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Extraction of Algal Lipids and Their Analysis by HPLC and Mass Spectrometry

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Abstract Algae are a promising source of biofuel but claims about their lipid content can be ambiguous because extraction methods vary and lipid quantitation often does not distinguish between particular lipid classes. Here we compared methods for the extraction of algal lipids and showed that 2-ethoxyethanol (2-EE) provides superior lipid recovery (>150–200 %) compared to other common extraction solvents such as chloroform:methanol or hexane. Extractions of wet and dry algal biomass showed that 2-EE was more effective at extracting lipids from wet rather than dried algal pellets. To analyze lipid content we used normal-phase HPLC with parallel quantitation by an evaporative light scattering detector and a mass spectrometer. Analysis of crude lipid extracts showed that all major lipid classes could be identified and quantified and revealed a surprisingly large amount of saturated hydrocarbons (HC). This HC fraction was isolated from extracts of bioreactor-grown algae and further analyzed by HPLC/MS, NMR, and GC/MS. The results showed that the sample consisted of a mixture of saturated, straight- and branched-chain HC of different chain lengths. These algal HC could represent an alternative biofuel to triacylglycerols (TAG) that could feed directly into the current petroleum infrastructure.

Keywords Algae · Biodiesel · Biofuels · Lipids · ELSD · HPLC/MS · GC/MS · Lipid extraction · Lipidomics

Abbreviations

APCI	Atmospheric pressure chemical ionization
BC	β-Carotene
CE	Cholesteryl ester
CHL	Chlorophylls & chlorophyll catabolites
DAG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
DMF	Dimethylformamide
ELSD	Evaporative light scattering detector
FAME	Fatty acid methyl ester
FFA	Free fatty acid
GL	Glycolipid
HC	Hydrocarbon
MAG	Monoacylglycerol
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
PHY	Phytol
PL	Phospholipid
TAG	Triacylglycerol
2-EE	2-Ethoxyethanol

Introduction

The need to reduce dependence on petroleum-based fuels as well as tackle the problem of global warming has led to

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increased interest in algae-based biofuels [1]. A number of microalgae are promising as sources of biofuel due to their rapid growth, high lipid content, and ability to be cultivated in non-arable land [2]. In addition, biofuel derived from algae would present a near carbon-neutral footprint. For the most part, the potential of using algae for biofuel depends on their content of triacylglycerol (TAG) yet reports of high algal lipid content can be ambiguous because of the way lipids are measured.

Most reports regarding lipid quantitation in algae are based on indirect methods such as gravimetric measurement of crude lipid extracts, Nile Red fluorescence or related dye-partition assays, or gas chromatography (GC) analysis of lipid-derived fatty acid methyl esters (FAME). Nile Red fluorescence has become one of the most popular methods for determining neutral lipids in algae [3]. Yet while it is visibly specific for lipid droplets, there are numerous anomalies that can affect lipid quantitation including distortions due to β-carotene (BC) [4], complex kinetics of the fluorescent signal [5] and issues with sensitivity or specificity [6]. At a minimum, Nile Red data must be confirmed by other more definitive techniques. With GC analysis, one normally first converts acyl constituents in the lipid extract to their corresponding FAME. Since FAME can be derived from both neutral and polar lipids, information concerning content of particular lipids such as TAG is lost in the conversion. High-performance liquid chromatography (HPLC) presents an alternative method for lipid analysis that can potentially resolve all the various classes of lipids in a crude lipid extract [7, 8]. When combined with mass spectrometry one can obtain a more detailed picture of particular lipid species within each class [9].

An accurate analysis of lipid content also assumes the complete extraction of lipids from algal biomass yet extraction methods can vary in their efficiency and specificity. The extraction of total lipids has generally followed the methods of Folch et al. [10] or Bligh and Dyer [11], the latter differing mainly in the chloroform:methanol:water ratios and the ratio of solvent to tissue or biomass. Despite their similarities, some studies have shown that in tissues with high lipid content, the Bligh and Dyer technique can yield only half the total lipid obtained by the Folch procedure [12]. Furthermore, these methods were developed for the extraction of lipids from animal tissues and one cannot automatically assume they would be equally effective with algae.

In this study, various solvent systems were evaluated for their effectiveness at extracting neutral lipids from algae. The lipid content of these extracts was analyzed using normal-phase HPLC coupled in parallel to an evaporative light scattering detector (ELSD) and quadrupole mass spectrometer. Analysis of these lipid extracts by HPLC revealed in addition to TAG, a surprisingly large amount of saturated hydrocarbon (HC), which was isolated and further characterized by NMR and GC/MS. These methods are relatively simple yet they provide a detailed and quantitative measure of the various lipid components in algae including saturated HC that are not often considered in assessing the potential of algae for biofuels.

