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The Effect of Extraction Methods on Fatty Acid and Carotenoid Compositions of Marine Microalgae *Nannochloropsis oculata* and *Chaetoceros gracilis*

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ABSTRACT

This study was conducted to assess three extraction methods for the determination of fatty acid compositions and carotenoids (lutein, zeaxanthin, β-carotene, and α-carotene) from marine microalgae, *Nannochloropsis oculata (NO)* and *Chaetoceros gracilis* (*CG*). For this purpose, three different extraction methods for the determination of fatty acids (dichloromethane:methanol, water:propan-2-ol:hexane and direct saponification-ethanol KOH) and carotenoids (hexane:ethanol:acetone:toluene, methanol:chloroform and methanol:tetrahydrofulran) were used. Two derivatization methods using different types of catalyst (acetyl chloride and boron trifluoride) were also used for the transmethylation of the fatty acids into corresponding methyl esters. The results of the fatty acid compositions showed that *NO* had a higher amount of n-3 and n-6 polyunsaturated fatty acid (PUFA), particularly eicosapentaenoic acid (EPA) (C20:5). *CG* was predominantly high in palmitic acid (C16:0) and palmitoleic acid (C16:1). The extraction method 1 (dichloromethane:methanol) and extraction method 2 (water: propan-2-ol: hexane) with acetyl chloride-catalyzed transmethylation were found to be the best methods for the determination of fatty acid compositions in *NO* and *CG,* respectively. A significantly higher ($P<0.05$) amount of carotenoids was found in *NO* as compared to *CG* using different extraction methods. Extraction method 1 (involving saponification procedure) yielded the best result for *NO* while extraction method 3 (methanol: tetrahydrofuran with no saponification procedure) generated higher amounts of carotenoids in *CG*. Overall, this study has shown that significantly high amounts of fatty acids and carotenoids could be obtained from these microalgae using these methods.

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INTRODUCTION

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms unified primarily by the lack of roots, leaves, and stems that characterize higher plants. They can be found almost anywhere, with water and sunlight as their fundamental requirements, including lakes, soils, rivers, hot springs, and the ocean. Microalgae contain high value compounds like fatty acids [γ- linolenic acid, arachidonic acid, eicosapentaenoic acid (EPA), docosahexaenoic acids (DHA), etc.], pigments (chlorophyll and carotenoids), vitamins (biotin, vitamins C and E, and others) (Converti *et al.,* 2009). *Chaetoceros gracillis*, a diatom in the class of Bacillariophycae and *Nannochloropsis occulata*, a unicellular green alga with spherical shape of the Eustigmatophyceae class plays an important role in the food chain system and it is also commonly used as live feed; thus, it is widely cultivated in fish hatcheries and shrimp farms (Gwo *et al.,* 2005).

DHA and EPA are constantly an area of interest in nutrition because they are essential for optimizing human health. DHA is important for the development of the brain and eyes in pre-term and young infants, as well as for supporting cardiovascular health in adults, whereas EPA is essential for the human metabolism and involved in the blood lipid equilibrium that prevents hypertriglyceridemia and anti-inflammatory activities (Kroes *et al.,* 2003; Ward & Singh, 2005; Fajardo *et al.,* 2007). Previously, fish was the principal dietary source of DHA and EPA. However, due to the serious environmental consequences and continuous exploitation, the declining sources of marine fish stocks and fish oil have prompted research into new sources of polyunsatured fatty acids (PUFAs) (Burja *et al.,* 2007). In addition, certain disadvantages of fish oil, such as the unpleasant odour, possible pollutants, and mixed fatty acid properties have also encouraged the search for alternative sources of PUFAs (Pulz & Gross, 2004).

In addition, microalgae contain a multitude of pigments, particularly chlorophyll and carotenoid. Carotenoids are essential to human health and important in commercial applications (Felti *et al.,* 2005). For example, β-carotene acts as pro-vitamin A and has been proven to prevent xeropthlamia (Puah *et al.,* 2005); astaxanthin acts as a natural colorant for muscle in marine fish and crustaceans; lutein, zeaxantin and canthaxantin for chicken skin coloration, pharmaceutical purposes, and also as a natural food additive (Del Campo *et al.,* 2000; Pulz & Gross, 2004).

