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Microalgae Lipid Characterization

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Supporting Information

ABSTRACT: To meet the growing interest of utilizing microalgae biomass in the production of biofuels and nutraceutical and pharmaceutical lipids, we need suitable analytical methods and a comprehensive database for their lipid components. The objective of the present work was to demonstrate methodology and provide data on fatty acid composition, lipid class content and composition, characteristics of the unsaponifiables, and type of chlorophylls of five microalgae. Microalgae lipids were fractionated into TAG, FFA, and polar lipids using TLC, and the composition of fatty acids in total lipids and in each lipid class, hydrocarbons, and sterols were determined by GC-MS. Glyco- and phospholipids were profiled by LC/ESI-MS. Chlorophylls and their related metabolites were qualified by LC/APCI-MS. The melting and crystallization profiles of microalgae total lipids and their esters were analyzed by DSC to evaluate their potential biofuel applications. Significant differences and complexities of lipid composition among the algae tested were observed. The compositional information is valuable for strain selection, downstream biomass fractionation, and utilization.

KEYWORDS: *chlorophylls, fatty acids, glycolipids, hydrocarbons, lipid characterization, microalgae, sterols*

INTRODUCTION

Algae contribute 40% of global photosynthesis and are tremendously diverse.[1] Green algae, for example, have approximately 16,000 recognized species. Certain types of microalgae have been recognized as commercially promising sources of materials for third-generation biofuel production as well as for nutraceutical and pharmaceutical applications due to their rapid growth under a range of environmental conditions, their high lipid content, and their unique lipid composition. The diversity of algae determines that they have an exceptionally wide range of lipid patterns, which is generally believed to be a result of the algae's adaptation to environmental conditions.[2] For instance, some microalgae that have gained increasing commercial attentions are capable of synthesizing high levels of long-chain polyunsaturated fatty acids in response to ecological factors.[3−5] The occurrence of unusual fatty acids or lipids makes microalgae distinguishable from higher plant or other animal products.

Although many microalgae species have been analyzed for their fatty acid compositions, and some have been determined for certain lipid classes such as polar lipids[2,3,5−8] or sterols,[9−15] few studies have presented the complete characterization of lipid components (both simple and complex lipids) of a particular microalga in one paper. Because of the large variation among the types of algae and cultivation conditions, it is often difficult to compare the results from different laboratories for a comprehensive profiling of lipid components of one microalga. There is a need to fully characterize the various lipid components within one alga species and demonstrate the appropriate analytical methodology that can be employed. Such information on complete lipid characterization of microalgae is essential for successful strain selection, downstream biomass extraction, and utilization for the production of biofuels and high-value compounds, as well as algae product marketing. The availability and sensitivity of modern instrumentations such as GC-MS and LC-MS provide powerful tools to gain in-depth knowledge of the lipid composition of microalgae.

The objectives of the present work were to provide data on the fatty acid composition, lipid class content and composition, composition of unsaponifiables (including sterols and hydrocarbons), and type of chlorophylls of five commercially important microalgae, *Chlamydomonas reinhardtii*, *Chlorella vulgaria*, *Nannochloropsis* sp., *Scenedesmus* sp., and *Schizochytrium limacinum*, and to demonstrate the importance of using combined analytical methods for these analyses.

MATERIALS AND METHODS

Microalgae Samples. The microalga *Nannochloropsis* sp. (referred to as *Nanno*) was purchased from Seambiotic Ltd. (Tel Aviv, Israel) as a frozen paste containing 16−18% dry matter. *Schizochytrium limacinum* SR-21 (ATCC MYA-1381, referred to as *Schizo*) and *Chlorella vulgaria* (UTEX 265, referred to as *Chlorella*) were provided by Professor Z. Wen at Iowa State University (Ames, IA, USA). *Schizo* was grown in a 5-L stirred-tank reactor using glycerol as the carbon source.[4] *Chlorella* was grown in photobioreactors in autotrophic conditions.[16] *Scenedesmus* sp. (referred to as *Scene*), native to Louisiana, was grown in raceway open ponds in Roanoke, LA, USA. Its growth condition and initial concentration process to obtain a dry matter content of 1.7% were described previously.[17] *Chlamydomonas reinhardtii* (21gr, CC-1690, *Chlamydomonas* Resource Center, St. Paul, MN, USA), referred to as *Chlamy*, was grown in 5-L photobioreactors in autotrophic conditions according to the procedure described by Vance and Spalding[17] with minor modifications.[18] All of the microalgae were further concentrated (or dewatered) using a laboratory centrifuge (Sorvall RC 3B Plus, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 5000 rpm and 10 °C to give a wet paste

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containing 12% dry matter. Then the concentrated microalgae were lyophilized to yield dry flakes containing 95−97% dry matter. The dry algae samples were stored at −26 °C and processed within 3 days.

Chemicals and Reagents. FAME mix standards (GLC 566) was purchased from Nu-Chek (Elysian, MN, USA). Cholesterol (≥99% GC), 5α-cholestane (≥97% GC), and hexadecane (99%) used in the determination of GC response factors were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other analytical grade solvents (isopropanol, chloroform, and methanol) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Proximate Analysis of Microalgae Biomass. Dry matter content was determined by weight difference after oven-drying at 110 °C for 5 h. Lipid content was determined gravimetrically after solvent extractions as described below, and protein content was determined by using the Dumas nitrogen combustion method with an ElementarVario MAXCN analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) and a nitrogen-to-protein conversion factor of 4.36.[19] Ash content was determined by heating the dry algae samples at 550 °C for 5 h and then weighing the residual ash (AOAC Official Method 923.03). All of the algae samples were washed with DI water to remove growth media before drying/dewatering prior to ash determination.

Microalga Lipid Extraction. Dry algae sample (20 g) was first extracted with isopropanol (300 mL) for 1 h with continuous stirring (300 rpm) to deactivate hydrolytic enzymes that might be present. After isopropanol extract was recovered by filtration, the residual wet solids were mixed with chloroform/methanol (300 mL, 2:1, v/v) and then homogenized using an Ultra-Turrax T25 homogenizer (Ika Works, Wilmington, NC, USA) at 10,000 rpm for 3 min, followed by a 2 h continuous stirring at 300 rpm and then a sequential extraction with chloroform/methanol at ratios of 2:1, 1:1, and 1:2 (v/v). Each extraction used 300 mL of solvent, a 300 rpm stirring rate, and a duration of 1 h. The extracts from isopropanol and chloroform/methanol extractions were combined, and the solvent was removed by rotary evaporation at 45 °C. The crude lipid was subjected to the modified Folch wash[20] twice using a mixed solvent of chloroform, methanol, and water in a ratio of 8:4:3. The lipids obtained from the chloroform phase were filtered through a PTFE filter disk (0.45 μm) and then dried by rotary evaporation at 45 °C and vacuum oven at 23 °C for 12 h. The quantity of extracted lipids was determined gravimetrically. Four replicates of extractions were performed to determine lipid content. Then the lipids of each alga were combined and dissolved in a small amount of chloroform and stored at −26 °C until further analysis.

Fractionation of Neutral Lipid, FFA, and Polar Lipid by TLC. Algae total lipids (30−40 mg) dissolved in chloroform were applied on a preparative TLC plate (silica gel G, 500 μm, Analtech, Newark, DE, USA), and the plate was developed in a mixed solvent of hexane/diethyl ether/acetic acid (70:30:1, by vol). The separation was visualized under ultraviolet light after spraying with 0.1% (w/v) 2′,7′-dichlorofluorescein in methanol. The bands of TAG and FFA were scraped off the plate and extracted four times with 10 mL of chloroform/methanol (2:1). The bands of other lipid components (including polar lipids, sterols, and chlorophyll derivatives) that were located below the FFA band, referred to as the polar lipid fraction, were extracted twice with 10 mL of chloroform/methanol (2:1) and then twice with 10 mL of chloroform/methanol/water (5:5:1). The solvent of combined extracts of each fraction was removed by rotary evaporation at 45 °C. Residual water was removed with a small amount of isopropanol by rotary evaporation. The recovered lipids were converted to FAME in the presence of 1 mg of nonadecanoic acid (internal standard) and analyzed by GC following the procedure described below. Because only fatty acids' GC peaks were quantified, sterols and chlorophyll derivatives in the polar lipid fraction did not interfere with FAME quantification. The weight percentage of FAME was converted to TAG and FFA equivalents using the factors given in AOCS Official Method Ce 1i-07. Three replicates of total lipids from each alga were fractionated by TLC. Because polar lipids of the examined algae contained a variety of molecular species, which varied by the type of algae, estimation of polar lipid content based on GC-FAME result would be inaccurate if converting it to one type of polar lipid. Instead, the weight percentage of glycolipids and phospholipids obtained from LC-MS quantification was used in the calculation.

Fatty Acid Composition. The fatty acid composition of the total lipids and those of neutral and polar lipid fractions were determined by GC analysis of FAME. The lipids (10−20 mg) were methylated with 2% sulfuric acid in methanol (3 mL) by incubation at 65 °C for 18 h with occasional shaking. The resulting FAME were extracted with 3 mL of hexane and washed with 10 mL of water. The hexane extract (1 μL) was injected into an Agilent 7890 GC (Santa Clara, CA, USA) equipped with a DBWAXtre capillary column (30 m × 0.25 mm × 0.25 μm; Agilent, Santa Clara, CA). Helium was used as a carrier gas with a flow rate of 1 mL/min. The split ratio was 50:1. The temperature of the injector and FID detector was 260 °C. GC oven temperature was programmed from 100 °C (1 min) to 240 °C (15 min) at a rate of 4 °C/min. GC peaks were identified by the mixed FAME standard (GLC 566) and confirmed by GC-MS. The relative percentage of fatty acids was reported on the basis of the peak area without using correction factors. Three replicates of FAME samples were prepared from each alga.