Materials and Methods

All solvents were ACS reagent or HPLC grade with 2-ethoxyethanol (2-EE; Acros, Bridgewater, NJ), dimethylformamide (DMF; Fisher, Waltham, MA), and nitromethane (Fluka, St. Louis, MO) being further purified by distillation before use. Isooctane was dried and stored over calcium hydride (Sigma-Aldrich, St. Louis, MO). For HPLC analysis, solvents were filtered and degassed before use. For HPLC standards, triolein, tripalmitin and diolein (1,2- and 1,3-diolein) were obtained from Nuchek Prep (Elysian, MN) whereas β-carotene, cholesteryl palmitate, dipalmitin (1,2- and 1,3-dipalmitin isomers), monoolein, palmitic acid, phytol, digalactosyl diglycerol (various acyl constituents), dioleoylphosphatidylethanolamine and phosphatidylcholine (various acyl constituents), and chlorophyll-*a* and -*b* were all obtained from Sigma-Aldrich. Mineral oil (Squibb), containing a mixture of saturated HC, was purchased locally. PTFE filters (Omnipore) were obtained from Waters Corp (Milford, MA).

Culture Maintenance and Harvesting

A species of saltwater *Chlorella* (KAS603, Kuehnle Agro Systems) was cultivated at room temperature (RT, 23 °C) in f/2 saltwater medium [13]. The medium was prepared with distilled water that was further purified through a Barnstead E-PURE filtration system. Cultures were grown in an airlift photo bioreactor [2] under cool white fluorescent lights on a 12:12 h, light:dark photoperiod and supplied with air from an aquarium diaphragm pump. Algae were harvested in the late-logarithmic growth phase (approximately 28 days post-inoculation) when the culture had attained a density of 0.2 g/L based on dry cell weight.

Lipid Extractions

For typical extractions, algal pellets were prepared in triplicate by centrifugation at 10,000 rpm (Sorvall Legend XTR with a F14S-6x250LE rotor, 15,244$\times g$), washed by centrifugation in distilled water, and heated to 60 °C in 25 mL of solvent for 30 min with continuous stirring. The suspension was then filtered through a 0.45-μm PTFE membrane and the isolated cell solids were extracted again

with a second 25-mL portion of solvent. To test for completion of lipid removal, in some cases samples were extracted a third time. After the second extraction, the remaining solids were collected, dried in a vacuum oven, and weighed. Filtrates were pooled and solvent was removed by rotary evaporation (<60 °C) and dried further to a constant weight using a vacuum pump (10^{-1} Torr). Extracts were re-suspended in 2:2:1:1 isooctane:toluene:acetone:methanol (v/v/v/v) to a final concentration of 2–10 mg/mL, filtered (0.2 μm PTFE) and analyzed by HPLC/ELSD at gain six.

The lipid extraction efficiency of 2-EE was compared to 2:1 chloroform:methanol either by heating in solvent (as described above) or by the method of Folch et al. [10]. Each analysis was carried out in triplicate. For the Folch extraction, the concentrated algal pellet (~200 mg) was disrupted in 20 volumes of 2:1 chloroform:methanol using a Dounce homogenizer. Particulates were removed from the homogenate by filtering through a 0.45 μm PTFE membrane. The clarified filtrate was transferred to a separatory funnel, and distilled water equal to 0.25 times the volume of chloroform:methanol was added in order to bring the composition to 8:4:3 chloroform:methanol:water (v/v/v). After shaking and phase separation, the lower organic phase was collected into a pre-weighed flask, solvent was removed (as described above), and the weight of the extract was determined. The extract was then dissolved in 2:2:1:1 isooctane:toluene:acetone:methanol (v/v/v/v) and filtered before analysis.

Lipid extraction efficiency of 2-EE was also compared to hexane for both dried and wet algal pellets. Here, 12 algal pellets (88 mg each) were divided into two groups; half were lyophilized (12 h, 10^{-1} Torr) and the other half remained wet. Three wet and three dried samples were extracted twice with 2-EE or by refluxing (68–72 °C) in *n*-hexane [14]. Otherwise, the procedure followed the typical extraction protocol described above.

HPLC–ELSD/MS Analysis of Crude Lipid Extracts

The HPLC system consisted of a Surveyor LC pump and autosampler plus (Thermo Finnigan), with an adjustable splitter (Analytical Scientific Instruments) that divided the column eluate 1:10 between a quadrupole mass spectrometer (Thermo Finnigan MSQ Plus) and an ELSD (Sedere Sedex 75), respectively. Tubing lengths to each detector were adjusted to provide coincident peaks. Xcalibur software controlled operation and data acquisition from the autosampler, pump and mass spectrometer. Analog data from the Sedex 75 ELSD was supplied to the Xcalibur software through an A/D data acquisition box (Agilent Technologies, SS420X) and a RS232 PCI data acquisition card (Sea Level Systems, 7406S) in the PC. Lipid standards and algal extracts were resolved using a YMC Pack PVA-Sil-NP (250 mm × 4.6 mm ID, 5 μm particle size; YMC America, Allentown, PA). The column was protected by a Waters Guard Pak™ guard column containing Nova-Pak™ silica inserts.