Although microalgae contain important bioactive components (particularly PUFA and carotenoids), the extraction methods, especially for algae, are not well established, as there are no standard extraction methods for the determination of the fatty acid content or carotenoids in microalgae (Wiltshire *et*

al., 2000). Lipids are mainly a mixture of esters, and therefore, the preparation of fatty acid methyl esters (FAME) consists essentially on the conversion of one ester to another (i.e. transesterification) by cleavage of an ester bond via an alcohol; when such an alcohol is methanol, the reaction is referred to as methanolysis or transmethylation (Liu, 1994). Transmethylation are reversible reactions which are normally accomplished in the presence of a catalyst, either an acid or a base. Reactions involving acidic catalysts require heat to accelerate the process. Commonly used acidic catalysts are (the Brønsted-Lowry acid) HCl, H_2SO_4, acetyl chloride and (the Lewis acid) BF3. Base-catalyzed methanolysis proceeds much more rapidly under mild temperature conditions than acid-catalyzed reactions. However, bases cannot catalyze the esterification of FFAs.

The nutritional value of microalgae is strongly dependent on its bioactive profile; therefore, methods that could get the highest value is preferred. Felti *et al.* (2005) suggested that the absence of a standard extraction method for carotenoid is actually attributed to the wide spectrum of the analyzed materials (foodstuff, plant, animal, and human samples) and the wide range of the carotenoids present. As a result, three extractions methods of fatty acid and carotenoids from marine microalgae, *Nannochloropsis oculata* and *Chaetoceros gracilis* were evaluated in the current study. The criteria used in choosing these methods were maximum extraction efficiency, ease of handling, and use of solvents of low toxicity.

MATERIAL AND METHODS

Microalgae Samples

Both the *Nannochloropsis oculata* and *Chaetoceros gracilis* samples were purchased from Reed Mariculture Inc. USA. The samples were collected using non-probability, convenient sampling method. Both the microalgae were purchased in two batches and prior to the extraction, these samples were freeze-dried, homogenized, and kept at -20^{o}C until further use.

Oil Extraction

The first extraction method used was adopted from Cequier-Sanchez *et al.* (2008). First, 500 mg of the samples were extracted by mixing 15 ml of dichloromethane-methanol 2:1 (v/v) contained in a beaker. The mixing was performed with occasional gentle hand agitation for 2 hours. Subsequently, the samples were filtered and transferred into a new test tube to which 3.13ml of an aqueous solution of potassium chloride (0.88%, w/v) was added with strong agitation, followed by centrifugation (Universal 320/320R Benchtop Centrifuges, Hettich Instruments, Germany) at 350g at 4^{0}C for 5 minutes. The aqueous upper phase was discarded and the organic phase was evaporated using a rotary evaporator (Büchi Rotavapor R-200, Switzerland).

The second extraction method was adopted from Schlechtriem *et al.* (2003) with a slight modification, in which hexane was used to substitute the cyclohexane. First, 500 mg samples were weighed into the Falcon tubes and mixed with 10ml of

propan-2-ol and 12.5 ml of hexane using a vortex for 30s. The tubes were placed in an ultrasonic bath at room temperature for 15 minutes. Then, 13.75ml of water was added to obtain a mixture of water: propan-2-ol:hexane (11:8:10 v/v/v). The mixture was mixed again using the vortex for 30s. The different phases were separated by centrifugation at 1800g for 10 minutes and the organic phase was transferred into a pre-weighed Flacon tube with a dropper. The organic phase containing the lipid fraction was separated at the top of the extraction mixture (the hexane phase). Subsequently, the second extraction with 12.5ml of hexane containing 13% v/v propan-2-ol was done. The mixture was vortexed and placed into the ultrasonic bath for another 15 minutes. After centrifugation, the hexane phase was added to the first extract. The tubes were placed in a water bath (50^0C) for about 15 minutes and the solvent was evaporated to dryness using a rotary evaporator.

The third extraction method was adopted from Burja *et al.* (2007) with a minor modification, in which 95% ethanol was used instead of 96% ethanol. First, the 500mg samples were weighed. Then, 38ml of 3mM potassium hydroxide in ethanol (95%) was added into a 150ml beaker. The beaker was passed under the flow of nitrogen, and shaken for 1 hour in a water bath set at 60°C. Thereafter, the samples were cooled to room temperature and filtered through filter paper. The biomass was washed with 10ml of ethanol and transferred into a new beaker, to which, 10ml of water was added. Unsaponifiables were extracted by adding 20ml of hexane and gently mixing twice. After the layers were separated, the pH was adjusted to 1 (from pH 13-14) by the addition of hydrochloric acid:water (1:1, v/v). The top layer, containing the fatty acid fraction, was recovered by two rounds of the addition of 10ml of hexane and a gentle mixing. Lastly, the solvent at the top layer was evaporated to dryness using a rotary evaporator.