The GC-MS system for the identification of fatty acids in each microalga included an Agilent 6890 GC and a Waters GCT time-of-flight mass spectrometer (Milford, MA, USA) operated in electron ionization (+) mode at 70 eV. Data were acquired with accurate mass precision (7000 resolution fwhm). The MS scanned from 45 to 650 Da each 0.5 s. Data were processed with MassLynx 4.0 (Waters Inc.) and compared with MS libraries Wiley 7th edition (2000) and NIST 2011. GC column specification, oven temperature program, injector temperature, carrier gas flow rate, and split ratio were the same as those used for fatty acid composition analysis.

Unsaponifiable Matters (USP). The percentage of USP in the total algae lipids was determined according to AOCS Official Method Ca 6b-53. Briefly, 2 g of total lipids was saponified with 95% ethanol and 50% KOH, and then the USP was extracted multiple times with diethyl ether. Total USP was determined by the weight of the ether extract after solvent removal and expressed as the weight percent of total lipids. The profile of USP was determined by GC-FID and GC-MS. For GC-FID analysis, USP dissolved in chloroform (3 mg/mL) was injected into an Agilent 7890 GC equipped with SAC-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm; Supelco, Bellefonte, PA, USA). The split ratio of GC injector was 50:1. The temperature of the injector and FID detector was 300 °C. GC oven temperature was programmed from 200 °C (1 min) to 300 °C (19 min hold) at a rate of 10 °C/min. The percentage of USP that were GC quantifiable was determined by using an internal standard, 5α-chloestane. GC response factors of hydrocarbons and sterols relative to 5α-chloestane were determined to be 0.862 for hexadecane and 0.775 for cholesterol, according to AOCS Official Method Ce 1j-07, and these factors were used in the calculations. Two replicates of microalgae total lipids were saponified.

The USP of *Chlamy*, *Chlorella*, *Nanno*, and *Scene* were fractionated on a silica gel TLC using a solvent system of hexane/diethyl ether/acetic acid (60:40:1, by vol). Six TLC fractions were collected from each microalga on the basis of their polarity or the position on the plates. Both the total USP and TLC fractionated USP of four green microalgae and the total USP of *Schizo* were then analyzed by GC-MS. The GC-MS unit for USP analysis was the same as that used for fatty acid analysis. GC column specification, oven temperature program, injector temperature, carrier gas flow rate, and split ratio in GC-MS were the same as those used for GC-FID analysis of USP.

Profiling of Polar Lipid (Glycolipids and Phospholipids) by TLC and ESI-MS/MS. A preliminary qualification of polar lipids was performed using normal phase TLC. First, the total lipids was applied on a normal phase SPE column (silica, 2000 mg, Alltech Extract-Clean column; Grace, Columbia, MD, USA), and then the cartridge was sequentially washed with 20 column volumes of hexane/diethyl ether (4:1), acetone, chloroform/methanol (1:1), and chloroform/methanol/water (5:5:1). The first fraction (hexane/diethyl ether) contained exclusively nonpolar lipids and was discarded. Other SPE fractions were applied on an analytical TLC plate (silica gel GHL, 250 μm;

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Table 1. Microalgae Biomass Composition on a Dry Weight Basis[a]

	Chlamy	*Chlorella*	*Nanno*	*Scene*	*Schizo*
lipid	24.2 (0.1)	17.9 (3.3)	25.0 (0.4)	10.5 (0.4)	56.7 (0.8)
protein[b]	34.2 (0.3)	28.2 (1.4)	32.2 (0.1)	24.6 (0.3)	12.4 (0.3)
ash	6.1 (0.2)	10.5 (0.3)	5.5 (0.3)	29.5 (0.3)	5.6 (0.1)
carbohydrates[c]	35.5	43.4	37.3	35.4	25.3

[a]Values in parentheses are standard deviations of three replicates. [b]Nitrogen-to-protein conversion factor of 4.36 is used (Gerde et al.[19]). [c]Carbohydrate content is calculated by subtracting lipid, protein, and ash contents from 100%.

Table 2. Microalgae Lipid Class Composition (Weight Percent)[a]

	Chlamy	*Chlorella*	*Nanno*	*Scene*	*Schizo*
neutral lipids	51.3	57.2	14.5	31.5	78.2
TAG	24.5 (2.2)	26.6 (2.8)	8.6 (1.5)	4.1 (0.8)	77.5 (6.4)
FFA	26.8 (2.3)	30.6 (2.1)	5.9 (0.6)	27.4 (1.3)	0.7 (0.6)
polar lipids[b]	9.7 (0.3)	0.7 (0.0)	24.6 (0.4)	0.7 (0.1)	0.9 (0.2)
USP[c]	13.1 (0.1)	13.2 (0.4)	14.6 (0.4)	18.7 (0.6)	1.9 (0.0)
chlorophyllides[c]	16.8	14.6	5.8	14.3	
others[d]	9.1	14.3	40.5	34.8	19.1

[a]Values in parentheses are standard deviations of three replicates for TAG and FFA and differences of two replicates for USP and polar lipids. [b]Polar lipids in this table are glycolipids and phospholipids quantified by HPLC-MS. [c]The wt % of phytol chain of chlorophylls is included in USP (unsaponifiable matters). Chlorophyllide (nonphytol moiety of chlorophylls) is calculated on the basis of the assumption that all of the chlorophyll pigments had the same molecular structure as chlorophyll *a*. [d]"Others" represents the differences between 100 and the known components.

Analtech, Newark, DE, USA). The developing solvent system was methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:4, v/v), which was based on a published method[21] with a minor modification of the ratio of aqueous KCl. Glycolipid standards MGDG, DGDG, and steryl glucoside (purchased from Matreya, Pleasant Gap, PA, USA), SQDG (purchased from Larodan, Sweden), phospholipid standards PC, PE, PG, and PI (purchased from Avanti, Alabaster, AL, USA), and soy lecithin (Fisher Scientific) were used in the comparison with microalgae lipid components. The spraying agent of 70% orcinol in sulfuric acid was used for the identification of glycolipids and steryl glucoside, and molybdenum blue was used for phospholipids.[20]

Polar lipid (i.e., glycolipids and phospholipids) analysis by LC-MS was performed by Kansas Lipidomics Research Center Analytical Laboratory (Manhattan, KS, USA). ESI-MS/MS analysis and quantification were conducted as described previously.[22] Microalgae lipid samples (total lipid extract) were introduced by continuous infusion into the electrospray ionization source on a triple-quadrupole MS/MS (API 4000; Applied Biosystems, Foster City, CA, USA). Lipid species were detected using sequential precursor and neutral loss scans. Lipids in each class were quantified in comparison with the two internal standards of that class using a correction curve determined between standards. The compositional data are presented as nanomoles (per weight) or weight percent of the total lipids analyzed. Two replicates of unfractionated total lipids of each microalga were analyzed.

Analysis of Chlorophyll Derivatives by TLC and LC/APCI-MS. The TLC fractionated polar lipid fraction of green microalgae were dissolved in iso-octane/toluene/acetone/methanol (5:5:1:1)[7] to a concentration of 1 mg/mL, and 2 μL was injected to an Agilent HPLC system equipped with the 1200 series Agilent binary pump system. A guard column (2.0 × 23 mm, s-0.5 μm; YMC, Allentown, PA, USA) and a YMC PVA-SIL column (150 mm × 2 mm, s-0.5 μm, 12 nm; YMC) were used in all separation experiments. The column temperature was maintained at 35 °C. The flow rate was 0.5 mL/min. The solvent composition and gradient program were modified on the basis of previous studies.[7,23,24] The solvent system consisted of isopropanol/hexane (2:98, by vol, with 0.1% of stoichiometric triethylamine/formic acid, solvent A1), isopropanol/acetonitrile/chloroform/acetic acid (84:8:8:0.025, by vol, solvent B1), and chloroform/isopropanol/methanol (39:21:40, by vol, solvent A2). The gradient elution program was as follows: 0–2.5 min, 100% A1; 2.5–40 min, 0–100% B1. After 40 min, the column was flushed with A2 (0–100%) for 15 min and reconditioned with A1 for 15 min. Mass spectrometry analysis was performed using the Agilent 6540 Accurate-Mass Q-TOF mass spectrometer with MassHunter Qualitative Analysis software for data acquisition and processing. The atmospheric pressure chemical ionization (APCI) source was set to operate in negative ion mode with a vaporizer temperature of 250 °C. Resolution was 40,000 fwhm, and mass accuracy was better than 2 ppm across the mass range. The instrument was tuned and calibrated with an Agilent tune mix prior to all LC-MS experiments.

Melting and Crystallization Temperatures of Microalgae Lipids and Their Methyl and Isopropyl Esters. The methyl and isopropyl esters of microalgae lipids were obtained by sulfuric acid (2%) catalyzed alcoholysis. The reaction conditions and purification procedure were the same as those used in FAME preparation described above. The melting and crystallization properties of lipid samples were determined by using a differential scanning calorimeter (Diamond DSC; PerkinElmer, Norwalk, CT, USA) equipped with an Intracooling II system. A sample of 3–5 mg was weighed in an aluminum pan (PerkinElmer) and sealed. Indium and *n*-decane were used as calibration standards. The temperature program started with a 1 min hold at 25 °C, followed by a rapid heating (40 °C/min) to 80 °C and a 5 min hold at 80 °C. Then the sample was cooled to −40 °C at a rate of 10 °C/min. After a 15 min hold at −40 °C, the sample was heated to 80 °C at a rate of 5 °C/min. The crystallization onset and completion of melting were recorded during the cooling and the last heating steps, respectively. Two replicates from each alga were measured.

Statistical Analysis. The number of replicates of each analysis was described above. Standard deviations were given for the mean values obtained with three or more replicates. Differences between two values were also given when there were only two replicates.