Two methods were developed for the separation of lipid classes. Mobile phases and gradients for these methods are given in Tables 1 and 2. The mass spectrometer was used mainly in conjunction with Method A (Table 1) due to the fact that toluene used in Method B (Table 2) produced a high background. The atmospheric pressure chemical ionization (APCI) source was normally set to operate in positive mode with a probe temperature of 400 °C. The ELSD temperature was set to 42 °C and the gain was set at six.

Isolation and Analysis of the Hydrocarbon Fraction

The HC fraction was isolated from the crude algal extracts using silica gel preparative TLC plates (20 cm × 20 cm × 1.0 mm GF; Analtech, Newark, DE). Typically, a number of lipids were isolated at the same time so the plate was developed using two sequential solvent systems. The first solvent consisted of 65:10:20:10:3 chloroform:methanol:acetone:acetic acid:water [15], which was used to develop the plate half way. After drying, the plate was developed with 80:20:1 hexane:diethyl ether:acetic acid [16] to the top. The HC band was then scraped from the plate, eluted with 2:1 chloroform:methanol (v/v), and the solvent removed under vacuum.

The isolated HC fraction was further characterized by GC/MS and NMR at core facilities located in the Department of Chemistry at the University of Texas at Austin.

Table 1 Method A solvents and gradient program

Time (min)	Flow rate (mL/min)	A (%)	B (%)	C (%)
0	1.5	100	0	0
5	1.5	98	2	0
15	1.5	75	25	0
19	1.5	20	80	0
24	1.5	0	100	0
32	1.3	0	50	50
38	1.0	0	15	85
43	1.0	0	0	100
52	1.0	0	100	0
54	1.0	0	100	0
60	1.5	90	10	0
64	1.5	100	0	0
74	1.5	100	0	0

A, isooctane + 0.005 % ethyl acetate; *B*, ethyl acetate; *C*, 3:3:1 isopropanol:methanol:water + 0.1 % acetic acid

Table 2 Method B solvents and gradient program

Time (min)	Flow rate (mL/min)	A (%)	B (%)	C (%)	D (%)
0	1.5	100	0	0	0
6	1.5	100	0	0	0
10	1.5	50	50	0	0
17	1.5	9	90	1	0
28	1.5	1	93	6	0
35	1.5	0	0	100	0
41	1.3	0	0	50	50
47	1.0	0	0	15	85
52	1.0	0	0	0	100
56	1.0	0	0	100	0
59	1.5	0	90	10	0
61	1.5	0	100	0	0
71	1.5	100	0	0	0
81	1.5	100	0	0	0

A, isooctane + 0.005 % ethyl acetate; *B*, toluene + 0.05 % nitromethane + 0.05 % ethyl acetate; *C*, 3: 1 ethyl acetate:acetone + 0.01 % acetic acid; D, 3:3:1 isopropanol:methanol:water + 0.1 % acetic acid

GC/MS analysis was conducted by injecting 1.0 µL of 0.2 mg/mL HC (in hexane) into a Restek Rxi®-5SilMS column (30 m, 0.25 mm ID, 0.25 µm df; Bellefonte, PA). The column temperature program began at 60 °C for 5 min, and then was increased by 15 °C/min to 320 °C, where it was maintained for 20 min. Afterwards, the temperature was increased to 340 °C (25 °C/min) and held for 2 min to complete the run. The GC was coupled to a quadrupole mass spectrometer using a chemical ionization source. The gas (CH_4) flow rate was 1.5 mL/min and the source temperature was 160 °C. For 1H-NMR, HC was dissolved at 5 mg/mL in $CDCl_3$ (99.8 atom % D, Acros) and the spectrum recorded using a Varian Unity + 300 instrument.

Results and Discussion

In order to measure lipid content and the extraction efficiency of various solvent systems, we first needed methods for accurately analyzing the complex mixture of lipids present in the extracts. In the end, we developed two HPLC methods, one which gave a low background in the mass spectrometer ("Method A"; Table 1) and a second method that gave better resolution of lipids but was primarily useful with the ELSD ("Method B"; Table 2). These methods omitted amines common in other HPLC solvent systems because they tended to form amine salts at the tip