Preparation of Fatty Acid Methyl Esters (FAMEs)

This acetyl chloride-catalyzed transmethylation method was adopted from Carvalho and Malcata (2005). The lipid extracts (2mg) were subjected to acid-catalyzed transesterification by dissolving them in 2ml of a freshly prepared mixture of acetyl chloride and methanol at a ratio of 5:100 (v/v), together with 1mg of tricosanoic acid as an internal standard. The reagents were placed in Teflon-capped Pyrex tubes, and the reaction continued at 100°C for 1 hour under pure nitrogen and darkness. After cooling to 30~40°C, 1ml of the extracting solvent (isooctane containing 0.01% butylated hydroxytoluene, BHT) was added, and the FAME solvent solution was mixed using a vortex for between 5 to 30s. The purification of the solution was achieved by adding 1ml of water, causing the formation of two immiscible phases, which were then allowed to separate. Subsequently, the upper extracted solvent phase was recovered and stored in sealed glass vials at -20^0C until GC analysis.

The BF3-catalyzed transmethylation

method was adopted from Carvalho and Malcata (2005), which was modified by the use of 10% (v/v) of BF3 in methanol instead of 12% (v/v) of BF3 in methanol. First, all the lipid extracts (2mg) were subjected to a preliminary alkaline hydrolysis with 0.5M sodium hydroxide at 100^{0}C for 5 minutes. Subsequently, it was dissolved in 2ml of 10% (v/v) BF3 in methanol, together with 1mg of tricosanoic acid as an internal standard. The reagents were placed in the Teflon-capped Pyrex tubes, and the reaction was allowed to continue at 100°C for 30 minutes under pure nitrogen and darkness. The subsequent procedure was similar to the acetyl chloride-catalyzed transmethylation procedure, as described above.

Gas Chromatography Analysis

The assay of FAME was analyzed using gas chromatography (Agilent 6890, ISA Agilent Tech, USA) equipped with a split/splitless injector, and Hewlett Packard EL-980 flame ionization detector (FID). The FID system was used to separate and quantify each FAME component. FAME was separated using DB-23 column (60m x 0.25mm ID, and 0.15 µm). The chromatography data were recorded and integrated using the chemistation software (version 6). The oven temperature was programmed to hold at 50^{0}C for 1 min, before it was increased to 175^{0}C with 25^{0}C/min, held for 4 minutes, and lastly increased to 230^{0}C with 4^{0}C/min and held for 5 mins. The temperature for the injector and detector was set at 250^{0}C and 280^{0}C, respectively. One microlitre of the sample volume was injected with a split ratio of 1:50µl and a column temperature of 110^{0}C. The carrier gas was helium gas (1.0 ml/min) which was controlled at 123.4kPa/Hz and the air used for FID was held at 275.6kPa.

Calculation of Fatty Acid

The identification of the fatty acid compositions for the sample was made by comparing the retention time of the sample FAMEs with those of Supelco 37 component FAMEs mixture (Sigma-aldrich, USA) for each chromatography peak. The quantification of the fatty acid was done using tricosanoic acid (C23:0) as an internal standard. The amount of the individual fatty acid was calculated using the expression: $C_i = C_p (A_i/A_p)$, where A is the chromatographic area units and C is the amount of fatty acid. Subscript p represents the internal standard and i refers to any fatty acid. The percentage of the individual fatty acid in the total amount of fats used was calculated as C_i/total amounts of fat x 100%.

Carotenoids Extraction

The first extraction method was adopted from Inbaraj *et al.* (2006). First, 400mg of freeze-dried microalgae was mixed with 12 ml hexane-ethanol-acetone-toluene (10:6:7:7 v/v/v/v) in a volumetric flask. After shaking for 1 hour, 0.8ml 40% methanolic potassium hydroxide was added and the solution was saponified at 25^{0}C in the dark for 16 hours. Then, 12ml of hexane was added to partition the carotenoids. The mixture was shaken for 1 min and 10% sodium sulfate solution was added. After

shaking for 1 minute, the upper layer was collected and the lower layer was repeatedly extracted twice with hexane. Finally, the upper extracts were pooled and evaporated to dryness using a rotary evaporator.

