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A study of fatty acid composition and tocopherol content of lipid extracted from marine microalgae, *Nannochloropsis oculata* and *Tetraselmis suecica*, using solvent extraction and supercritical fluid extraction

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Abstract

This study was conducted to investigate and compare the fatty acids and tocopherols of lipid extracted from marine microalgae, *Nannochloropsis oculata* (NO) and *Tetraselmis suecica* (TS) using solvent extraction and supercritical fluid extraction (SFE). Fatty acids and tocopherols were determined in the extracted lipid as functions of the temperature (40, 80°C) and pressure (3000, 5000, 7000, 9000psi). Dichloromethane/methanol and hexane were the chosen conventional solvent for fatty acids and tocopherols extraction respectively. The results obtained showed that there were differences in the fatty acid composition of various lipid extracts of NO and TS. Extracts of NO were high in myristic acid (C14:0) (17-35%), palmitic acid (C16:0) (14-47%) and palmitoleic acid (C16:1) (11-42%) whereas extracts of TS were high in C14:0 (21-34%) and C16:0 (29-49%). Eicosapentaenoic acid (EPA) was detected only under certain SFE conditions in NO but was not detected in TS. α-, β- and γ-tocopherol were detected in various SFE extracts of NO but only α- and β-tocopherol were detected in TS. Hexane extraction of both NO and TS resulted in the detection of only α-tocopherol. In conclusion, the use of different extraction methods resulted in different compositions and concentrations of fatty acids and tocopherols in the microalgae studied.

Introduction

Microalgae, which comprise of all the eukaryotic photosynthetic microorganisms, have been used by the indigenous populations for centuries. In recent years, they have been studied for numerous commercial applications, ranging from animal and human nutrition to cosmetic products (Spolaore *et al*., 2006; Mendes, 2007). They contain rich amount of high value compounds such as fatty acids especially polyunsaturated fatty acids (PUFAs) and to a lesser extent vitamins, therefore, unleashing their potential as functional food ingredients. *Nannochloropsis oculata* (NO) and *Tetraselmis suecica* (TS) are both microalgae which have been used as aquatic feed since long ago and have been reported to contain valuable amount of eicosapentaenoic acid (EPA) and vitamin E, respectively (De Roeck-Holtzhauer *et al*., 1991; Hodgson *et al*., 1991).

The conventional source of EPA and docosahexaenoic acid (DHA) – marine-derived oily fish, are exposed to risk of being polluted with heavy metals, leading to consumption of fish in high amount for these health beneficial PUFAs may no longer be a wise choice. Besides, vegetarianism is gaining popularity worldwide, be it due to health benefits, religious, cultural, economic or ecological factor. This type of dietary practice has limited EPA and DHA intake from animal sources, especially from rich sources such as fish, and the consumer would depend largely on the limited conversion of α-linolenic acid (ALA) from vegetable oil and seeds into EPA and DHA in the body. It has been reported that the conventional sources of vitamin E which consist of higher plant materials normally contain low concentration of tocopherol with high proportion of less active tocopherol homologues. Studies have also suggested that intake of synthetically produced vitamin E has less beneficial effects compared with those from natural sources (Mendiola *et al*., 2008; Ogbonna, 2009). In addition, the used of conventional extraction methods to extract bioactive compound is

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not only time consuming, laborious, low selectivity and/or low extraction yield, but also uses large amounts of toxic solvents which present hazardous pollution problem to the environment (Herrero *et al*., 2006).

Recognising the problems aforementioned and the potential of microalgae in providing valuable amount of PUFAs and vitamin E coupled with agreement in that an environmental friendly extraction method is currently the direction forward among the food industries, therefore, this study was conducted to investigate and compare the fatty acids and tocopherols of lipid extracted from marine microalgae, NO and TS, using solvent extraction and the environmental friendly supercritical fluid extraction (SFE).

Materials and Methods

Microalgae samples

Two samples of microalgae were used, *Nannochloropsis oculata* and *Tetraselmis suecica*. *Nannochloropsis oculata* was purchased from Reed Mariculture Inc. USA whereas *Tetraselmis suecica* was a gift from Marine Science Research Centre, Universiti Putra Malaysia (UPM), Port Dickson. The samples were collected using non-probability, convenient sampling method. Prior to extraction, these samples undergone freeze-drying, homogenization process and then kept in freezer at -20^{0}C.