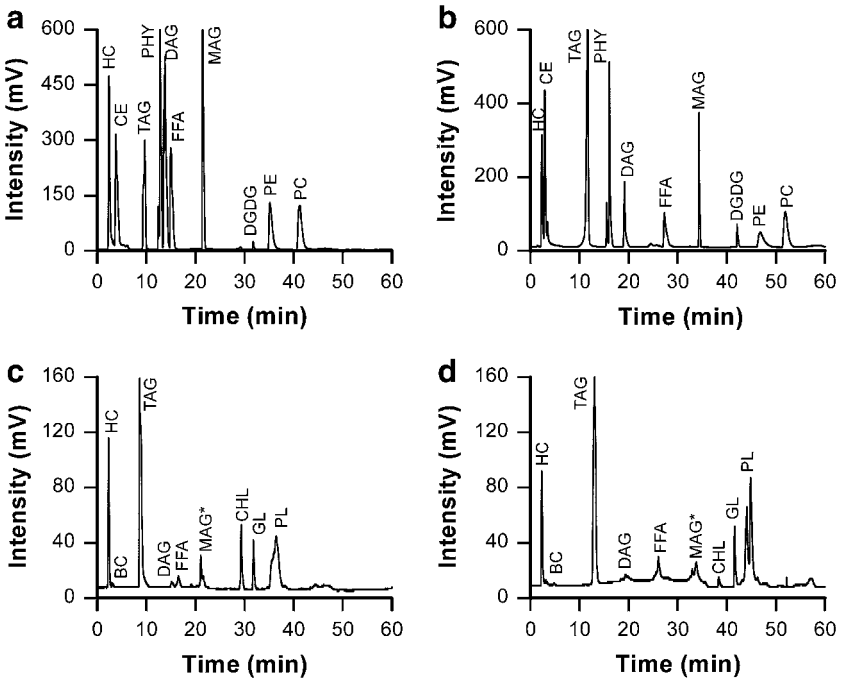

Fig. 1 HPLC separation of lipids by methods A and B. HPLC/ELSD chromatograms of lipid standards are shown for Method A (**a**) and for Method B (**b**). The standards are, in order of their elution time: mineral oil (HC), cholesteryl palmitate (CE), tripalmitin (TAG), phytol (PHY), dipalmitin (DAG), palmitic acid (FFA), monoolein (MAG), digalactosyl diglyceride (various acyl groups; DGDG), dioleoylphosphatidylethanolamine (PE) and phosphatidylcholine (various acyl groups; PC). For comparison, a crude algal lipid extract was also analyzed by Method A (**c**) and by Method B (**d**). Additional compounds identified in the crude extract include for Method A; *β*-carotene (BC, seen as a shoulder to the *right* of the HC peak), chlorophyll-*a* (20 min) and chlorophyll-*b* (22 min) eluting with monoacylglcyerol (MAG*), and chlorophyll catabolites (CHL, 29 min). Glycolipids (GL) and phospholipids (PL) were also detected

of the ELSD atomizer and inside the instrument resulting in signal spikes or an elevated background. With Method A, a set of lipid standards was fairly well resolved with the exception of diacylglycerol (DAG) and free fatty acid (FFA) (Fig. 1a). However, these components were easily distinguished in the mass spectra. Separation of these same lipid standards using Method B provided better resolution of all lipid components including DAG and FFA (Fig. 1b).

