilo, Miagao, e-mail: rtmueda@yahoo.com
Mellie June Santander Paulines, University of the Philippines Visayas, College of Arts and Sciences, Department of Chemistry, Philippines, 5023, Iloilo, Miagao, e-mail: mj.paulines@yahoo.com
Pilar Mercedes Azarcon Retiracion, University of the Philippines Visayas, College of Arts and Sciences, Department of Chemistry, Philippines, 5023, Iloilo, Miagao, e-mail: pemz_13@yahoo.com.ph
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