Instrumentation

Gas chromatography (GC) (Agilent Technologies; HP Agilent 6890), Ultra Performance Liquid Chromatography (UPLC) (Agilent Technologies; Agilent 1290 Infinity LC system) and Supercritical carbon dioxide extractor (Supercritical Fluid Technologies; SFT-100)

Chemicals

Dichloromethane (Fisher Scientific, UK), methanol (Fisher Scientific, UK), potassium chloride (KCI) (HmbG Chemicals, Germany), butylhydroxytoluene (BHT) (Sigma, USA), sodium hydroxide (NaOH) (Sigma-Aldrich, USA), 12% boron trifluoride/methanol complex (BF_3/MeOH) (ACROS ORGANICS, Belgium), isooctane (Fisher Scientific, UK), sodium chloride (NaCI) (Merck, Germany), ethanol (HmbG Chemicals, Germany), ascorbic acid (Sigma, USA), hexane (J.T. Baker, USA), HPLC grade hexane (Fisher Scientific, UK), HPLC grade isopropanol (Fisher Scientific, UK), 37 FAMEs (Supelco, USA), tocopherols standard (CALBIOCHEM, Germany), nitrogen gas (SIG, Malaysia), and liquid carbon dioxide (MOX, Malaysia)

Supercritical fluid extraction (SFE)

A laboratory-scale supercritical fluid extraction system was used for this study. For each experiment, the extraction vessel was filled with 500 mg of microalgae. The CO_2 with 99.8% purity was fed into the extractor with fixed solvent flow rate of 5-6 ml/min and the extraction time was fixed to 4 hours (4 cycles of 40 minutes static extraction followed by 20 minutes of dynamic extraction). The pressure within the extraction vessel was built up with constant CO_2 flow rate at 24 ml/min and regulated by automated back pressure regulator. The supercritical fluid was initiated after the desirable temperature and pressure were achieved. After the extraction was completed, the CO_2 was depressurized and the oil was collected. The extraction yield was determined gravimetrically by measuring the weights of the collection vials before and after extract collection. The extract obtained was divided into two portions and further analysis was done following the procedure of fatty acid analysis and tocopherol analysis.

The samples were extracted at 8 different extraction parameters (temperature [^{o}C]/pressure [psi]: 40/3000; 40/5000; 40/7000; 40/9000; 80/3000; 80/5000; 80/7000; 80/9000). These extraction conditions were chosen by considering previous works reported for the extraction of lipids and vitamin E from other microalgae species (Perretti *et al*., 2003; Andrich *et al*., 2005; Andrich *et al*., 2006; Mendiola *et al*., 2007; Mendiola *et al*., 2008; Couto *et al*., 2010).

Fatty acid analysis

The conventional lipid extraction was performed following the dichloromethane/methanol method described by Cequier-Sanchez *et al*. (2008). AOCS Official Method Ce 1b-89 was used to prepare Fatty Acid Methyl Ester (FAME) (Wood, 1993). The assay of FAME was analysed using a gas chromatography equipped with a split/splitless injector, and detector Hewlett Packard EL-980 flame ionization detection (FID). FID system was used to separate and quantify each FAME component. FAME was separated using HP-88 capillary column (100 m x 0.25 mm id x 0.2 μm of film thickness) (Agilent J&W Scientific GC Column, USA). Chromatography data was recorded and integrated using chemistation software (version 6). The oven temperature was programmed as such: 120^{o}C, 1 min, 10^{o}C/min to 175^{o}C, 10 min, 5^{o}C/min to 210^{o}C, 5 min, 5^{o}C/min, and to 230^{o}C, 5 min. Temperature for injector and detector was set at 250^{o}C and 280^{o}C respectively. One microliter of

sample volume was injected with split ratio 1:50 µl. The carrier gas used was hydrogen, with head pressure maintained at 2.0 ml/min constant flow. The detector gases used were hydrogen (40 ml/min), air (450 ml/min) and helium make-up gas (30 ml/min). Fatty acids were identified by comparing the retention times of FAME mixture with a pure standard of 37 FAMEs, ranging from C4 to C24 chain lengths. Fatty acid quantification was expressed as area percentage (%) of total fatty acids quantified.

Tocopherol analysis

The tocopherol extraction was performed following a method described by Chen *et al*. (1998). Oil extracted from SFE was prepared for Ultra Performance Liquid Chromatography (UPLC) analysis according to the following procedures. Five milligrams of oil was weighed into a test tube and 1 ml of hexane was added. The test tube was vortexed for 30 seconds and centrifuged at room temperature for 10 minutes at 1000 x g. Finally, a portion of the hexane layer was transferred to a sample vial and 20 µl was injected into the UPLC equipped with an automatic injector and a fluorescent detector (λ_{exc} = 290 nm and λ_{em} = 330 nm). The separation was carried out in an analytical High Performance Liquid Chromatography (HPLC) column, Purospher STAR Si (250 mm x 4.6 mm id; 5 µm particle size) (Merck, Germany). The mobile phase was a mixture of hexane and isopropanol (99.3:0.7, v/v) degassed and eluted at a constant flow of 1.4 ml/min. The tocopherol identification was made by comparing with external standards. The concentration of individual tocopherol was calculated based on standard curve. The total tocopherol content was calculated by summing each tocopherol concentration.

Statistical analysis

Data was analysed using SPSS (version 15.0). All the data was expressed as mean ± standard deviation. One-way ANOVA analysis with Tukey HSD post hoc test was used to compare the mean amounts of fatty acids and tocopherols of two different extraction methods whereas independent sample t-test was used to compare the mean amounts of fatty acids and tocopherols between the *Nannochloropsis oculata* and *Tetraselmis suecica*. All analyses were performed at the significance level of $p<0.05$.