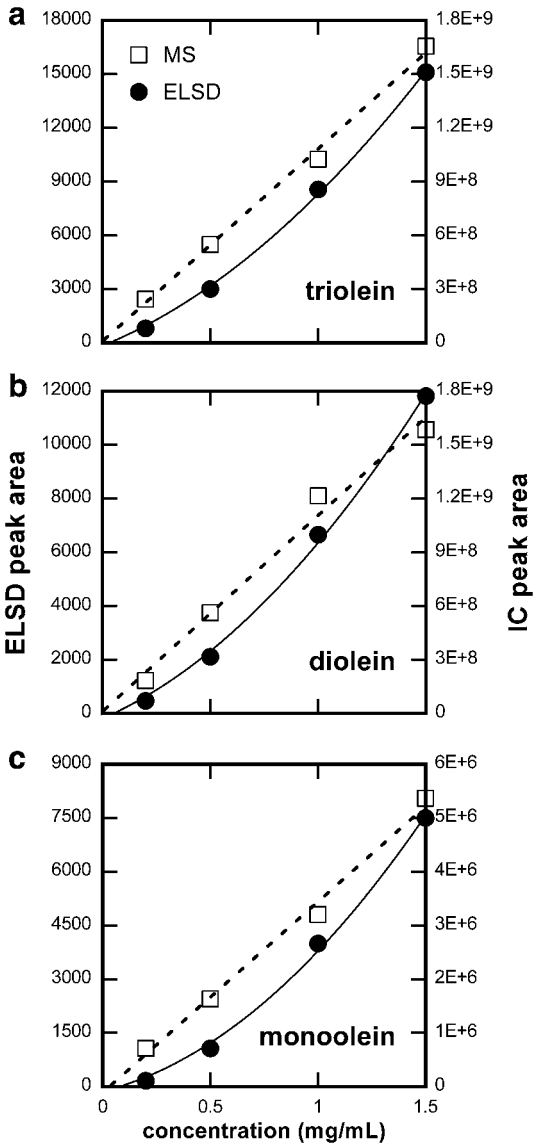

Fig. 2 Simultaneous detection and quantitation of lipids using an ELSD and mass spectrometer. Lipid standards were used to determine the ELSD and mass spectrometer concentration-response functions for triolein (**a**), diolein (**b**), and monoolein (**c**). The ELSD data, given as signal peak area versus concentration, was nonlinear and best fitted with a polynomial curve (*solid line*). The mass spectrometer data, reported as the total ion count for the $[M + H]^+$ ion, gave a linear response (*dashed line*). The R^2 values for the ELSD measurements were >0.999 for all three samples. For the mass spectrometer, R^2 values were >0.99 except for diolein, which had an R^2 value >0.98

When these methods were used to analyze a crude algal lipid extract, both Method A (Fig. 1c) and Method B (Fig. 1d) showed reasonably good separation of neutral lipids, fatty acids and polar lipids. In these extracts, several components eluted between the dead time ($T_0 = 1.46$ min) and TAG. The largest of these peaks, eluting at 2.5 min, comigrated with mineral oil, and was tentatively identified

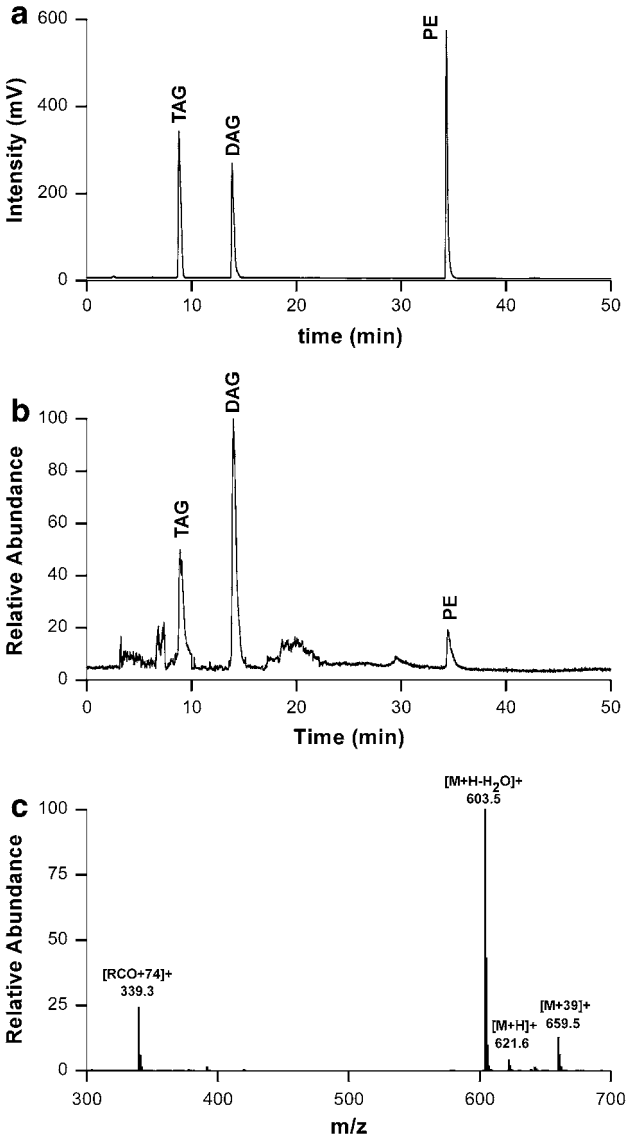

Fig. 3 Simultaneous detection of lipids by ELSD and mass spectrometer. The ELSD chromatogram, total ion chromatogram, and mass spectrum are shown to illustrate the simultaneous detection of a lipid species. A mixture of triacylglycerol (TAG), diacylglycerol (DAG), and phosphatidylethanolamine (PE) standards was resolved by HPLC, and chromatograms are shown for the ELSD (**a**) and the mass spectrometer (**b**). The mass spectrum corresponding to the DAG peak time (**c**) reveals a characteristic signature composed of the intact molecular ion $[M + H]^+$, ions generated by the dehydration product $[M + H–H_2O]^+$ or the loss of a fatty acyl group from the glycerol backbone $[RCO + 74]^+$, and a molecular species, possibly a potassium adduct, at $[M + 39]^+$

as saturated HC, closely followed by BC, and, on occasion, terpenoid esters eluting in the 5–6 min range.

Initially, the fast eluting components were not well resolved. Better separation was obtained when isooctane was dried with calcium hydride just prior to use. Isooctane alone gave relatively broad peaks that were sharpened by the addition of a small amount of freshly distilled nitromethane (0.02 %) or ethyl acetate (0.005 %). The best resolution was obtained using dry isooctane containing nitromethane (Supplementary Data S1) but there are safety concerns when attempting to dry nitromethane since it reacts with strong bases (such as calcium hydride), and can explode on contact with molecular sieves. Thus for routine separations, we used 0.005 % ethyl acetate.