The second extraction method was adopted from Reboul *et al.* (2006). First, 400mg of frozen-dried microalgae was added into 8ml of methanol containing 0.57% magnesium carbonate. Subsequently, the samples were homogenised for 30s using a vortex. Then, 8ml of chloroform, containing 0.005% butylated hydroxyl toluene (BHT), was added. The samples were homogenized for 30s more in the vortex blender. After a rest of 15 minutes, 8ml of distilled water was added into the samples and centrifuged (2000g for 10 minutes). The lower phases of the samples were collected and the remaining upper phases were extracted by the addition of 6ml of tetrahydrofuran. After that, the mixture was vortexed for 30s, and 6 ml of dichloromethane was also added. It was then vortexed for another 30s, after which 4ml of distilled water was added and the mixture was further re-vortexed for 30s. After centrifugation (2000g for 10 minutes at room temperature), the lower phase was collected and pooled with the previously collected phase. Lastly, the collected lower phase solvent was evaporated to dryness using a rotary evaporator.

The final extraction method was adopted from Marinova and Ribarova (2007). First, the pigments were extracted from a 400mg sample to which 0.04 g magnesium carbonate was added, with 6ml extraction solvent methanol:tetrahydrofuran (1:1, v/v) containing 0.1% butyl hydroxytoluene (BHT). The mixture was vortexed for about 3 minutes, and then centrifuged for 3 minutes at 1400g and the supernatant was collected. The pellet was re-extracted following the same procedure until the supernatant became colourless. The combined supernatants were evaporated to dryness using a rotary evaporator.

All the extraction procedures were performed under subdued light to avoid degradation loss of the pigments. The residue was dissolved in methanol at a concentration of 100mg/ml. Prior to the HPLC analysis, the sample solution was filtered using a Whatman polytetrafluroethylene (PTFE) 0.22µm syringe filter and the filtrate was injected into a HPLC valve with a 1ml syringe.

Analysis of Carotenoids

The carotenoids were analyzed by using HPLC (Agilent Series 1100, Model G131 3A, Agilent Technologies, Germany) equipped with degasser, quaternary pump, auto sampler and photodiode array detector. The carotenoids were separated by HPLC using a 150 × 4.6 mm, 3 µm C30 analytical column (Waters Co., Milford, MA, USA). The mobile phase system comprised methanol–methyl tert-butyl ether (MTBE) – water (81:15:4 v/v/v) (A) and methanol/ MTBE (10:90 v/v) (B) in the following gradient conditions 100% of A and 0% B, to 50% A and 50% B in 45 minutes, followed by 100% B within 15 minutes. The column temperature was set at 25^{0}C. The volume

injected into the HPLC was set as 20µl and the flow rate during the separation was set as 1ml/min. The wavelength used for the photodiode array detector in measuring the carotenoids was 450nm. The elution time was 45 minutes for a sample and the post time was 5 minutes.

The standard for the carotenoids were prepared from a stock solution of β-carotene, α-carotene, zeaxanthin and lutein. The identification of carotenoids was made by comparing with these standards, and the spiking test was also carried out to confirm the identification of certain peaks. The carotenoids were quantified using a calibration curve that was prepared using pure standards in the range of 0.025-5µg/ml.

Data Analysis

The computer software Statistical Package for Social Sciences version 16 (SPSS 16) was used to analyze the data in this study. The analysis was done in triplicates and the results were expressed as mean ± standard deviation. Meanwhile, the two-way ANOVA was used to compare the differences in the mean amounts of fatty acid and carotenoid from the microalgae, *Nannochloropsis oculata* and *Chaetoceros gracilis* using various extraction methods and also two different transmethylation methods (fatty acid only). The analysis was considered at a significance value of $p<0.05$.

RESULTS AND DISCUSSION

Percentage of Extraction Yields (Total Oil)

As shown in Table 1, extraction method 1 (dichloromethane:methanol) showed the highest amount of the extraction yields for both *NO* and *CG*, with the mean values of 48.61% and 36.81%, respectively. Extraction method 3 using direct saponification with ethanolic KOH gave the least amount of extraction yields for both *NO* and *CG*, with the percentage value of 10.45% and 14.67%, respectively. A two-way ANOVA was conducted to examine the sample differences and the extraction methods on the extraction yields. There was a significant interaction between the various methods used and the extraction yields, $p = .027$. However, no significant differences were seen between the two microalgae in the extraction yield. The higher extraction yields of method 1 (dichloromethane: methanol) might be attributed to the presence of methanol,

TABLE 1
Total oil yield using different extraction methods from microalgae *Nannochloropsis oculata (NO)* and *Chaetoceros gracilis (CG)*

Extraction methods	*Nannochloropsis oculata (NO)*	*Chaetoceros gracilis (CG)*
Method 1(Dichloromethane:methanol)	486 ± 236[a]	368 ± 56[a]
Method 2(Water: propan-2-ol: hexane)	366 ± 151[a]	316 ±18[a]
Method 3 (Direct saponification-ethanol)	105 ± 14[b]	147 ± 21[a]

Each value is the mean ± standard deviation of triplicates expressed as g kg^{-1}dry weight. Within a column, means followed by the same letter are not significantly different ($p>0.05$).
No significant differences were observed between *NO* and *CG*.

a primary alcohol with the most active hydroxyl group (highly polar), which could stimulate the disruption of hydrogen bonds between lipid carbonyl, hydroxyl, and the amino groups, and the compounds of the nonextractable residue (Ruiz-lopez *et al.,* 2003).