RESULTS AND DISCUSSION

Proximate Composition. Of the examined microalgae in this study, *Chlamy* (taxonomic class: Chlorophyceae), *Chlorella* (taxonomic class: Trebouxiophyceae), and *Scene* (taxonomic class: Chlorophyceae) are freshwater green algae; *Nanno* (taxonomic class: Eustigmatophyceae) is a saltwater green alga; and *Schizo* (taxonomic class: Thraustochytriaceae) is a saltwater heterotrophic alga.[25,26] Microalgae biomass composition is given in Table 1. *Scene* and *Schizo* had the lowest (11%)

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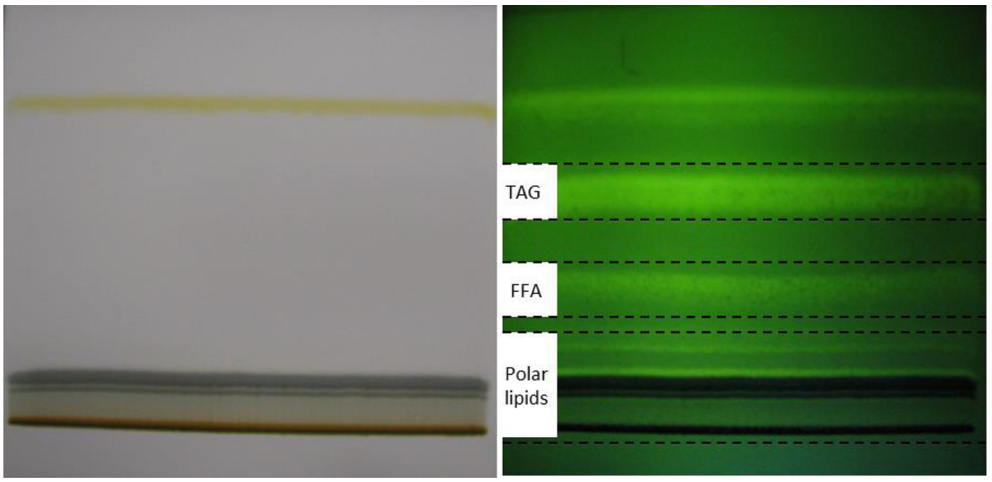

Figure 1. Separation of neutral and polar lipids of *Nanno*. The images on the left and right were taken before and after the fluorescence spray. The dashed lines illustrate how the fractions/silica strips were taken. The developing solvent was hexane/diethyl ether/acetic acid (70:30:1, by vol).

and highest lipid contents (57%), respectively. *Chlamy*, *Chlorella*, and *Nanno* had lipid contents in the range of 18−25%, similar to that of common oilseeds, such as soybeans. Their lipid contents were not unusual as compared to the literature.[3,5,7,25] *Scene* had an exceptionally high level of ash content (30%), followed by the 11% of *Chlorella*. The other three species contained 5−6% ash. Green microalgae contained higher protein contents (25−34%) than *Schizo* (12% proteins). Protein contents of *Chlamy*, *Chlorella*, and *Scene* were generally lower than the values summarized by Becker.[27] It is noted that protein content was not only affected by the growth conditions but also varied with the determination method.[19]

Microalgae Lipid Class Composition. *Nanno* contained the least amount of neutral lipids (15%) but the greatest amount of polar lipids (25%) among the green microalgae (Table 2). Because sterols and hydrocarbons are discussed separately, neutral lipids in this paper are defined as the simple acyl lipids, which include TAG and FFA. *Schizo* was mainly composed of TAG (78%) with <1% of polar lipids. All of the green microalgae had appreciable amounts of chlorophylls (6−17%) and USP (13−19%). *Chlamy* and *Chlorella* had similar lipid class compositions in terms of neutral lipids (51−57%), USP (13%), and chlorophylls (15−17%). Freshwater species were found to contain 27−31% FFA in this study, which more likely resulted from lipid degradation during storage and processing[28] rather than the algae responding to the change of or detrimental growth condition.[29] The relative ratio of TAG/FFA/polar lipids varied by algae species.[2,6] Even for the same species, the compositions of fatty acids and complex lipids in algae heavily fluctuate depending on growth conditions such as light, temperature, nitrogen level, salt stress and the growth stages at which they are harvested.[30,31] Detailed reviews on lipid metabolism in response to various growth factors were given by Harwood and Jones[2] and Guschina and Harwood.[3] For the above reasons, a compositional comparison of fatty acids and complex lipids of the microalgae examined with literature values is not given here. "Others" in Table 2 might include, but is not limited to, the acyl chains of sterol esters, carotenoids, and other polar lipids, such as diacylglycerol trimethylhomoserine ether (DGTS), which were reported as a major component in green algae, and chlorosulfolipids, which were found in freshwater species.[2] The polar lipid composition was very complex as seen by TLC. The lipid components with high polarity covered nearly the entire lower half of the TLC plate. The TLC images of separation of neutral and polar lipids of *Nanno* are given in Figures 1 and 2.

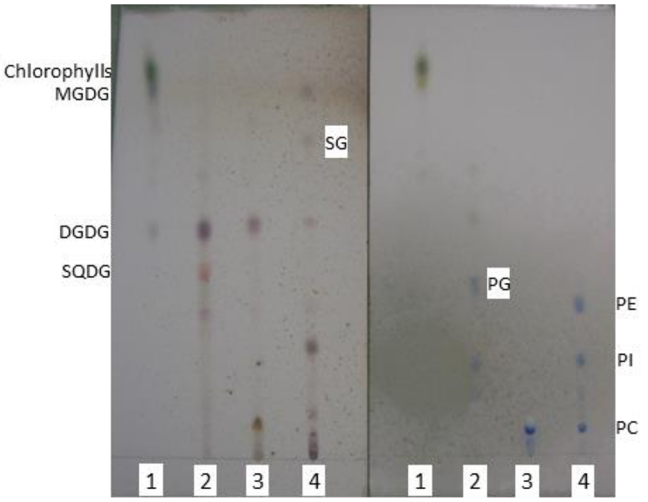

Figure 2. TLC separation of polar lipids of *Nanno*. The plates on the left and right were sprayed with 70% orcinol in sulfuric acid and molybdenum blue, respectively. The samples on the two plates were the same, which were the fractions collected after SPE separation of *Nanno* total lipids. Lanes: 1, acetone fraction; 2, chloroform/methanol (1:1) fraction; 3, chloroform/methanol/water (5:5:1) fraction; 4, soy lecithin. The developing solvent was methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:4, by vol).

FAME Composition of Total Lipids, TAG, FFA, and Polar Lipids. Table 3 presents the FAME composition of unfractionated total lipids of five microalgae, as well as those in their TAG, FFA, and polar lipid fractions separated by normal phase TLC. Table 4 summarizes the distribution of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) of five microalgae as well as their n-3 fatty acid contents.

Major fatty acids in *Chlamy* were C16:0 (27%), C16:4 (6%), C18:1*c* (16%), C18:2 (16%), and C18:3 (10%), representing 75% of total fatty acids. Although there were some differences in the proportion of individual fatty acids when TAG, FFA, and polar lipid fractions were compared with the total lipids of

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Table 3. FAME Composition (relative weight percent) of Unfractionated Total Lipids, TAG, FFA, and Polar Lipids[a]

	Chlamy				*Chlorella*				*Nanno*			
	total lipids	TAG	FFA	polar lipids	total lipids	TAG	FFA	polar lipids	total lipids	TAG	FFA	polar lipids
C8:0									0.3 (0.0)			
C10:0									0.1 (0.0)			0.1
C12:0									0.3 (0.0)	0.8	0.6	0.2
C14:0	1.4 (0.5)	0.3	0.2	5.6	1.3 (0.1)	0.3	0.1	0.2	6.0 (0.2)	5.9	5.4	6.2
C14:1	0.6 (0.2)				0.2 (0.0)				0.2 (0.0)			
C15:0br[b]						0.1	0.1	0.1	0.5 (0.1)			
C15:0									0.3 (0.0)	0.3		0.6
C16:0	26.7 (0.3)	29.2	28.5	25.8	20.0 (0.8)	22.8	21.4	23.7	19.4 (0.1)	18.9	7.6	21.7
C16:1 isomer	4.7 (0.2)	3.5	5.7	3.8	4.0 (0.1)	3.7	3.5	3.5	1.3 (0.6)		0.8	2.8
C16:1	0.2 (0.0)	0.1	0.2		0.4 (0.0)	0.3	0.4	1.5	24.3 (0.1)	25.7	18.7	24.7
C16:2	4.1 (0.1)	3.0	5.1	3.5	6.0 (0.0)	4.2	7.1	6.3	0.3 (0.1)	0.2	0.1	0.3
C16:3	1.9 (0.4)	0.9	0.8	1.1	12.5 (0.5)	9.8	12.2	12.2	1.1 (0.2)			0.9
C16:4	5.6 (0.4)	4.3	5.4	6.8								
C17:0	2.4 (0.1)	0.5	2.8	3	0.2 (0.0)	0.4	0.3	0.8	0.2 (0.1)	0.2	0.1	0.3
C17:1					0.2 (0.0)	0.1	0.2		0.7 (0.1)	0.6	0.5	0.8
C18:0	2.8 (0.1)	4.0	2.7	3.8	1.2 (0.0)	2.4	0.6	5.3	0.4 (0.1)	1.7	0.5	0.6
C18:1*c*	16.3 (0.7)	21.9	14.8	13.4	9.2 (0.3)	12.2	7.8	8.4	3.2 (0.0)	1.9	1.9	3.7
C18:1*t*	2.6 (0.1)	3.2	2.1	2.8	0.9 (0.0)	0.9	0.9	2.6	0.3 (0.0)	0.4	0.4	0.3
C18:2	16.4 (0.0)	15.1	18.1	12.3	22.6 (0.0)	26.5	21.8	17.8	3.8 (0.0)	1.3	2.8	2.4
C18:3γ	4.1 (0.0)	5.2	3.5	3.5					0.5 (0.0)			
C18:3	10.3 (0.3)	7.7	10.4	10.6	21.0 (0.8)	15.7	23.4	18.0				
C18:4												
C20:0	0.3 (0.3)	0.5	0.2	0.5	0.2 (0.0)	0.3	0.1					
C20:1	0.4 (0.0)	0.7	0.2	0.5								
C20:3n-6									0.4 (0.0)	0.6	0.4	0.3
C20:4									6.1 (0.1)	6.8	9.0	5.8
C20:5									30.1 (0.3)	34.4	51.1	28.0
C22:0					0.1 (0.1)	0.1						
C22:5												
C22:6												
C24:0					0.2 (0.1)	0.1	0.1					