To determine the concentration-response factors for the mass spectrometer and ELSD, initially, a series of TAG standards was tested at each ELSD gain setting (Supplementary Data S2). When the data points were fitted with straight lines, the R^2-values are fairly good at higher concentrations of samples. However, due to the nonlinear response of the ELSD [7, 8, 17], they got progressively worse at lower concentrations of the lipid standards. Quantification was simplified by standardizing the extraction procedure such that most algal extracts could be analyzed at a single ELSD gain setting (gain 6). This permitted calibration using one set of lipid standards containing all of the different lipids to be quantified. Figure 2 shows sample lipid calibration curves for the mass spectrometer and ELSD obtained in parallel using the splitter. As expected, the mass spectrometer shows a linear response whereas the ELSD shows a non-linear response to the same concentration set. For the ELSD traces, a second-order polynomial gave the best fit of the data.

The use of a splitter to couple the HPLC with the ELSD and mass spectrometer provided complementary methods of detection to verify lipid species in complex mixtures. The chromatograms and mass spectrum in Fig. 3 illustrate the simultaneous detection of diolein by ELSD and positive-mode APCI-MS around 15 min. Mass assignments for other lipid standards were also confirmed (Table 3, Supplementary Data S3). For most lipids, the best response of the mass spectrometer was obtained using APCI in the positive-ion mode; however, some easily deprotonated lipids, such as FFA, were more readily detected in the negative-ion mode.

Having developed methods to analyze and quantify the various lipids present in crude algal lipid extracts, we proceeded to evaluate methods for lipid extraction. Initially extractions were carried out using the Folch method, based on 2:1 chloroform:methanol, which typically performs better than other methods [18–20]. However, it was noted that after extraction with this solvent, algae remained green despite the fact that chlorophyll dissolves in a number of organic solvents. For example, polar solvents, such as methanol, isopropanol and acetone, were all effective at extracting chlorophyll although they were not effective at extracting most lipids (Supplementary Data S4).

Since the polarity and HPLC retention time of chlorophyll is similar to some of the neutral lipids (between DAG and MAG; see Fig. 1), it was reasoned that complete lipid extraction from algae should remove chlorophyll as well. Therefore, decolorization of extracted material became a preliminary indicator of solvent extraction efficiency. Efforts to improve the effectiveness of 2:1 chloroform:methanol extraction by sonication, pulverization using

Table 3 Ions observed with mass assignments

Analyte (M)	Ion (*m/z*)	Mass assignments	RT (min)
Mineral oil (mixture)	N/A	N/A	2.5
Cholesteryl palmitate (16:0)	625.67	$[M + H]^+$ $[M-RCO_2]^+$	3.5
	369.44		
Triolein (18:1/18:1/18:1)	885.93	$[M + H]^+$	9
	603.55	$[M-RCO_2]^+$	
Diolein (18:1/18:1/0:0)	621.55	$[M + H]^+$	15
and (18:1/0:0/18:1)	603.54	$[M + H-H_2O]^+$	
	339.42	$[M-RCO_2]^+$	
Palmitic Acid (C16:0)[a]	255.34	$[M-H]^-$	16
Monoolein (18:1/0:0/0:0)	357.39	$[M + H]^+$	21
DGDG (18:2/18:2/0)[b]	941.87	$[M + H]^+$	32
	779.77	$[M + H-C_6H_{11}O_5]^+$	
	617.60	$[M + H-2(C_6H_{11}O_5)]^+$	
PE (18:1/18:1/0)	744.80	$[M + H]^+$	34
	603.61	$[M + H-C_2H_7NO_4P-H_2O]^+$	
PC (18:1/18:1/0)	807.69	$[M + H]^+$	40
	603.56	$[M + H-C_5H_{13}NO_4P-H_2O]^+$	

The mean retention times (RT) are reported for Method A and have a standard range about the mean ± 0.75 min

[a] Detected in negative mode APCI

[b] Mixture of DGDG species from wheat flour; values are listed for dilinoleoyl digalactosyl

a FastPrep^{TM} (MP Biochemicals, Solon, OH) bead mill, or refluxing in solvent each gave about a 10 % increase in extracted TAG but the algae still remained dark green.