Fatty Acid Composition of Nannochloropsis Oculata (NO) and Chaetoceros Gracilis (CG) Using Different Extraction Methods

In terms of the extraction efficiency of the fatty acid content (weight %), extraction method 1 (dichloromethane: methanol) coupled with acetyl chloride catalyzed transmethylation appeared to be the most efficient method for *NO*, as compared to other methods (Table 2). This was because this particular method could generate higher fatty acid content than other methods using both the acetyl chloride and BF3-catalyzed transmethylation methods. Since *NO* consists of a polysaccharide cell wall, the solubility of its cell matrix towards the non-polar solvent may enable the penetration of solvents into it and subsequently allow the oil to dissolve and be extracted for transmethylation. In addition, this method is also simpler and easier in its procedure as compared to two other methods. Indeed, a previous study has shown that dichloromethane, a less hazardous solvent, was an effective extraction solvent for fatty acid research (Cequier-Sanchez, 2008).

Nevertheless, extraction method 2 (water:propan-2-ol:hexane), coupled with acetyl chloride catalyzed transmethylation, appeared to be the most suitable method for the determination of fatty acid for *CG*, particularly C16 and C18 fatty acids (Table 3). The use of additional cell disruption treatment (ultrasonic bath) in this extraction procedure was notably useful in *CG*, a genus of diatoms, as they have the unique characteristic of a silica-based rigid cell wall, which may be difficult to break (Scala *et al.,* 2002). The use of an ultrasonic bath was related to the destruction of cell walls and the enhancement of mass-transfer through the cell wall due to the collapse of the bubbles produced by cavitation (Macias-Sanchez *et al.,* 2009). In this way, its extraction efficiency could be enhanced. Moreover, the use of this particular combination of solvents was also highly recommended in terms of its safety, low toxicity, and low cost (Smedes, 1999). Although hexane was used to substitute cyclohexane in this method, their almost similar properties would not create much difference in the result.

Among the three extraction methods used, method 3, which involved a direct saponification using ethanolic potassium hydroxide, gave the least number and amount (weight %) of fatty acid compositions in both the acetyl chloride and BF3-catalyzed transmethylation methods. This result disagrees with previous study which claimed that this method was an efficient technique to increase the extraction of fatty acid from biomass (Burja *et al.,* 2007). However, a study by Wang *et al.* (2000) found a lower concentration of fatty acids on chicken egg yolk by using the direct saponification extraction method compared to other methods (direct-methylation, chloroform-

TABLE 2
Fatty acid composition of *Nannochloropsis oculata (NO)* using different extraction and derivatization transmethylation) methods