	Scene				*Schizo*			
	total lipids	TAG	FFA	polar lipids	total lipids	TAG	FFA	polar lipids
C8:0								
C10:0								
C12:0					0.1 (0.0)	0.1		0.1
C14:0	1.9 (0.6)	1.3	0.5	1.3	4.4 (0.2)	5	4.9	3.6
C14:1	0.3 (0.0)							
C15:0br[b]	1.2 (0.3)	0.6	0.1	0.0				
C15:0	0.3 (0.0)		0.2		1.3 (0.1)	1.5	0.7	1.1
C16:0	31.1 (0.0)	15.8	39.5	15.2	60.1 (1.5)	66.2	71.7	67.2
C16:1 isomer	1.7 (0.1)	0.7	1.3	0.0				
C16:1	0.8 (0.0)	1.1	0.6	1.1	0.1 (0.0)	0.1		0.3
C16:2	0.8 (0.0)	0.7	0.9	0.7				
C16:3	2.4 (0.3)	0.4	1.6	2.6	0.1 (0.0)	0.1		0.4
C16:4	5.5 (0.0)	1.6	6.2	3.5				
C17:0	0.3 (0.0)		0.4	0.5	0.5 (0.0)	0.6	0.7	0.6
C17:1	0.2 (0.0)	1.3	0.1					
C18:0	1.6 (0.0)	2.8	2.0	2.7	1.5 (0.1)	1.8	6.9	2.3
C18:1*c*	10.5 (0.1)	16	10.3	15.5				0.1
C18:1*t*	2.4 (0.1)	2.8	2.0	2.7			0.2	0.1
C18:2	11.1 (0.0)	17.4	10.7	16.9				
C18:3γ	1.5 (0.0)				0.1 (0.0)			
C18:3	21 (0.2)	33.2	20.5	32.4	0.1 (0.0)			0.2
C18:4	4.4 (0.0)	3.5	2.5	3.4	0.1 (0.0)	0.1		
C20:0					0.1 (0.0)	0.1	2.1	0.1
C20:1	0.4 (0.0)	0.5	0.3		0.1 (0.0)			
C20:3n-6					0.1 (0.0)			
C20:4					0.4 (0.0)	0.3		0.1

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Table 3. continued

	Scene				*Schizo*			
	total lipids	TAG	FFA	polar lipids	total lipids	TAG	FFA	polar lipids
C20:5					0.5 (0.0)	0.3		0.6
C22:0	0.2 (0.2)	0.2	0.1	0.5	0.1 (0.0)	0.1		
C22:5					5.1 (0.2)	4.2	3	3.9
C22:6					25.6 (1.6)	19.4	9.7	19.3
C24:0	0.6 (0.0)		0.2	0.0				

[a]Values in parentheses are standard deviations of three replicates. Standard deviations of other fractions are generally <1% and are not presented. [b]"br" stands for "branched".

Table 4. Distribution of Saturated, Monounsaturated, and Polyunsaturated Fatty Acids, and n-3 Fatty Acids Content (Weight Percent) of Unfractionated Total Lipids

fatty acid	*Chlamy*	*Chlorella*	*Nanno*	*Scene*	*Schizo*
saturated (SFA)	33.6	23.2	27.5	37.2	68.1
monounsaturated (MUFA)	24.8	14.9	30.0	16.3	0.1
polyunsaturated (PUFA)	42.4	62.1	42.3	46.7	32.1
n-3	12.2	33.5	37.3	23.4	31.8

Chlamy, the general distributions of saturated versus unsaturated fatty acids and MUFA versus PUFA were the same. The polar lipid fraction contained slightly higher levels of C14:0 and C16:4, but reduced levels of C18:1*c* and C18:2, which was opposite to the TAG fraction. Fatty acid distribution in the FFA fraction was similar to that in total lipids.

Chlorella was rich in C16:0 (20%), C16:3 (13%), C18:1*c* (9%), C18:2 (23%), and C18:3 (21%), which accounted for 86% of the total. *Chlorella* contained the highest amount of PUFA and total unsaturated fatty acids. *Chlorella*'s TAG, FFA, and polar lipid fraction had similar fatty acid compositions.

Nanno was rich in C14:0 (6%), C16:0 (19%), C16:1 (24%), C20:4 (6%), and C20:5 (30%), totaling 85%. *Nanno* also contained the highest amount of n-3 fatty acids, at about 37%. There was a reduced level of C18 fatty acids in *Nanno*. Compared to the total lipids, the FFA fraction contained less C16:0 and C16:1, but had more than half of its fatty acids as C20:5. *Nanno*'s polar lipid and TAG fractions had fatty acid distributiond similar to its total lipids.

Scene was rich in C16:0 (31%), C16:4 (6%), C18:1*c* (11%), C18:2 (11%), and C18:3 (21%), accounting for 80% of the total. The unusual n-3 fatty acid C18:4 was present in *Scene* at 4% level. Studies have shown that C18:4 was as effective as marine oil derived long-chain n-3 fatty acids in providing the health benefits in humans relating to cardiovascular disease.[32] *Scene* had the highest amount of SFA among the four green microalgae. The FFA fraction of *Scene* had fatty acids similar to the total lipids. The TAG and polar lipids had lower levels of C16:0 and C16:4 and higher levels of C18 fatty acids.

Schizo has a narrow distribution of fatty acids, with 68% SFA and 32% PUFA. The main fatty acids were C14:0 (4%), C16:0 (60%), C22:5 (5%), and C22:6 (26%). The unique fatty acid distribution of *Schizo* made this species an ideal substrate for the production of functional lipids because its relatively simple fatty acid composition would facilitate the fractionation or purification of long-chain PUFA. The TAG, FFA, and polar lipid fractions all had high proportions of C16:0 and low levels of C22:5 and C22:6, which suggests that long-chain PUFA might have been oxidized during the TLC fractionation, or some of them might be present in other lipid classes that were not identified. The ability of efficient accumulation of DHA and DPA has made *Schizo* a commercial interest for producing purified long-chain n-3 PUFA as a nutritional ingredient in foods.[33]

The similarity of fatty acid composition of FFA to that of total lipids for most species suggests that these FFAs were released from lipid degradation as mentioned earlier. There are

Table 5. Glycolipids and Phospholipids Composition (Weight Percent) of Unfractionated Total Lipids by LC-MS[a]

	Chlamy	*Chlorella*	*Nanno*	*Scene*	*Schizo*
total glycolipids	9.02	0.43	11.99	0.35	0.07
DGDG	4.28 (0.34)	0.34 (0.03)	9.09 (0.53)	0.13 (0.01)	0.02 (0.01)
MGDG	4.29 (0.15)	0.08 (0.00)	1.71 (0.12)	0.19 (0.06)	0.05 (0.09)
SQDG	0.46 (0.1)	0.01 (0.01)	1.19 (0.04)	0.03 (0.01)	0
total phospholipids	0.64	0.28	12.59	0.34	0.80
PG	0.22 (0.01)	0.13 (0.00)	2.89 (0.12)	0.11 (0.04)	0.11 (0.01)
LPG	0.02 (0.01)	0.00 (0.01)	0.29 (0.00)	0.03 (0.02)	0.05 (0.01)
LPC	0	0	1.05 (0.02)	0.01 (0.00)	0
LPE	0	0	0.13 (0.01)	0	0
PC	0.01 (0.01)	0.06 (0.00)	5.10 (0.14)	0.12 (0.00)	0.55 (0.04)
PE	0.01 (0.00)	0	0.34 (0.02)	0.02 (0.00)	0
PI	0.36 (0.03)	0.07 (0.00)	2.77 (0.05)	0.04 (0.01)	0.06 (0.00)
PS	0.01 (0.00)	0.01 (0.00)	0	0.00 (0.01)	0.01 (0.01)
PA	0.01 (0.00)	0	0.01 (0.00)	0.01 (0.00)	0
total polar lipids	9.66	0.71	24.58	0.69	0.87

[a]Values in parentheses are the differences of two replicates.