Results and Discussion

Extraction yield

The yield of NO increased as pressure rises from 3000 psi to 9000 psi at 40°C. However, at 80°C, the yield decreased when pressure increases to 9000 psi. Similar trend has been observed for TS as well (Table 1). The rise in yield at 40°C as pressure increases is because as the pressure goes up the solvating power of the supercritical fluid CO_2 will be higher as well. However, when temperature rises, CO_2 density decreases and overrides the effect of increase solvating power as imposed by increasing pressure. This may explain the decrease in yield as pressure increases at 80°C.

Table 1. The extraction yield (mg/g freeze dried microalgae) of *Nannochloropsis oculata* (NO) and *Tetraselmis suecica* (TS) under different extraction methods

	mg/g freeze dried microalgae	
	Nannochloropsis oculata (NO)	*Tetraselmis suecica (TS)*
SFE condition (°C/psi)		
40/3000	47.30 ± 6.93[a]	49.40 ± 7.64[a]
40/5000	52.60 ± 5.37[a]	47.50 ± 3.81[a]
40/7000	59.30 ± 3.25[a]	45.20 ± 0.84[a]
40/9000	60.90 ± 5.80[a]	69.90 ± 2.97[a]
80/3000	71.00 ± 7.35[a]	51.20 ± 0[a]
80/5000	67.50 ± 0.99[a]	51.60 ± 10.18[a]
80/7000*	67.30 ± 0.14[a]	44.40 ± 1.13[a]
80/9000*	65.70 ± 2.40[a]	49.60 ± 1.98[a]
Solvent extraction		
Dichloromethane/methanol	665.33 ± 16.97[b]	480.67 ± 161.22[b]
Hexane	163.00 ± 10.85[c]	160.34 ± 14.62[a]

Each value is the mean ± standard deviation of duplicate (n=2) expressed as mg/g freeze dried microalgae.
The different superscript letters in each column indicate a significant difference ($p<0.05$).
The superscript (*) indicates a significant difference ($p<0.05$) between NO and TS at that particular condition.

At constant pressure, the yield of NO and TS increased as temperature rises, except at 7000 psi and 9000 psi of TS where an inverse trend was observed. Temperature affects the extraction process by two aspects named solute diffusivity and solute solubility (Hu *et al*., 2007). When the temperature rises, solute diffusivity increases, thus the extraction yield improves. Besides, the vapour pressure of solute also increases with temperature, resulting in an increase of its solubility, and therefore the yield of the extractable lipid. The exception at 7000 psi and 9000 psi of TS could be due to the stronger effect of temperature on CO_2 density resulting in backward shift of extraction yield.

The solvent extraction method, namely dichloromethane/methanol (DCM/MeOH) and hexane extraction, resulted in higher yield of both NO and TS compared to SFE. This is probably due to the polarity of solvent. Whereas supercritical CO_2 extracts pure oil consisted mainly of non-polar compounds, the organic solvent may extract other compounds as well, such as pigments (Cequier-Sanchez *et al*., 2008).

Table 2. Fatty acid composition of *Nannochloropsis oculata* (NO) under supercritical fluid extraction (SFE) and dichloromethane/methanol (DCM/MeOH) extraction