Fatty Acid Composition	*Nannochloropsis oculata (NO)*					
	Acetyl chloride- catalyzed transmethylation			BF3-catalyzed transmethylation		
	Method 1	Method 2	Method 3	Method 1	Method 2	Method 3
C8:0	2.78 ± 0.72^{a}	ND	ND	1.60 ± 0.06^{b}	1.78 ± 0.31^{b}	ND
C12:0	2.25 ± 0.14	ND	ND	ND	ND	ND
C13:0	9.04 ± 0.11^{a}	7.52 ± 0.03^{b}	1.80 ± 0.11^{c}	1.91 ± 0.07^{d}	1.10 ± 0.02^{e}	ND
C14:0	25.62 ± 0.23^{a}	12.13 ± 0.09^{b}	ND	7.40 ± 0.04^{c}	2.98 ± 0.10^{cd}	1.43 ± 0.07^{d}
C14:1	ND	ND	ND	ND	ND	3.22 ± 0.05
C16:0	159 ± 1.56^{a}	76.12 ± 0.23^{b}	16.47 ± 0.17^{c}	60.78 ± 0.48^{d}	18.91 ± 0.1^{e}	9.62 ± 0.05^{c}
C16:1	198 ± 0.61^{a}	81.32 ± 1.32^{b}	14.13 ± 0.12^{c}	71.61 ± 0.47^{d}	16.79 ± 0.2^{e}	31.36 ± 0.39^{f}
C17:0	4.44 ± 1.51^{a}	4.99 ± 0.18^{a}	ND	ND	ND	ND
C17:1	6.67 ± 0.26^{a}	2.50 ± 0.06^{a}	ND	2.04 ± 0.08^{b}	ND	ND
C18:0	7.26 ± 0.28^{a}	8.91 ± 0.23^{b}	2.65 ± 0.31^{c}	4.24 ± 0.15^{d}	1.96 ± 0.08^{e}	ND
C18:1n9trans	ND	ND	ND	ND	ND	ND
C18:1n9cis	59.86 ± 0.83^{a}	42.71 ± 0.29^{b}	5.67 ± 0.13^{c}	19.16 ± 0.23^{d}	7.51 ± 0.40^{e}	3.27 ± 0.10^{f}
C18:2n6cis	43.86 ± 0.58^{a}	30.12 ± 0.12^{b}	3.79 ± 0.20^{c}	14.84 ± 0.11^{d}	6.65 ± 0.17^{e}	2.96 ± 0.10^{f}
C18:3n6	6.76 ± 0.39^{a}	03.04 ± 0.03^{b}	ND	ND	ND	6.27 ± 0.18^{c}
C18:3n3	2.23 ± 0.37^{a}	ND	ND	ND	ND	ND
C20:4n6	34.15 ± 0.54^{a}	12.1 ± 0.40^{b}	ND	10.86 ± 0.03^{c}	1.36 ± 1.18^{d}	0.88 ± 0.76^{d}
C20:5	351 ± 3.37^{a}	152 ± 0.11^{b}	19.27 ± 0.23^{c}	106 ± 0.81^{d}	34.28 ± 0.24^{e}	15.59± 0.15^{c}

Method 1: (dichloromethane: methanol), Method 2: (water:propan-2-ol:hexane), Method 3: (Direct saponification- ethanolic KOH).
Each value is the mean ± standard deviation of triplicates expressed as g $kg^{-1}$of the total oil.
Within a row, means followed by the same letter are not significantly different (p>0.05).
ND = not detected

TABLE 3
Fatty acid composition of *Chaetoceros gracilis (CG)* using different extraction and derivatization (transmethylation) methods

Fatty Acid Composition	*Chaetoceros gracilis (CG)*					
	Acetyl chloride transmethylation			BF3-catalyzed transmethylation		
	Method 1	Method 2	Method 3	Method 1	Method 2	Method 3
C8:0	3.12 ± 0.62^{a}	ND	ND	1.41 ± 0.27^{b}	0.67 ± 0.59^{b}	0.87 ± 0.76^{b}
C12:0	2.03 ± 2.04	ND	ND	ND	ND	ND
C13:0	5.83 ± 103^{a}	14.46 ± 0.07^{b}	0.93 ± 0.81^{c}	2.26 ± 0.09^{d}	1.50 ± 0.22^{d}	ND
C14:0	77.03 ± 0.10^{a}	80.70 ± 0.55^{a}	11.45 ± 5.57^{b}	19.73 ± 0.10^{c}	47.34 ± 0.19^{d}	4.04 ± 0.11^{e}
C14:1	2.42 ± 0.11^{a}	2.33 ± 0.02^{a}	ND	0.93 ± 0.04^{b}	1.60 ± 0.10^{c}	ND
C16:0	60.60 ± 1.78^{a}	129 ± 0.47^{b}	13.52 ± 7.60^{c}	12.13 ± 0.12^{d}	70.38 ± 0.13^{e}	8.50 ± 0.10^{c}
C16:1	107 ± 0.28^{a}	103 ± 0.88^{a}	19.29 ± 7.38^{b}	25.71 ± 0.55^{c}	92.70 ± 0.09^{d}	6.54 ± 0.17^{e}
C17:0	20.38 ± 0.34^{a}	18.89 ± 0.29^{ab}	3.50 ± 1.59^{c}	4.80 ± 0.06^{d}	17.95 ± 0.07^{b}	1.33 ± 0.14^{e}
C17:1	28.72 ± 0.22^{a}	25.55 ± 4.34^{a}	6.28 ± 2.36^{b}	6.55 ± 0.25^{c}	22 ± 0.20^{b}	2.14 ± 0.06^{c}
C18:0	8.13 ± 0.04^{a}	20.95 ± 0.43^{b}	ND	1.64 ± 0.03^{c}	12.52 ± 0.12^{d}	ND
C18:1n9trans	ND	5.68 ± 0.28^{a}	ND	ND	6.62 ± 0.14^{b}	ND
C18:1n9cis	11.80 ± 0.18^{a}	173 ± 0.29^{b}	5.79 ± 0.70^{c}	ND	3.38 ± 0.19^{d}	2.42 ± 0.04^{e}
C18:2n6cis	7.76 ± 0.09^{a}	121 ± 0.21^{b}	3.08 ± 0.18^{c}	1.05 ± 0.02^{d}	5.13 ± 0.15^{e}	1.59 ± 0.11^{d}
C18:3n6	ND	ND	ND	ND	1.22 ± 0.05^{a}	ND
C18:3n3	ND	4.64 ± 0.10^{a}	ND	ND	ND	ND
C20:4n6	7.83 ± 8.74^{a}	ND	ND	ND	2.44 ± 0.11^{b}	ND
C20:5	18.05 ± 0.35^{a}	14.93 ± 0.28^{ab}	3.82 ± 3.32^{c}	3.52 ± 0.06^{d}	17.45 ± 0.12^{b}	ND