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Table 6. Molecular Species of Glycolipids and Phospholipids (Nanomoles per Milligram of Total Lipids)[a]

lipid molecular species (total acyl carbons:total double bonds)	mass	*Chlamy*	*Chlorella*	*Nanno*	*Scene*	*Schizo*
DGDG (34:6)	926.6	1.12	1.09	0.29	0.05	0.01
DGDG (34:5)	928.6	4.36	0.79	23.59	0.08	0.03
DGDG (34:4)	930.6	7.40	0.45	0.24	0.17	0.00
DGDG (34:3)	932.6	8.73	0.27	1.77	0.47	0.01
DGDG (34:2)	934.6	6.20	0.15	1.68	0.13	0.00
DGDG (34:1)	936.6	15.00	0.46	0.80	0.12	0.03
DGDG (36:6)	954.6	1.14	0.19	24.52	0.10	0.01
DGDG (36:5)	956.6	0.81	0.12	41.77	0.14	0.00
DGDG (36:4)	958.6	0.52	0.06	0.00	0.04	0.00
DGDG (36:3)	960.6	0.26	0.01	0.23	0.03	0.04
DGDG (36:1)	964.7	0.09	0.01	0.05	0.03	0.08
DGDG (38:6)	982.6	0.00	0.00	0.57	0.01	0.00
DGDG (38:5)	984.6	0.01	0.00	0.13	0.00	0.00
DGDG (38:3)	988.7	0.00	0.00	0.13	0.01	0.00
total DGDG		45.64	3.60	95.77	1.38	0.21
MGDG (34:6)	764.5	9.45	0.31	0.13	0.21	0.04
MGDG (34:5)	766.5	14.03	0.37	11.58	0.19	0.00
MGDG (34:4)	768.5	11.29	0.12	0.00	0.32	0.01
MGDG (34:3)	770.5	8.54	0.06	0.22	0.68	0.00
MGDG (34:2)	772.6	6.14	0.02	0.23	0.10	0.00
MGDG (34:1)	774.6	4.11	0.02	0.06	0.12	0.00
MGDG (36:6)	792.5	0.58	0.04	1.65	0.04	0.02
MGDG (36:5)	794.5	0.64	0.01	6.59	0.07	0.00
MGDG (36:4)	796.6	0.39	0.01	0.05	0.06	0.00
MGDG (36:3)	798.6	0.37	0.00	0.06	0.03	0.00
MGDG (36:1)	802.6	0.08	0.03	0.58	0.13	0.08
MGDG (38:6)	820.6	0.01	0.04	0.47	0.20	0.34
MGDG (38:5)	822.6	0.00	0.03	0.17	0.02	0.11
MGDG (38:4)	824.6	0.02	0.00	0.03	0.11	0.05
MGDG (38:3)	826.6	0.00	0.00	0.13	0.09	0.00
total MGDG		55.65	1.06	21.95	2.37	0.65
SQDG (32:2)	789.5	0.01	0.00	0.65	0.00	0.00
SQDG (32:1)	791.5	0.07	0.00	13.84	0.01	0.00
SQDG (32:0)	793.5	4.46	0.08	0.26	0.23	0.01
SQDG (34:3)	815.5	0.39	0.01	0.03	0.02	0.00
SQDG (34:2)	817.5	0.33	0.00	0.09	0.05	0.00
SQDG (34:1)	819.5	0.42	0.00	0.11	0.03	0.00
total SQDG		5.68	0.09	14.98	0.34	0.01
PG (32:1)	738.5	0.09	0.12	5.87	0.07	0.00
PG (32:0)	740.5	0.74	0.39	0.28	0.13	1.33
PG (34:4)	760.5	0.13	0.20	0.03	0.28	0.00
PG (34:3)	762.5	0.34	0.30	0.15	0.08	0.01
PG (34:2)	764.5	0.44	0.43	0.27	0.10	0.00
PG (34:1)	766.5	0.82	0.24	0.27	0.20	0.01
PG (36:6)	784.5	0.25	0.00	25.03	0.14	0.00
PG (36:5)	786.5	0.00	0.00	5.04	0.00	0.00
PG (36:4)	788.5	0.00	0.00	0.17	0.02	0.00
PG (36:2)	792.5	0.01	0.03	0.06	0.23	0.00
PG (36:1)	794.6	0.00	0.00	0.00	0.10	0.02
total PG		2.82	1.71	37.17	1.35	1.37
LPG(16:1)	500.3	0.10	0.01	4.13	0.15	0.05
LPG(16:0)	502.3	0.25	0.05	1.46	0.14	1.00
LPG(18:1)	528.3	0.07	0.01	0.12	0.33	0.01
total LPG		0.42	0.07	5.71	0.62	1.06
LPC (16:1)	494.3	0.00	0.00	10.03	0.02	0.00
LPC (16:0)	496.3	0.00	0.01	1.61	0.05	0.08

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Table 6. continued

lipid molecular species (total acyl carbons:total double bonds)	mass	*Chlamy*	*Chlorella*	*Nanno*	*Scene*	*Schizo*
LPC (18:3)	518.3	0.00	0.01	0.95	0.00	0.00
LPC (18:2)	520.3	0.00	0.00	5.16	0.01	0.00
LPC (18:1)	522.3	0.00	0.01	3.08	0.03	0.00
total LPC		0.00	0.03	20.83	0.11	0.08
LPE (16:1)	452.3	0.00	0.00	1.80	0.02	0.00
LPE (16:0)	454.3	0.01	0.01	0.29	0.01	0.03
LPE (18:2)	478.3	0.00	0.00	0.33	0.01	0.00
LPE (18:1)	480.3	0.00	0.00	0.34	0.01	0.00
total LPE		0.01	0.01	2.76	0.05	0.03
PC (32:0)	734.6	0.00	0.00	0.43	0.04	0.06
PC (34:4)	754.5	0.00	0.08	3.54	0.02	0.01
PC (34:3)	756.5	0.02	0.16	13.92	0.08	0.01
PC (34:2)	758.6	0.06	0.12	14.52	0.13	0.01
PC (34:1)	760.6	0.04	0.04	3.37	0.22	0.01
PC (36:6)	778.5	0.01	0.05	6.75	0.02	0.09
PC (36:5)	780.5	0.01	0.06	7.39	0.02	0.05
PC (36:4)	782.6	0.01	0.10	4.18	0.08	0.02
PC (36:3)	784.6	0.01	0.06	3.61	0.09	0.01
PC (36:2)	786.6	0.02	0.05	1.19	0.57	0.01
PC (36:1)	788.6	0.01	0.01	0.42	0.13	0.02
PC (38:6)	806.6	0.00	0.01	3.65	0.04	5.13
PC (38:5)	808.6	0.00	0.01	1.48	0.03	1.07
PC (38:4)	810.6	0.00	0.01	0.19	0.02	0.01
PC (38:3)	812.6	0.00	0.01	0.29	0.01	0.01
PC (38:2)	814.6	0.00	0.03	0.77	0.03	0.01
PC (40:5)	836.6	0.00	0.00	0.18	0.00	0.03
PC (40:4)	838.6	0.00	0.00	0.07	0.00	0.24
total PC		0.19	0.80	65.95	1.53	6.80
PE (32:3)	686.5	0.00	0.00	0.12	0.00	0.00
PE (32:2)	688.5	0.00	0.00	1.46	0.02	0.00
PE (32:1)	690.5	0.02	0.00	0.31	0.02	0.00
PE (34:4)	712.5	0.00	0.00	0.10	0.00	0.00
PE (34:3)	714.5	0.00	0.00	0.16	0.00	0.00
PE (34:2)	716.5	0.03	0.02	0.15	0.02	0.00
PE (36:6)	736.5	0.00	0.00	0.48	0.01	0.00
PE (36:5)	738.5	0.00	0.00	0.65	0.00	0.00
PE (36:4)	740.5	0.00	0.00	0.17	0.01	0.00
PE (38:6)	764.5	0.00	0.00	0.53	0.00	0.03
PE (38:5)	766.5	0.00	0.00	0.27	0.01	0.01
total PE		0.05	0.02	4.40	0.09	0.04
PI(32:3)	822.5	0.00	0.03	0.13	0.01	0.00
PI (32:2)	824.5	0.00	0.05	3.25	0.02	0.01
PI (32:1)	826.5	0.01	0.02	20.45	0.09	0.03
PI (32:0)	828.5	0.12	0.01	0.27	0.12	0.62
PI (34:4)	848.5	0.02	0.03	0.17	0.00	0.00
PI (34:3)	850.5	0.16	0.11	0.61	0.02	0.01
PI (34:2)	852.5	0.40	0.23	3.21	0.04	0.01
PI (34:1)	854.5	3.08	0.08	3.57	0.06	0.01
PI (36:6)	872.5	0.02	0.01	0.58	0.00	0.02
PI (36:5)	874.5	0.01	0.02	0.75	0.01	0.03
PI (36:4)	876.5	0.04	0.05	0.12	0.02	0.02
PI (36:1)	882.6	0.20	0.10	0.01	0.02	0.02
total PI		4.06	0.74	33.12	0.41	0.78

[a]Values are the averages of two replicates. The difference of two replicates is not included. Lipid molecular species that had concentrations below 0.1 nmol/mg are not included.

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Table 7. Composition (Weight Percent) of Unsaponifiable Matters[a]

	Chlamy	*Chlorella*	*Nanno*	*Scene*	*Schizo*
GC quantifiable of total USP	76.5 (1.0)	68.3 (0.3)	47.7 (1.6)	79.9 (4.1)	36.8 (1.2)
of GC quantifiable USP					
hydrocarbons	0.4 (0.0)	3.7 (0.6)	12.9 (0.3)	20.9 (0.4)	0.5 (0.0)
phytol	83.2 (0.4)	80.1 (1.6)	41.1 (0.3)	47.3 (6.8)	0
sterols	13.7 (1.4)	7.8 (4.6)	34.9 (0.2)	22.9 (0.1)	90.0 (0.3)
others	2.7	8.4	11.1	8.9	9.8

[a]Values in parentheses are the differences of two replicates.

a few discrepancies; that is, fatty acids of the three fractions do not account for that in total lipid extracts. This suggests the presence of other acyl lipids, most likely in the "others" fraction in Table 2, that have contributed significantly to the total lipid fatty acid composition.