Fatty acid	SFE condition (°C/psi)								DCM/MeOH
	40/3000	40/5000	40/7000	40/9000	80/3000	80/5000	80/7000	80/9000	
C8:0	0.41 ± 0.09^{a}	0.52 ± 0.13^{a}	0.57 ± 0.11^{ab}	0.98 ± 0.43^{abc}	1.13 ± 0.35a^{abc}	1.48 ± 0.26^{cd}	1.95 ± 0.77^{d}	1.48 ± 0.23^{cd}	1.41 ± 0.14^{bcd}
C10:0	0.38 ± 0.10^{a}	1.00 ± 0.22^{ab}	0.84 ± 0.34^{ab}	1.33 ± 0.55^{ab}	0.74 ± 0.34^{a}	1.16 ± 0.64^{ab}	1.28 ± 0.18^{ab}	1.17 ± 0.69^{ab}	1.99 ± 0.93^{b}
C12:0	1.74 ± 0.42^{a}	2.99 ± 1.35^{a}	1.50 ± 0.98^{a}	3.05 ± 0.45^{a}	2.06 ± 0.95^{a}	1.57 ± 0.94^{a}	2.05 ± 0.16^{a}	2.49 ± 0.35^{a}	1.48 ± 1.10^{a}
C14:0	17.00 ± 4.98^{a}	30.89 ± 7.81^{ab}	18.61 ± 3.82^{a}	35.22 ± 5.98^{b}	20.44 ± 7.76^{a}	19.38 ± 5.19^{a}	28.05 ± 1.95^{ab}	25.20 ± 7.57^{ab}	22.52 ± 5.28^{ab}
C14:1	4.13 ± 3.02^{ab}	nd	3.32 ± 1.37^{b}	5.70 ± 3.68^{ab}	4.30 ± 3.97^{ab}	5.77 ± 1.55^{ab}	11.05 ± 0.85^{a}	8.01 ± 4.06^{ab}	5.23 ± 2.72^{ab}
C16:0	14.24 ± 3.02^{a}	31.00 ± 9.53^{ab}	21.74 ± 8.78^{a}	31.90 ± 8.91^{ab}	27.97 ± 8.68^{ab}	17.48 ± 4.22^{a}	37.88 ± 1.98^{ab}	31.87 ± 10.72^{ab}	47.23 ± 23.41^{b}
C16:1	39.93 ± 16.36^{ab}	26.12 ± 14.95^{ab}	32.71 ± 10.84^{ab}	15.23 ± 3.51^{ab}	30.20 ± 17.01^{ab}	41.94 ± 12.42^{a}	10.81 ± 3.51^{b}	21.49 ± 13.25^{ab}	15.68 ± 14.36^{ab}
C18:0	1.23 ± 0.74^{ab}	2.87 ± 1.52^{ab}	0.92 ± 0.61^{b}	2.34 ± 0.72^{ab}	1.09 ± 0.38^{b}	1.42 ± 1.09^{ab}	2.23 ± 0.56^{ab}	3.47 ± 1.47^{a}	nd
C18:1n9c	1.25 ± 0.17^{a}	1.64 ± 0.46^{a}	2.35 ± 0.43^{a}	2.16 ± 0.69^{a}	3.87 ± 2.82^{a}	2.07 ± 1.46^{a}	2.82 ± 0.69^{a}	2.39 ± 0.69^{a}	2.50 ± 1.86^{a}
C18:2n6c	0.18 ± 0.07^{a}	0.41 ± 0.19^{a}	0.15 ± 0.07^{a}	0.39 ± 0.12^{a}	0.38 ± 0.18^{a}	0.38 ± 0.18^{a}	0.21 ± 0.01^{a}	0.42 ± 0.08^{a}	nd
C18:3n6	1.33 ± 1.00^{a}	2.56 ± 0.59^{a}	1.90 ± 0.46^{a}	1.70 ± 0.99^{a}	1.75 ± 0.78^{a}	1.03 ± 0.33^{a}	1.68 ± 0.67^{a}	2.01 ± 0.27^{a}	0.97 ± 0.08^{a}
C20:4n6	1.38 ± 0.55^{a}	nd	0.27 ± 0.14^{a}	nd	3.19 ± 1.32^{b}	1.17 ± 0.37^{a}	nd	nd	nd
C20:5n3	16.80 ± 4.91^{a}	nd	15.11 ± 4.26^{a}	nd	2.90 ± 2.44^{b}	5.15 ± 0.90^{b}	nd	nd	0.99 ± 0.39^{b}
∑SFA	35.00 ± 7.91^{a}	69.27 ± 15.91^{bc}	44.18 ± 14.26^{ab}	74.82 ± 3.49^{c}	53.44 ± 15.58^{abc}	42.49 ± 10.56^{ab}	73.44 ± 2.44^{c}	65.68 ± 12.00^{bc}	74.63 ± 18.38^{c}
∑MUFA	45.31 ± 13.45^{a}	27.76 ± 15.42^{a}	38.38 ± 11.12^{a}	23.09 ± 3.84^{a}	38.37 ± 11.89^{a}	49.78 ± 10.17^{a}	24.68 ± 2.12^{a}	31.89 ± 11.66^{a}	23.41 ± 18.75^{a}
∑PUFA	19.69 ± 6.30^{a}	2.97 ± 0.60^{b}	17.43 ± 3.83^{a}	2.09 ± 1.04^{b}	8.22 ± 4.17^{b}	7.73 ± 0.95^{b}	1.89 ± 0.75^{b}	2.43 ± 0.41^{b}	1.96 ± 0.46^{b}

Abbreviations used: ∑SFA, Total saturated fatty acid; ∑MUFA, Total monounsaturated fatty acid; ∑PUFA, Total polyunsaturated fatty acid; nd, not detected.
Each value is the mean ± standard deviation of quadruplicate (n=4) expressed as area percentage (%).
The different superscript letters in each row indicate a significant difference ($p<0.05$).

Table 3. Fatty acid composition of *Tetraselmis suecica* (TS) under supercritical fluid extraction (SFE) and dichloromethane/methanol (DCM/MeOH) extraction