Method 1: (dichloromethane: methanol), Method 2: (water:propan-2-ol:hexane), Method 3: (Direct saponification- ethanolic KOH).
Each value is the mean ± standard deviation of triplicate expressed as g $kg^{-1}$of total oil.
Within a row, means followed by the same letter are not significantly different (p>0.05).
ND = not detected.

methanol extraction, and postextraction saponification), although the reason was unknown.

Acetyl chloride-catalyzed transmethylation generated a higher amount (weight %) of fatty acid compared to BF3-catalyzed transmethylation in both the microalgae. This might be due to the highly basic condition of acetyl chloride, which could cause severe disruption to cell integrity, making *in situ* methyl ester derivation efficient (Tran *et al.,* 2009). Furthermore, the use of acetyl chloride-catalyzed transmethylation procedure has several advantages as compared to the most commonly performed methanolic BF3 method, such as longer shelf-life (without the need for refrigeration), lower cost, and smaller amount of catalyst required (5% acetyl chloride versus 10% BF3) (Carvalho & Malcata, 2005).

Percentage of Extraction Yields (Carotenoids)

Table 4 shows that extraction method 1 (he xane:ethanol:acetone:toluene) generated the highest extraction yields for both *NO* (74.54 ± 4.75%) and *CG* (69.28 ± 14.71%). This was probably due to the longer period of contact time (1 hour) between the cellular component to be extracted and the solvent mixtures in extraction method 1 as compared to the other two methods (Henriques *et al.*, 2007). The two-way ANOVA showed significant differences between the samples ($p = 0.022$) on the extraction yields. Overall, it could be seen that all the extraction methods used generated higher extraction yields in *NO* than in *CG*. However, the difference in the extraction yields was small among these microalgae, particularly between extraction methods 2 and 3.

Carotenoids Concentration of the Different Extracts of Nannochloropsis Oculata (NO) and Chaetoceros Gracilis (CG)

As shown in Table 5, β-carotene was found to be the highest, followed by zeaxanthin, α-carotene, and lutein in the *NO* using different extraction methods. However, extraction methods 1 and 2 were the only methods that could detect lutein

TABLE 4
Extraction yield of carotenoids using different extraction methods from microalgae *Nannochloropsis oculata (NO)* and *Chaetoceros gracilis(CG)*

Extraction methods	*Nannochloropsis oculata (NO)*	*Chaetoceros gracilis (CG)*
Method 1 (Saponification) (hexane:ethanol:acetone:toluene)	745 ± 48[a]	693 ± 147[a]
Method 2 (No saponification) (methanol:chloroform)	682 ± 25[a]	515 ±12[b]
Method 3 (No saponification) (methanol:tetrahydrofuran)	636 ± 39[a]	502 ± 25[a]

Each value is the mean ± standard deviation of triplicates expressed as g kg^{-1}dry weight.
Within a row, means followed by the same letter are not significantly different ($p>0.05$).
No significant differences were observed between the 3 extraction methods.

and α–carotene in *NO*, respectively, while zeaxanthin was not detected in extraction method 2. For zeaxanthin and β-carotene contents in *NO*, extraction method 1, which involved the saponification step, generated the highest concentration (µg/100g dry weight) as compared to the other methods. The functions of saponification include hydrolyzing the carotenoid esters and removing the chlorophyll and unwanted lipids on microalgae, which may interfere with chromatographic separation (Howe *et al.*, 2006). Since microalgae were high in their lipid content, saponification was necessary to achieve better results (better identification and higher concentration) as compared to the other two methods, which do not employ saponification.