As reviewed by Harwood and Jones,[2] freshwater algae typically contain fatty acids similar to those of plants, but with a relatively high proportion of C16 fatty acids and a reduced level of C18 unsaturated fatty acids. Among the photosynthetic species examined, freshwater microalgae *Chlamy*, *Chlorella*, and *Scene* had low levels (<1%) of long-chain fatty acids that have more than 20 carbons, but an appreciable quantity (9−19%) of 16-carbon polyunsaturated fatty acids, which were not usually found in plant oils. The two saltwater microalgae, *Nanno* and *Schizo*, had much higher levels of PUFA of C20 (37%) and C22 (31%), respectively, than those found in fish oil, egg yolk, or other available sources. They are good sources of specialty oil. Small quantities of branched-chain fatty acids were found in *Chlorella*, *Nanno*, and *Scene*, in agreement with previous findings for *Chlorella* and *Scenedesmus* species.[34] All of the algae examined contained significant amounts of n-3 fatty acids (12−37%). In particular, *Chlorella*, *Nanno*, and *Schizo* had one-third of their fatty acids as n-3 fatty acids.

Polar Lipids (Glycolipids and Phospholipids) Composition by LC-MS and TLC. MGDG, DGDG, SQDG, and PG are the major components of chloroplast lipids in microalgae.[2,35] They function as membrane structural lipids and signaling molecules in the cells. These four classes of polar lipids represented 96, 79, 61, and 67% of the total polar lipids of the green microalgae *Chlamy*, *Chlorella*, *Nanno*, and *Scene*, respectively, as quantified by LC-MS (Table 5). TLC analysis confirmed the presence of MGDG, DGDG, SQDG, PC, PE, PI, and PG in the green microalgae by comparison with commercial standards and the literature.[20] The decrease of the ratio of water in the developing solvent from 9 (as used in the literature[20]) to 4 enabled the TLC separation of PG and SQDG and moved PE further below PG. An example of the separation of polar lipids of *Nanno* is given in Figure 2. Steryl glucoside, which was previously found in *Nanno*,[5] was not detected in the present work as examined by the TLC method.

Nanno contained the greatest amount of total polar lipids, 3 times more than that of *Chlamy*, the second ranked. It has been reported that phospholipids are generally present at minor quantities in microalgae,[6] as observed for *Chlamy* in this study. However, the two saltwater species, *Nanno* and *Schizo*, had more phospholipids than glycolipids. *Chlorella* and *Scene* had roughly the same amount of phospholipids as glycolipids. Unlike other species containing one or two types of major phospholipids, *Nanno* contained significant amounts of PC, PG, PI, and LPC. The high level of phospholipids in *Nanno* was suggested to relate to EPA biosynthesis.[3] The proportion of individual glycolipid varied with species. DGDG was dominant in *Nanno*. In addition, *Nanno* contained the highest level of SQDG (12%) among the microalgae examined. The ratio of MGDG to DGDG in *Chlamy* was close to 1, and SQDG represent only 5% of polar lipids in *Chlamy*. *Schizo* had a very low level of polar lipids, which were mainly phospholipids. The MS spectra of the polar lipids of *Nanno*, as an example, are given in the Appendix I (Supporting Information).

Glycolipids have been shown to have various biological properties including antiviral, antitumor, anti-inflammatory, and immune-suppressive activities.[36] Some of the biological properties of MDGD and DGDG might be dependent on the structure of sugar and acyl moieties.[37] Table 6 reports the molecular species of glycolipids and phospholipids determined by LC-MS. On the basis of the fatty acid composition in Table 3, we could reasonably infer the major acyl groups of each individual polar lipid. For *Nanno*, the dominant SQDG isoform (32:1) is composed mainly of the 16:1/16:0 diacyl group, in agreement with the result of He et al.[8] The principal MGDG isoform (34:5) mainly contains the 14:0/20:5 diacyl group. A minor MGDG isoform (36:5) contains the 16:0/20:5 diacyl group. DGDG is rich in 16:0/20:5, 16:1/20:5, and 14:0/20:5 fatty acids. The major PG isoform (36:6) mainly comprises 16:1/20:5. Two minor PG isoforms contain 16:1/16:0 and 16:0/20:5. The principal LPG, PE, and LPE contain the 16:1 acyl group. LPC mainly comprises 16:1 and 18:2 (minor) acyl groups. The major PC isoforms, 34:3 and 34:2, mainly comprise the 16:1/18:2 and 16:1/18:1 or 16:0/18:2 diacyl groups. The dominant PI isoform (32:1) is composed mainly of the 16:1/16:0 diacyl group.

For *Chlamy*, the major SQDG contains 16:0 fatty acid. The major DGDG and MGDG isoforms have 34 carbons and 1−6 double bonds distributed in two acyl groups, which covers all of the C16 and C18 major fatty acids in *Chlamy* total lipids.

The principal isoform (38:6) of PC, which is the most abundant polar lipid of *Schizo*, is composed of the 16:0/22:6 diacyl group, in agreement with the previous study.[38]

The acyl groups of polar lipids in *Chlorella* and *Scene* are not separately discussed here. In general, SQDG, LPG, PE, LPE, LPC, PC, and PI prefer SFA and MUFA, whereas MGDG, DGDG, and PG prefer PUFA, confirming previous results.[3,6,8]

Unsaponifiable Matters. *Scene* had the highest level of USP (19% of total lipids), followed by *Nanno* (15%), *Chlorella* and *Chlamy* (13% for both), and *Schizo* (2%) (Table 2). These values differed from the literature data to different extents.[5,9] The composition of USP was determined by GC-MS. Table 7 shows that a significant portion of USP was not GC quantifiable (with a SAC-5 column). GC-quantifiable USP of freshwater microalgae *Chlamy*, *Chlorella*, and *Scene* accounted for 68−80% of their total USP, much higher than the 37−48% of saltwater microalgae *Nanno* and *Schizo*. Wang and Wang[5] reported up to 70 and 30% of GC-unquantifiable USP in *Nanno* and *Schizo*, respectively. The GC-unquantifiable USP were nonvolatiles

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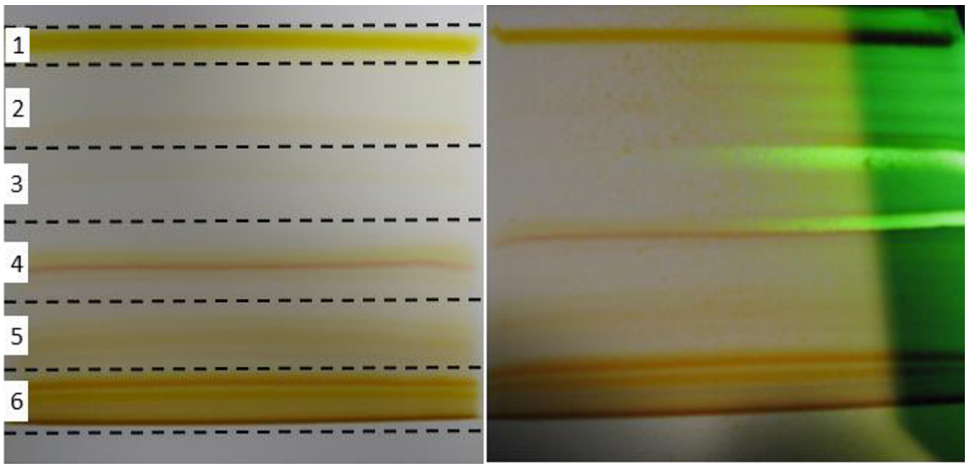

Figure 3. Preparative TLC image of unsaponifiables of *Nanno*. The image on the left was taken before fluorescence spray was applied. The dashed lines illustrate how the six fractions were taken. The image on the right was visualized under UV light after the right half of the plate was sprayed with fluorescence indicator. The developing solvent was hexane/diethyl ether/acetic acid (60:40:1, by vol).

under our GC conditions, present mainly in TLC fractions 5 and 6 (Figure 3), and had color from orange to red. They showed peaks upon HPLC normal phase elution (data not shown). Many of these unidentified USP were likely carotenoids. Carotenoids such as β-carotene and xanthophylls are commonly found in microalgae, as they are not only utilized in photosynthesis but also function as antioxidants.[7,9] The USP of *Scenedesmus quadricauda*, for example, contained 6% carotenoids and 0.5% β-carotene.[9] We did not further characterize these compounds, so the HPLC chromatograms are not included.

TLC fractionation of USP provided information on the relative polarity of the various compounds, which further assisted in structure identification. The preparative TLC separation of *Nanno* USP as illustrated in Figure 3 demonstrates the complexity of microalgae USP. A comparison of the USP of five examined microalgae is shown in the analytical TLC image of Figure 4.

The major USP components of green microalgae were hydrocarbons (TLC fraction 1, Figure 3), phytol (TLC fractions 2 and 3), and sterols (TLC fraction 4), in agreement with the findings of Paoletti et al.[9] Phytol is a diterpene alcohol and is the hydrocarbon tail of chlorophylls. The presence of phytol is attributed to the degradation of chlorophyll during saponification of the total lipids.[10] The level of phytol in the USP can be used as a quantitative indicator of the pigment. *Chlamy* and *Chlorella* contained the highest amount of phytol, representing 80−83% of their GC quantifiable USP, 2-fold higher than those of *Nanno* and *Scene*, which had phytol contents ranging from 40 to 50% (Table 7). *Scene* had the greatest amount of hydrocarbons (21%), whereas *Chlamy* and *Schizo* contained nearly no hydrocarbons. Sterols of the examined green microalgae accounted for 8−35% of total GC quantifiable USP. *Schizo* had a very different USP composition in that 90% of its GC-quantifiable USP was sterols despite the total USP of *Schizo* accounting for only 2% of its total lipids. In all of the species examined, higher amounts of sterols than hydrocarbons were found, in agreement with Paoletti et al.[9]

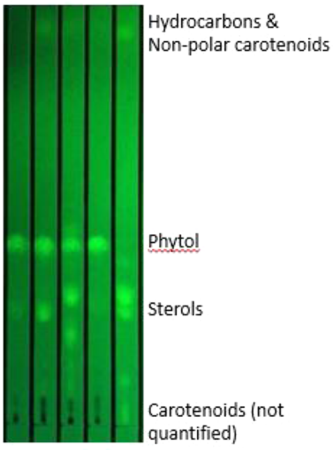

Figure 4. Analytical TLC image of unsaponifiables of microalgae. Lanes (from left to right): *Chlamy*, *Scene*, *Nanno*, *Chlorella*, and *Schizo*. The developing solvent was hexane/diethyl ether/acetic acid (60:40:1, by vol).