Fatty acid	SFE condition (°C/psi)								DCM/MeOH
	40/3000	40/5000	40/7000	40/9000	80/3000	80/5000	80/7000	80/9000	
C8:0	0.52 ± 0.05^{a}	0.62 ± 0.19^{a}	0.55 ± 0.29^{a}	0.59 ± 0.32^{a}	0.38 ± 0.02^{a}	0.48 ± 0.09^{a}	0.43 ± 0.36^{a}	0.51 ± 0.38^{a}	nd
C10:0	0.52 ± 0.09^{a}	0.63 ± 0.27^{a}	0.75 ± 0.33^{a}	1.16 ± 0.54^{a}	0.54 ± 0.16^{a}	0.68 ± 0.50^{a}	0.83 ± 0.32^{a}	0.62 ± 0.21^{a}	0.66 ± 0.29^{a}
C12:0	0.76 ± 0.11^{a}	1.13 ± 0.17^{ab}	0.86 ± 0.22^{a}	3.26 ± 2.56^{b}	0.99 ± 0.55^{ab}	0.91 ± 0.34^{ab}	1.29 ± 0.27^{ab}	1.26 ± 0.30^{ab}	1.96 ± 1.33^{ab}
C14:0	21.15 ± 4.61^{a}	24.09 ± 6.51^{a}	30.24 ± 5.91^{a}	26.10 ± 7.82^{a}	25.08 ± 4.85^{a}	22.53 ± 4.84^{a}	20.66 ± 3.38^{a}	26.05 ± 6.00^{a}	34.40 ± 12.81^{a}
C14:1	9.18 ± 1.03^{a}	6.13 ± 1.70^{a}	10.73 ± 2.13^{a}	6.96 ± 4.67^{a}	7.73 ± 3.14^{a}	6.98 ± 1.63^{a}	5.35 ± 1.62^{ab}	10.41 ± 1.41^{a}	0.26 ± 0.03^{b}
C16:0	47.78 ± 5.11^{a}	45.99 ± 10.25^{a}	34.32 ± 12.82^{a}	39.38 ± 13.32^{a}	41.25 ± 11.12^{a}	33.98 ± 12.36^{a}	49.31 ± 4.22^{a}	29.36 ± 6.12^{a}	47.15 ± 18.57^{a}
C16:1	7.22 ± 1.03^{ab}	9.18 ± 3.74^{ab}	6.99 ± 2.61^{ab}	8.42 ± 4.72^{ab}	9.12 ± 3.42^{ab}	20.12 ± 14.74^{a}	5.76 ± 2.09^{b}	14.30 ± 2.79^{ab}	4.36 ± 0.30^{ab}
C18:0	3.29 ± 1.53^{a}	3.96 ± 1.15^{ab}	3.24 ± 1.13^{a}	5.47 ± 2.44^{abc}	5.50 ± 1.91^{abc}	3.07 ± 1.66^{a}	9.42 ± 1.92^{c}	8.25 ± 2.73^{bc}	nd
C18:1n9c	7.93 ± 4.47^{a}	4.88 ± 0.67^{a}	4.51 ± 3.00^{a}	4.56 ± 0.56^{a}	6.88 ± 4.41^{a}	7.73 ± 1.39^{a}	5.56 ± 1.55^{a}	3.19 ± 1.14^{a}	5.98 ± 3.85^{a}
C18:2n6c	0.42 ± 0.11^{a}	1.46 ± 0.85^{a}	1.15 ± 0.58^{a}	1.01 ± 0.80^{a}	1.35 ± 0.73^{a}	0.90 ± 0.40^{a}	0.58 ± 0.20^{a}	3.09 ± 4.42^{a}	0.25 ± 0.01^{a}
C18:3n6	1.23 ± 0.28^{a}	1.91 ± 1.11^{ab}	6.65 ± 4.82^{b}	3.08 ± 1.65^{ab}	1.19 ± 0.78^{a}	2.63 ± 1.26^{ab}	0.81 ± 0.19^{a}	2.95 ± 1.28^{ab}	4.99 ± 2.42^{ab}
∑SFA	74.02 ± 4.35^{abc}	76.42 ± 3.62^{abc}	69.96 ± 6.96^{abc}	75.96 ± 6.68^{abc}	73.74 ± 6.62^{abc}	61.65 ± 14.59^{c}	81.94 ± 5.21^{ab}	66.05 ± 7.42^{bc}	84.17 ± 6.44^{a}
∑MUFA	24.33 ± 4.41^{abc}	20.19 ± 2.37^{abc}	22.23 ± 4.81^{abc}	19.94 ± 5.34^{abc}	23.73 ± 7.14^{abc}	34.83 ± 14.93^{a}	16.67 ± 4.67^{bc}	27.90 ± 4.47^{ab}	10.60 ± 4.07^{c}
∑PUFA	1.65 ± 0.15^{ab}	3.37 ± 1.55^{ab}	7.80 ± 5.31^{a}	4.09 ± 2.19^{ab}	2.54 ± 1.47^{ab}	3.53 ± 1.12^{ab}	1.39 ± 0.60^{b}	6.04 ± 4.58^{ab}	5.24 ± 2.48^{ab}

Abbreviations used: ∑SFA, Total saturated fatty acid; ∑MUFA, Total monounsaturated fatty acid; ∑PUFA, Total polyunsaturated fatty acid; nd, not detected.
Each value is the mean ± standard deviation of quadruplicate (n=4) expressed as area percentage (%).
The different superscript letters in each row indicate a significant difference ($p<0.05$).