Just like *NO*, *CG* was found to be the highest in the amount (g/kg dry weight) of β-carotene, followed by lutein and zeaxanthin using different extraction methods. However, α-carotene was not detected in *CG* with either of these extraction methods. This does not indicate the absence of α-carotene in *CG* because the failure to detect it might be due to other possible reasons such as the presence of light and oxygen while handling the samples or storing that would have contributed to its degradation. As shown in Table 6, extraction method 2 was the only method that could not

TABLE 5
Carotenoid concentrations (g kg^{-1} dry weight) of *Nannochloropsis oculata (NO)* using different extraction methods

Carotenoids	Method 1 (Saponification) (hexane:ethanol:acetone: toluene)	Method 2 (No saponification) (methanol : chloroform)	Method 3 (No saponification) (methanol: tetrahydrofuran)
Lutein	1.55 ±0.01	ND	ND
Zeaxanthin	3.74 ± 0.03^{a}	ND	2.67 ± 0.08^{b}
β-carotene	9.58 ± 0.002^{a}	9.46 ± 0.05^{b}	8.69 ± 0.04^{c}
α-carotene	ND	2.16 ± 0.004	ND

Each value is the mean ± standard deviation of triplicates expressed as g kg^{-1} dry weight.
Within a row, means followed by the same letter are not significantly different ($p>0.05$).
ND = not detected.

TABLE 6: Carotenoid concentrations (g kg^{-1}dry weight) of *Chaetoceros gracilis (CG)* using different extraction methods

Carotenoids	Method 1 (Saponification) (hexane:ethanol:acetone: toluene)	Method 2 (No saponification) (methanol:chloroform)	Method 3 (No saponification) (methanol: tetrahydrofuran)
Lutein	1.33 ± 0.003^{a}	ND	1.57 ± 0.02^{b}
Zeaxanthin	0.58 ± 0.01^{a}	0.75 ± 0.003^{b}	8.68 ± 0.02^{c}
β-carotene	7.945 ± 0.002^{a}	ND	8.08 ± 0.01^{b}
α-carotene	ND	ND	ND

Each value is the mean ± standard deviation of triplicates expressed as g kg^{-1}dry weight.
Within a row, means followed by the same letter are not significantly different ($p>0.05$).
ND = not detected.

detect the presence of lutein and β-carotene in *CG*. Hence, extraction method 2 was less suitable for the determination of carotenoid for *CG* as compared to extraction methods 1 and 3. However, extraction method 3 could generate a higher concentration of carotenoids (g/kg dry weight) compared to extraction method 1. In the present study, the saponification step in extraction method 1 might not have much impact on carotenoids determination of *CG* since the absence of the saponification step in extraction method 3 yielded a better result for carotenoids.

Moreover, the different cell matrix of these microalgae might have contributed to the difference in the concentrations of carotenoid in them. As described earlier in the determination of the fatty acid composition, the cellular structure of *NO* was distinctly different to *CG*. Hence, *CG* with its unique characteristic of a silica-based rigid cell wall might cause incomplete extraction of the biochemical compounds by the solvents alone, without any additional treatment (e.g. ultrasound bath, enzymes, microwave-assisted, etc.). This is in accordance with a published study, whereby an efficient disruption treatment of the membrane was required in order to achieve the efficient extraction of carotenoids as there was no standard technique can guarantee a maximization of the extraction yield (Valduga *et al.*, 2009). Unlike *CG*, the polysaccharide cell wall of *NO* might also be easier to penetrate using compatible solvents, and subsequently allow the extraction of the desired biochemical compounds.

CONCLUSION

The comparison of various extraction methods on both fatty acids and carotenoids revealed that they produced extracts with different characteristics as well as quantitative differences. For fatty acid determination, the utilization of method 1 (dichloromethane:methanol) appeared to be the most efficient method for *NO*. Nevertheless, extraction method 2 (water:propan-2-ol:hexane), which involved additional treatment (ultrasonic bath), appeared to be a more suitable method for fatty acid determination in *CG*. As for carotenoids, extraction method 1, which uses the saponification step to remove chlorophyll, unwanted lipids and the involvement of more solvent mixtures (2 polar and 2 non-polar solvents), generated the highest concentration (µg/100g dry weight) in *NO*. However, extraction method 3 generated the highest concentrations in *CG*. Overall, this study has shown that using the right extraction method, high amounts of fatty acids and carotenoids could be obtained from the microalgae.

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