Table 8 presents the markedly different distribution pattern of hydrocarbons in these microalgae. Squalene was detected in all of the algae except for *Nanno* and was the only hydrocarbon found in *Schizo*, consistent with the previous result.[5]

Chlamy was mainly composed of odd-chain monounsaturated hydrocarbons, that is, 82% heptadecene and 18% nonadecene.

Chlorella had 88% of heptadecane and 13% of squalene. This differs from the previous result on a saltwater *Chlorella*, which had a wide range of hydrocarbons from C14 to C30 including branched-chain species.[7] However, two earlier papers showed that C17 alkane and alkene were the major components in *Chlorella* sp., albeit they were of different strains.

The major components in *Scene* were pentacosene (63%), heptadecane (6%), $C_{25}H_{48}$ (22%), and $C_{27}H_{54}$ (6%). The abundance of C25 hydrocarbons and the presence of a small amount of C_{27} alkene in *Scene* were also observed in previous studies except for the discrepancy on the level of unsaturation.[9,39]

Nanno had a variety of hydrocarbons from C14 to C30 including both saturated and unsaturated and even- and odd-chain species. The most abundant hydrocarbons in *Nanno* were heptadecane (8%), $C_{25}H_{48}$ (14%), hexacosene (7%), $C_{27}H_{52}$ (20%), and $C_{30}H_{44}$ (18%). Because *Nanno* had 35% sterols (mainly cholesterol) but no squalene, it is reasonable to consider the presence of certain types of biological precursor of

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Table 8. Composition (Relative Weight Percent) of Hydrocarbons of the Five Microalgae[a]

RT[b] (min)	hydrocarbon[c]	formula	*Chlamy*	*Chlorella*	*Nanno*	*Scene*	*Schizo*
2.0	tetradecane	$C_{14}H_{30}$			tr[d]		
2.7	hexadecane	$C_{16}H_{34}$			0.8 (0.0)		
3.1	8(3)-heptadecene	$C_{17}H_{34}$	81.8 (1.5)		2.7 (0.2)		
3.1	heptadecane	$C_{17}H_{36}$		87.5 (1.3)	8.1 (0.2)	5.7 (0.6)	
3.6	9-octadecene	$C_{18}H_{36}$			tr		
3.7	octadecane	$C_{18}H_{38}$			3.3 (0.1)		
4.2	5-nonadecene	$C_{19}H_{38}$	18.1 (1.5)		2.6 (1.4)		
4.3	nonadecane	$C_{19}H_{40}$			3.9 (0.0)		
5.7	9-henicosene	$C_{21}H_{42}$			0.7 (0.1)		
5.7	henicosane	$C_{21}H_{44}$			0.9 (0.2)		
7.2	*	$C_{23}H_{46}$			2.1 (0.0)		
8.7	12-pentacosene	$C_{25}H_{50}$			3.5 (0.0)	63.0 (0.3)	
9.0	*	$C_{25}H_{48}$			14.0 (0.3)	21.5 (0.2)	
9.5	1-hexacosene	$C_{26}H_{52}$			6.8 (0.3)	1.7 (0.0)	
10.0	*	$C_{27}H_{52}$			19.8 (0.3)	2.0 (0.0)	
10.2	*	$C_{27}H_{54}$			6.0 (0.2)	5.5 (0.0)	
10.3	*	$C_{27}H_{50}$			5.4 (0.2)		
10.8	*	$C_{27}H_{54}$			2.0 (0.1)		
11.2	squalene	$C_{30}H_{50}$	tr	12.7 (1.0)		0.60	100
11.5	*	$C_{30}H_{44}$			17.5 (0.0)		

[a]Values in parentheses are the differences of two replicates. The double bond position of unsaturated hydrocarbons is not confirmed. [b]RT, retention time. [c]Structures of compounds marked with an asterisk (*) are not completely resolved so that only their chemical formulas are given. [d]tr, trace

Table 9. Composition (Relative Weight Percent) of Sterols of the Green Microalgae[a]

RT[b](min)	compound[c]	MW or formula	*Chlamy*	*Chlorella*	*Nanno*	*Scene*	*Schizo*
12.6	n/a	376[d]	1.4 (0.1)	2.7 (0.6)			
13.5	n/a	390[d]	1.2 (0.1)				
14.0	cholesterol	$C_{27}H_{46}O$	2.7 (0.1)	tr[e]	83.5 (0.1)	tr	70.8
14.6	anthraergostatetraenol	$C_{28}H_{42}O$		4.5 (0.1)			
14.7	lathosterol	$C_{27}H_{46}O$					8.3
15.1	ergosterol	$C_{28}H_{44}O$	38.1 (2.4)	65.5 (7.0)			
15.1	cholesta-3,5-dien-7-one	$C_{27}H_{42}O$					1.6
15.2	5,6-dihydroergosterol	$C_{28}H_{46}O$	5.3 (0.4)				
15.9	ergosta-5,24(28)-dien-3β-ol, or ergosta-5,8-dien-3β-ol	$C_{28}H_{46}O$		7.2 (6.7)			3.7
16.1	γ-ergostenol	$C_{28}H_{48}O$	11.9 (1.5)	15.2 (0.2)		27.9 (0.0)	
	Δ7-stigmasterol	$C_{29}H_{48}O$	24.6 (1.3)				1.8
16.5	chondrillasterol	$C_{29}H_{48}O$	5.3 (0.5)			68.1 (0.0)	7.1
16.5	fucosterol	$C_{29}H_{48}O$			7.0 (0.0)		
16.6	isofucosterol	$C_{29}H_{48}O$			9.5 (0.1)		
17.4	stigmasta-7,16-dien-3β-ol	$C_{29}H_{48}O$					3.6
17.4	Δ7-chondrilla-stenol	$C_{29}H_{50}O$	2.7 (0.1)			4.1 (0.0)	
17.6	n/a	$C_{30}H_{50}O$					2.3
17.8	9,19-cyclolanost-24-en-3β-ol	$C_{30}H_{50}O$	6.7 (1.1)	5.0 (0.0)			0.9

[a]Values in parentheses following means are the differences of two replicates. We did not have enough *Schizo* samples to do the second replicate of saponification. [b]RT, retention time. [c]The mass spectra of compounds marked with "n/a did not match those given by MS NIST library, so that only their formula or MW is given. The sterol compounds are listed in the order of their GC retention time on the SAC-5 column. [d]Mass pattern similar to anthraergostatetraenol. [e]tr, trace.

sterols that have structures similar to squalene. This compound could be $C_{30}H_{44}$, which accounted for 18% of hydrocarbons in *Nanno* as shown in Table 8.

Sterol distribution in microalgae greatly varies with the microalgae classes, genera, and species.[40] The occurrence of sterols in these microalgae in relation to their evolutionary history and growth conditions has been extensively discussed in the literature cited.[40] Sterols are essential components of the membranes of eukaryotic organisms, having various biological functions.[40] Beneficial health effects of phytosterol have been reported.[41] A recent study has shown that sterol extract from *Schizochytrium* sp. had cholesterol-lowering activity.[42] The great diversity of sterol distribution in microalgae has long been recognized,[10,11,40] as observed in the present study. As shown in Table 9, saltwater microalgae *Nanno* and *Schizo* were rich in cholesterol, whereas freshwater microalgae *Chlamy*, *Chlorella*, and *Scene* were mainly composed of C28−C30 phytosterols, in agreement with previous reviews.[12,40] Both cholesterol and phytosterols were found in the species examined. Ergosterol (ergosta-5,7,22-trien-3β-ol) was the major sterol in *Chlamy* and *Chlorella*. *Chlamy*, *Chlorella*, and *Scene* contained 12−28% of γ-ergostenol (5α-ergost-7-en-3β-ol). Additionally, *Chlamy* contained 25% of Δ7-stigmasterol (stigmasta-5,7,22-trien-3β-ol), 5% of chondrillasterol (5α-stigmasta-7,22-dien-3β-ol), 3% of