The DCM/MeOH method extracted significantly higher ($p<0.05$) yield than hexane and this could be due to the addition of methanol to the fraction of dichloromethane. Methanol is a highly polar primary alcohol which can extract more polar substances by stimulating disruption of hydrogen bond between

Table 4. Tocopherol concentration (mg/g extract) of *Nannochloropsis oculata* (NO) under different extraction methods

	Tocopherol concentration (mg/g extract)			
	α-tocopherol	β-tocopherol	γ-tocopherol	Total
SFE condition (°C/psi)				
40/3000	10.353 ± 0.253^{a}	0.009 ± 0.003^{a}	nd	10.360 ± 0.252^{a}
40/5000	7.370 ± 0.735^{b}	0.028 ± 0.018^{ab}	0.044 ± 0.005^{a}	7.431 ± 0.766^{b}
40/7000	8.173 ± 1.663^{ab}	0.033 ± 0.020^{ab}	0.017 ± 0.014^{b}	8.224 ± 1.690^{ab}
40/9000	7.138 ± 0.284^{b}	0.025 ± 0.005^{ab}	0.017 ± 0.004^{b}	7.180 ± 0.284^{b}
80/3000	9.879 ± 1.257^{a}	0.028 ± 0.008^{ab}	0.019 ± 0.004^{b}	9.926 ± 1.257^{a}
80/5000	8.542 ± 1.246^{ab}	0.039 ± 0.005^{b}	0.017 ± 0.006^{b}	8.600 ± 1.257^{ab}
80/7000	9.079 ± 0.660^{ab}	0.033 ± 0.011^{ab}	0.018 ± 0.004^{b}	9.130 ± 0.649^{ab}
80/9000	8.913 ± 0.646^{ab}	0.041 ± 0.012^{b}	0.020 ± 0.004^{b}	8.974 ± 0.635^{ab}
Hexane extraction	4.722 ± 0.405^{c}	nd	nd	4.722 ± 0.405^{c}

Abbreviation used: nd, not detected.
Each value is the mean ± standard deviation of quadruplicate (n=4) expressed as mg/g extract.
The different superscript letters in each column indicate a significant difference ($p<0.05$)

Table 5. Tocopherol concentration (mg/g extract) of *Tetraselmis suecica* (TS) under different extraction methods

	Tocopherol concentration (mg/g extract)		
	α-tocopherol	β-tocopherol	Total
SFE condition (°C/psi)			
40/3000	0.585 ± 0.072^{ab}	0.016 ± 0.002^{ab}	0.601 ± 0.074^{ab}
40/5000	0.501 ± 0.160^{b}	0.013 ± 0.003^{a}	0.514 ± 0.162^{b}
40/7000	0.612 ± 0.176^{ab}	0.016 ± 0.006^{ab}	0.628 ± 0.182^{ab}
40/9000	0.540 ± 0.138^{b}	0.018 ± 0.010^{ab}	0.558 ± 0.147^{b}
80/3000	0.963 ± 0.290^{a}	0.045 ± 0.023^{c}	1.008 ± 0.310^{a}
80/5000	0.793 ± 0.052^{ab}	0.019 ± 0.002^{abc}	0.812 ± 0.054^{ab}
80/7000	0.845 ± 0.237^{ab}	0.026 ± 0.014^{abc}	0.871 ± 0.252^{ab}
80/9000	0.789 ± 0.126^{ab}	0.039 ± 0.010^{bc}	0.828 ± 0.131^{ab}
Hexane extraction	0.719 ± 0.171^{ab}	nd	0.719 ± 0.171^{ab}

Abbreviation used: nd, not detected.
Each value is the mean ± standard deviation of quadruplicate (n=4) expressed as mg/g extract.
The different superscript letters in each column indicate a significant difference ($p<0.05$).

lipid carbonyl, hydroxyl, and amino groups and compounds of the non extractable residue, compared to the non-polar hexane, therefore resulting in higher yield (Ruiz-Lopez *et al*., 2003).

Fatty acid composition

The results obtained in this study showed that myristic acid (C14:0), palmitic acid (C16:0) and palmitoleic acid (C16:1) were the dominant fatty acids in NO (Table 2). The high proportion of C16:0 and C16:1 in NO as detected in this study was in accordance with the findings reported by Zhukova and Aizdaicher (1995). However, C14:0 was not one of the dominant fatty acids in their study where it has been reported to be 3.9%, a value far lesser than 17-35% obtained in this study. In fact, the study conducted by Hodgson *et al*. (1991) who examined the effects of growth cycle towards fatty acid composition of NO, revealed that C14:0 was not one of the dominant fatty acids detected, ranging from 1.0% to 9.9% only.

Besides C16:0 and C16:1, Zhukova and Aizdaicher (1995) also reported in their study that eicosapentaenoic acid (EPA) was the other dominant fatty acid, constituting 29.7% of total fatty acids. However, the lower amount (0.99-16.80%) of EPA detected in SFE and DCM/MeOH extracts of this study do not support the claim of NO being a good source of EPA. Apart from EPA, Nannochloropsis sp. has also being reported to contain DHA (Andrich *et al*., 2005). However, the failure in detecting any DHA in this study was in accordance with other studies (Hodgson *et al*., 1991; Zhukova and Aizdaicher, 1995).