In testing other solvents, it was noted that hot DMF (60 °C) dissolved most of the biomass leaving a white residue and gave higher lipid recovery than chloroform:methanol solvents. However, the high boiling point of DMF made it difficult to remove from the sample. Another solvent, 2-EE also effectively decolorized algae. Of all the solvents tested, it gave the best total lipid recovery based

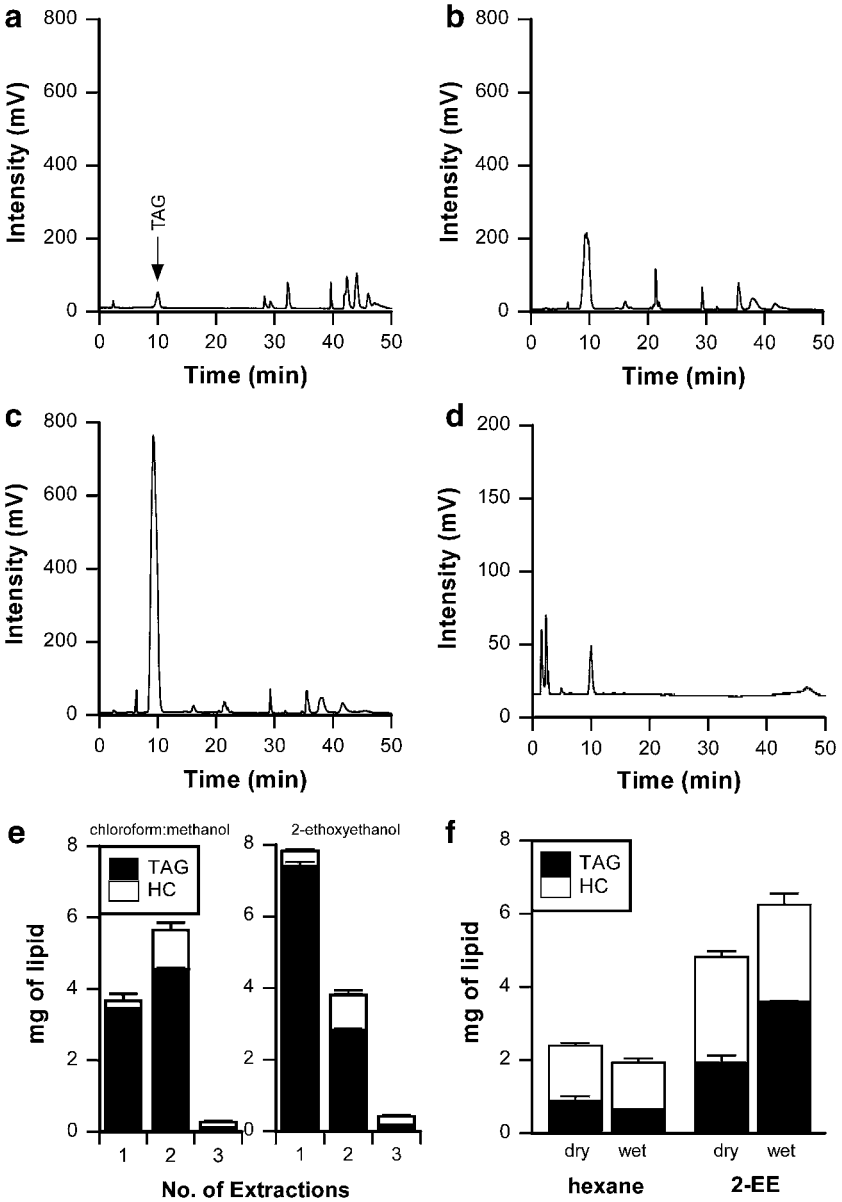

Fig. 4 Analysis of extraction methods. ELSD chromatograms are shown for the crude lipid extracts obtained by (**a**) using the Folch method, (**b**) by refluxing in 2:1 chloroform:methanol, (**c**) by heating in 2-EE at 60 °C (**c**), and, (**d**) by refluxing dried algae in hexane. While refluxing wet algae in chloroform:methanol recovered more lipid than using the Folch technique, the biomass retained on the filter was still green whereas after extraction with 2-EE, the biomass was a pale brownish color (see Supplementary Data S5a, b). None of the extraction techniques recovered all the algal lipid after a single extraction. When the algae were successively extracted three times with separate portions of either 2:1 chloroform:methanol or 2-EE, the amount of lipid recovered became negligible by the third extraction (**e**). Since hexane is commonly used for extracting neutral lipids, usually from dry material, lipid extracts using 2-EE and hexane were compared for both wet and dried algae (**f**). HPLC/ELSD analysis showed that 2-EE extracted substantially more TAG than hexane for both wet and dry samples but, in contrast to hexane, 2-EE was more effective on wet samples. This result was supported by photographs of the extracted biomass shown in Supplementary Data S5c–f. Substantial chlorophyll still remained with the biomass in all cases except when 2-EE was used to extract the wet algal pellet (Supplementary Data S5d). The mean values with *error bars* are shown ($n = 3$)

on gravimetric measurements and the best neutral lipid recovery based on HPLC analysis.