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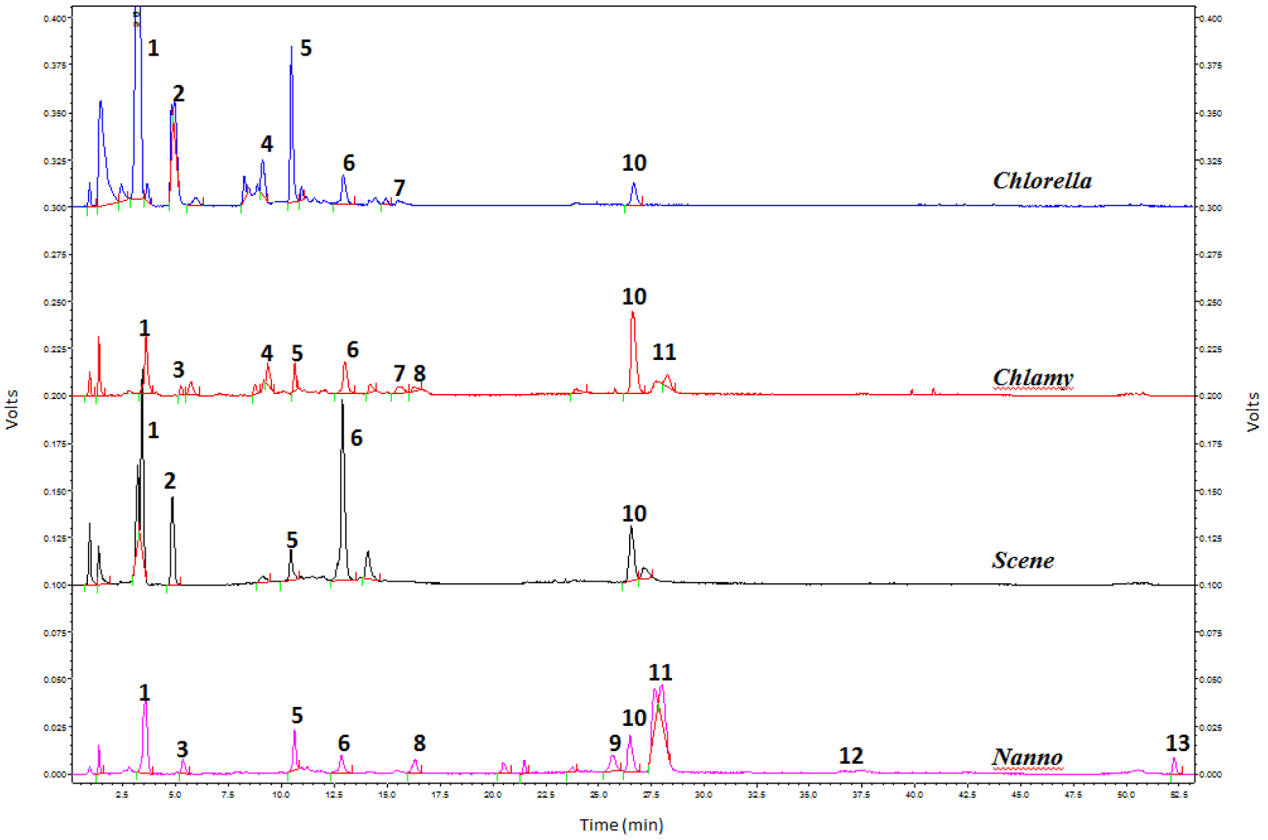

Figure 5. HPLC normal phase separation of polar lipid fractions of four green microalgae (qualitative). Peaks: 1, pheophytin *a*; 2, pheophytin *b*; 3, hydroxypheophytin *a*; 4, hydroxypheophytin *b*; 5, unidentified; 6, chlorophyllide *b*; 7, hydroxychlorophyllide *b*; 8, MGDG; 9, PG; 10, DGDG; 11, SQDG; 12, PI; 13, PC.

each of cholesterol and Δ7-chondrillastenol (stigmast-7-en-3-ol, (3*β*,5*α*,24S)-), and 5% of 5,6-dihydroergosterol (ergosta-7,22-dien-3*β*-ol), which is generally in agreement with the results of Paoletti et al.[10] on five Chlorophyceae. The characteristics of predominant chondrillasterol (68%) and *γ*-ergostenol (28%) of *Scene* in the present work are consistent with those found in *S. obliquus* examined by Patterson.[11] The principal sterols of *Chlorella* were ergosterol (66%) and *γ*-ergostenol (15%), differing from previous results showing the predominance of C29 sterols.[11−14,40] However, Rzama et al.[15] also found that ergosterol was the principal sterol in *C. vulgaris*. *Nanno* contained 84% of cholesterol as its major sterol, with the remaining being fucosterol and its isomer. Fucosterol was identified as the dominant sterol of brown algae,[11] but it was also found in some green algae in a small amount.[40] The other saltwater microalga, *Schizo*, had a more diverse distribution of sterols when compared to *Nanno*. Besides the dominant cholesterol (71%), *Schizo* contained a few C29 phytosterols as minor components. Although the relative percentages of sterols in *Schizo* differed from the reported values, the types of sterols were generally similar to those in the literature.[42]

Other GC-quantifiable USP in Table 7 included compounds having intermediate polarity (TLC fraction 2, Figure 3), such as 6,10,14-trimethyl-2-pentadecanone, which was found in *Chlamy*, *Nanno*, and *Scene* in small quantities, and some unidentified substances. A trace amount of *α*-tocopherolquinone was detected in the USP of *Nanno*. It is a membrane-bound quinone and previously found in blue-green algae.[43] Straight-chain alcohols as reported by Paoletti et al.[10] were not detected. Fatty amides were previously found in freshwater green algae.[44] In the present work, *m*/*z* 59 and 72 were observed in *Chlamy*'s USP (TLC fraction 5, Figure 3), which are the characteristic amide peaks resulting from McLaffery rearrangement of *γ*-cleavage,[45] indicating the presence of a trace of fatty amide in *Chlamy*.

Chlorophylls and Related Metabolites. Chlorophylls are the principal pigments involved in photosynthesis. They are natural food coloring agents and valuable bioactive compounds showing antimutagenic activity and other health benefits.[46,47] Green microalgae contain a great amount of chlorophyll and its metabolites for photosynthesis, suggesting a potential opportunity for value-added coproduct if an effective extraction and fractionation method is developed.[48] In addition, the separation of chlorophylls from the acyl lipids of microalgae also will improve the quality of the produced biodiesel.[49]

Pheophytins and chlorophyllides are derived from chlorophylls after loss of the central Mg atom and the phytol chain, respectively. They are both essential metabolites in chlorophyll biosynthesis and breakdown. The large amount of phytol that was detected in GC-MS analysis of USP suggested that chlorophylls were present in these green microalgae at a significant level. LC/APCI revealed the presence of the following degradation products of chlorophylls: pheophytin *a* [(*m*/*z* 870 ([M − H]$^-$)], pheophytin *b* [(*m*/*z* 884 ([M − H]$^-$)], hydroxypheophytin *a* [(*m*/*z* 886 ([M − H]$^-$)], hydroxypheophytin *b* [(*m*/*z* 900 ([M − H]$^-$)], chlorophyllide *b* [(*m*/*z* 627 ([M − H]$^-$)], and hydroxychlorophyllide *b* [(*m*/*z* 643 ([M − H]$^-$)] (Figure 5). The *m*/*z* values of the molecular ions found from the LC/APCI agreed with the reported values.[50,51] The detection of these compounds suggested that

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Table 10. Temperature of Completion of Melting and Onset of Crystallization of Microalga Total Lipids and Their Methyl and Isopropyl Esters[a]

	methyl ester		isopropyl ester		total lipids	
	melting (°C)	crystallization (°C)	melting (°C)	crystallization (°C)	melting (°C)	crystallization (°C)
Chlamy	14.1	−1.0	−5.0	−12.5	48.0	10.0
Chlorella	10.8	−3.8	−4.1	−14.8	30.1	1.5
Nanno	5.4	−4.0	−10.3	−14.0	66.7	47.1
Scene	38.4	31.0	32.5	25.5	57.2	38.9
Schizo	26.2	17.5	13.2	1.8	53.8	25.9

[a]Values are the means of duplicate samples. All differences between the two replicates of melting temperature are <1 °C, and all differences between the duplicates of crystallization are <3 °C.

both chlorophyll *a* and chlorophyll *b* were present in these green microalgae. Peak 5 in Figure 5 was not identified. It had the same retention time as chlorophyll *a* [(*m*/*z* 928 ([M + Cl]$^-$)], but an *m*/*z* of 902. Quantification of these individual pigments was not carried out. Further structural confirmation with MS/MS is needed. None of chlorophylls or its metabolites were found in *Schizo*.

DSC Melting and Crystallization Temperatures of the Total Lipids of Microalgae and Their Methyl and Isopropyl Esters. Knowledge of the melting and crystallization behavior of microalgae lipids is useful in determining their suitable applications. Table 10 shows that melting points of total lipids of the microalgae examined ranged from 30 to 67 °C, with the highest melting temperature from *Nanno*. The high melting temperatures are caused by the saturated acyl lipids as well as the high melting components in USP such as sterols. When used as biodiesel, total lipids would be converted to methyl (or isopropyl) esters. Monoesters of fatty acids have lower melting and crystallization temperatures than triacylglycerides, phospholipids, and glycolipids. Methyl and isopropyl esters of the microalgae had much reduced melting temperatures compared to their total lipids. The greatest reduction was found in *Nanno*, with the lowest melting point of 5 °C. The current melting temperatures of the esters of these algae would not be satisfactory in biofuel application. The presence of sterols, chlorophyll, and other substances that are soluble in organic solvent would act as crystallization initiators, adversely affecting the quality of the fuels. Appropriate fractionation technology has to be developed to remove these high-melting components. The melting and crystallization behavior of microalgae lipids also have an impact on how they are utilized in food and functional health products.

In summary, lipid characterizations of five microalgae including freshwater and saltwater types are presented. A combination of instrumental analyses was used to obtain the complete lipid profile. All of the examined microalgae are good sources of n-3 fatty acids. *Nanno* and *Chlamy* contained significant amounts of bioactive glycolipids. Freshwater microalgae are rich in phytosterols, whereas saltwater species are rich in cholesterol. The compositional information and methodology presented in this paper are valuable references for future study on algae strain selection and algae cultivation optimization, as well as for the production of value-added coproducts from microalgae.

■ ASSOCIATED CONTENT

Supporting Information

Appendix I. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Chlamy	*Chlamydomonas reinhardtii*
Chl	chlorophyll
Chld	chlorophyllide
Chlorella	*Chlorella vulgaria*
DGDG	digalactosyldiacylglycerol
DGTS	diacylglycerol trimethylhomoserine ether
FFA	free fatty acid
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LPG	lysophosphatidylglycerol
LPI	lysophosphatidylinositol
MGDG	monogalactosyldiacylglycerol
MUFA	monounsaturated fatty acid
Nanno	*Nannochloropsis* sp.
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
SFA	saturated fatty acid
Scene	*Scenedesmus* sp.
Schizo	*Schizochytrium limacinum* SR-21
SG	steryl glucoside
SQDG	sulfoquinovosyl diacylglycerol
TAG	triacylglycerols
USP	unsaponifiable matters
TLC	thin layer chromatography

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