On the other hand, the results of this study also showed that TS was high in C14:0 and C16:0 (Table 3). The high proportion of C16:0 detected in this study was in accordance with the results reported for *Tetraselmis* sp. (Zhukova and Aizdaicher, 1995). However, similar to NO, C14:0 was not one of the dominant fatty acids in TS as reported in their study where it consisted of only 0.6% of total fatty acids, a value lesser than 21-34% obtained in this study. Besides, Fabregas *et al*. (2001) also reported a low range of C14:0 in TS which was 0.90-2.14% of total fatty acids.

TS contains appreciable amount of EPA, ranging from 4.71% to 8.60% (Montaini *et al*., 1995; Zhukova and Aizdaicher, 1995; Fabregas *et al*., 2001). However, it was not detected in this study. The reason could be due to the different culturing condition of TS in this study from those adopted in other studies. It has been reported that the fatty acid composition of microalgae is highly changeable as a function of culture conditions adopted (Dunstan *et al*., 1993).

Among the SFE conditions adopted, extracts of 40°C contain significantly higher ($p<0.05$) amount of EPA than that of 80°C. At 40°C, the percentage of EPA in NO decreased but not significantly from 16.80 ± 4.91% to 15.11 ± 4.26% as the pressure increases from 3000 psi to 7000 psi. At 80°C, the percentage of EPA insignificantly increased two fold from 2.90 ± 2.44% to 5.15 ± 0.90% as pressure increases from 3000 psi to 5000 psi. From the aforementioned results, it can be deduced that pressure actually has little effect towards the concentration of EPA. However, the effect is obvious for the temperature change. The significantly lower ($p<0.05$) amount of EPA at 80°C could be due to thermal oxidation. This is because EPA, being long chain PUFA, is extremely sensitive to heat. High temperature as 80°C could have oxidised or degraded most of the EPA in the extracts. In fact, the study conducted by Andrich *et al*. (2005) found that at lower temperature (40 and 55°C), from 40

MPa to 70 MPa (5802 psi to 10153 psi), the content of EPA in Nannochloropsis sp. was almost similar. Thus, 80°C may not be a suitable temperature for high quality oil extraction in microalgae.

Comparing the EPA content of NO extracted between SFE and solvent methods, it was found that the content obtained in extracts of SFE were higher, with extracts of 40°C significantly higher ($p<0.05$) than the DCM/MeOH extract. PUFAs such as EPA are easily oxidised when exposed to air or when dissolved in organic solvent and this may explain the lowest concentration of EPA obtained in DCM/MeOH method (Okuyama *et al*., 2008). Besides, the use of boron trifluoride (BF_3) as the methylation agent could be responsible for the overall lower content of EPA or PUFAs detected because it has been associated with production of artifacts and loss of reasonable amounts of PUFA during the assay (Carvalho and Malcata, 2005). The same may go for the lower content of C18:2n6c and C18:3n6 in DCM/MeOH extract compared to SFE extracts of TS.

When the total saturated fatty acid (SFA), total monounsaturated fatty acid (MUFA) and total polyunsaturated fatty acid (PUFA) were being compared respectively between NO and TS, generally it can be concluded that NO contains lower amount of total SFA but higher amount of total MUFA and total PUFA than TS among SFE extracts. At 40°C/3000psi, 40°C/7000psi and 80°C/7000psi, NO contained significantly lower ($p<0.05$) amount of total SFA, and higher amount of total MUFA and total PUFA (total PUFA content was not significantly different at 80°C/7000psi compared to that of TS). Considering that MUFA and PUFA were of more pronounced health benefits, thus, NO oil will have higher potential than that of TS.

Tocopherol

Tocopherol composition of NO and TS were different where α-, β- and γ-tocopherol were detected in various SFE extracts of NO (except under 40°C/3000psi where γ-T was not detected) (Table 4) but only α- and β-tocopherol were detected in such of TS (Table 5). The reported tocopherol composition for *Tetraselmis* sp. in Huo *et al*. (1997) study was different from this study where α, γ, and δ but no β isomers were detected in their study. Vismara *et al*. (2003), on the other hand, detected only α and γ isomers in TS. To date currently, there is no study that looked comprehensively at tocopherol composition of NO. Nevertheless, Brown *et al*. (1999) and Durmaz (2007) have studied the α-tocopherol content in *Nannochloropsis* sp. and NO, respectively, under different growth condition.

Theoretically, the concentration of solutes is expected to rise as the pressure increases because pressure enhances the density and therefore the solvating power of supercritical CO_2 which allowed it to dissolve more solutes (Kwon *et a*l., 2010). However, this is not the observed trend for both NO and TS. As the pressure increases from 3000 psi to 9000 psi, a decrease of α-tocopherol concentration can be observed at 5000 psi and 7000 psi, with concentration at 9000 psi lower than that at 3000 psi, for both 40°C and 80°C. This could be due to the stronger diluting effect of extractable competitive materials with increasing pressure which resulted in lower concentration of the desirable solutes, α-tocopherol.

Temperature affects the extraction of vitamin E (α-tocopherol) positively (Mendiola *et al*., 2008). This is because increasing the temperature will lower the viscosity and enhances the diffusivity resulting in better solubility of solute (Herrero *et al*., 2006). Mendiola *et al*. (2008) has suggested that higher temperature, such as 80°C but lower pressure will result in higher concentration of tocopherol. The results obtained in this study correlated well with the aforementioned statement and principle. All the 80°C extracts of NO and TS yielded a higher concentration of α-tocopherol than their 40°C counterparts, except for 40°C/3000psi. Therefore the results are in agreement with the fact that high temperature favours and increases the extractable tocopherol as reported for other sample (Carluccia *et al*., 2001).

The concentration of α-tocopherol as obtained by hexane extraction was lower and in fact was the lowest when compared with extracts of SFE, for NO. This could possibly be due to the extraction condition, which was performed at ambient condition compared to the adjustable conditions in SFE. The low amount and absence of other tocopherols in hexane extraction could be due to the use of single non-polar solvent that may have limited solubility towards tocopherols which are slightly polar in nature. Generally, non-selective extraction of hexane will increase the extraction of other materials, therefore, imposing diluting effect of such (Athukorala *et al*., 2009).

Although hexane extraction gave the lowest tocopherol concentration, it yielded the highest amount of tocopherol per gram of freeze dried microalgae. This is because the lipid yield in hexane extraction is a lot higher than in SFE. This could be due to the sonication step that facilitates the lipid extraction as such procedure mechanically breaks the cell wall structure and improves material transfer (Abdullah *et al*., 2010).

Across all the SFE conditions and hexane

extraction, the α-tocopherol concentration of NO is significantly higher ($p<0.05$) than that of TS. However, its concentration is still considered lesser than other microalgae sources such as Spirulina platensis, which consisted of 31.61 mg of α-tocopherol per gram of extract, tripled the highest concentration of NO (10.353 ± 0.253 mg/g extract) at similar SFE condition (220 bar [3200 psi] and 83.3°C) (Mendiola *et al*., 2008). This would suggest that neither NO nor TS is a good source of vitamin E or specifically α-tocopherol.

Besides, the amount of α-tocopherol as in freeze dried material (μg/g freeze dried microalgae) of both NO (769.597 ± 66.003 μg/g freeze dried microalgae) and TS (115.272 ± 27.376 μg/g freeze dried microalgae) obtained in this study using hexane extraction is lower than those reported values too. TS have been reported to be a potential vitamin E producer where De Roeck-Holtzhauer *et al*. (1991) reported a yield of 6323 μg/g dry matter. Besides, Fabregas and Herrero (1990) also reported α-tocopherol value of 421.8 μg/g dry weight in TS. NO, on the other hand, have also been reported to contain 2326 μg/g dry weight of α-tocopherol (Durmaz, 2007). Lower values as obtained in this study could be due to the effect of harvesting phase and culturing condition adopted. It has been reported that the phase of harvesting, light intensity, nitrogen amount and many other environmental factors could affect the production of valuable amount in the microalgae, and vitamin E concentration is one of such (Huo *et al*., 1997; Bandara *et al*., 2003; Donato *et al*., 2003). This might suggest that the TS and NO used in this study were not harvested during their peak vitamin E production period.

Optimal condition

In order for commercialization, extract which is high in PUFA and α-tocopherol will be the favourable. Thus, when analysing the aforementioned results, it can be concluded that for NO, such extract can be obtained under the mildest SFE condition, markedly at 40°C/3000psi. This is because under this parameter, the total PUFA content was the highest apart from having high content of total MUFA and lowest content of total SFA across the SFE conditions and DCM/MeOH extracts. Under 40°C/3000psi, the α-tocopherol concentration was also significantly higher ($p<0.05$), therefore, offering a valuable extract option.

As for TS, 40°C/7000psi is perhaps the optimal condition if based on the results obtained in this study. Under this parameter, the total PUFA content was the highest, with the total MUFA and total SFA contents similar across different SFE conditions. In this condition, the α-tocopherol concentration was not the highest but was not significantly different with the other condition.

Conclusions

The use of different extraction methods have resulted in different compositions and concentrations of fatty acids and tocopherols in the microalgae studied. Compared with the solvent method, generally, the SFE method yielded a better profile of oil which is higher in PUFA and MUFA proportion, lower in SFA proportion, and with higher concentration of total tocopherol/α-tocopherol. Comparing between the two microalgae studied, NO was found to contain better fatty acid profile, especially with the existence of long chain PUFA, EPA, which was failed to be detected in TS in this study. Apart from that, NO also contains more tocopherol isomer and higher concentration of total tocopherol, about ten times higher than that of TS. Additionally, extraction of NO using SFE is also more economically feasible because the high quality oil aforementioned can be obtained at the mildest condition, which is 40°C/3000psi. However, it is worth noting that the extracted amount of tocopherol from NO was not high enough to allow an economic industrial size extraction. Perhaps optimization of microalgae culture condition, which can affect the fatty acids and tocopherols content, before optimizing SFE condition, may serve as a route to be evaluated further.

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