To illustrate the differences among extraction solvents, algal lipids were extracted by three different methods; the traditional Folch method, refluxing in 2:1 chloroform:methanol without the subsequent wash steps, and heating (60 °C) in 2-EE. Each extract was then analyzed by HPLC/ELSD (Fig. 4a–c). A comparison of these chromatograms shows that the traditional Folch extract contained the least amount of TAG (Fig. 4a) whereas the 2-EE extract contained the most TAG (Fig. 4c). Refluxing in 2:1 chloroform:methanol (Fig. 4b) gave intermediate amounts of TAG, suggesting that in preparing the Folch extract, some losses may have occurred during the washing step. In keeping with the idea that decolorization is related to extraction efficiency, photographs of extracted biomass show that 2-EE completely decolorized the algal biomass whereas both of the samples extracted with chloroform:methanol remained green (Supplementary Data S5 a and b).

While 2-EE extracted more lipid than chloroform:methanol, none of these solvents were able to exhaustively extract all the algal lipids in a single pass. Fig. 4e shows the progressive recovery of TAG and HC from algae that were extracted three times by either refluxing in chloroform:methanol or heating in 2-EE. Relatively little lipid was recovered by the third extraction, indicating that at least two sequential extractions are needed. It is possible that even better results could be obtained with specialized equipment such as the accelerated solvent extraction (ASE) system provided by Dionex [21]. However, the algae used for these studies have a diameter of $\sim$2 μm and can even squeeze through 1 μm pores, making it uncertain whether these systems would be useful.

Since hexane is a solvent commonly used to recover neutral lipids from plants and algae, the effectiveness of hexane and 2-EE were also compared. For these studies, extractions were carried out on equivalent amounts of either wet or dry algae, the latter being prepared by lyophilizing frozen algal pellets under vacuum ($\sim 10^{-1}$ Torr) for 12 h. Samples were either refluxed in hexane or heated (60 °C) in 2-EE. The HPLC/ELSD chromatogram of the hexane extract (Fig. 4d) shows that while hexane was more selective than 2-EE (Fig. 4c), it also extracted substantially less TAG and HC, regardless of whether the sample had been dried or not (Fig. 4f).

As one might expect, hexane was more effective at extracting lipids from dried as opposed to wet biomass. On the other hand, while 2-EE was more effective with wet samples, it still recovered more neutral lipids from the dried material than hexane. This somewhat counterintuitive finding has been a consistent observation for both 2-EE and chloroform:methanol solvents where drying the algae seems to generate structures that are more resistant to extraction [22]. The comparison between hexane and 2-EE extraction is supported by photographs of the residual algal solids showing the degree of decoloration (Supplementary Data S5). Wet algal solids extracted with hexane remained green (Supplementary Data S5c) but when extracted with 2-EE, the sample was completely decolorized (Supplementary Data S5d). In contrast, dried algae remained green regardless of whether samples were extracted with hexane (Supplementary Data S5e) or with 2-EE (Supplementary Data S5f).

Comparing 2-EE extracts to those obtained by other solvent systems has shown that 2-EE consistently extracts greater amounts of both TAG and HC. While traces of HC in algal lipid extracts have occasionally been reported, in 2-EE extracts it was often found to be 3–5 % of the algal dry weight. Furthermore, production of HC by algae often shows a reciprocal relationship to TAG. This is illustrated in Fig. 5, which compares the same concentration of lipid extract obtained from KAS603 late (Fig. 5a) and early (Fig. 5b) in the growth cycle.

To investigate the composition of the HC material, preparative TLC was used to isolate the HC from a crude algal extract (Fig. 6a). Analysis of the HC fraction by HPLC/ELSD showed one peak with a retention time of 2.5 min, which coincided with the HC peak in the original sample (Fig. 6b). Further analysis of this fraction by

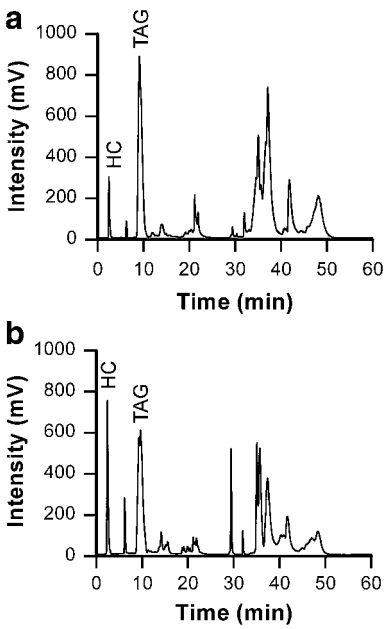

Fig. 5 Algal TAG and HC-like lipid. Algal lipid content, and especially the amount of TAG and HC-like lipid varies greatly with growth conditions. In general, the amount of HC often varies inversely with the amount of TAG. This is illustrated in ELSD chromatograms comparing extracts of the same algae in late (**a**) and early (**b**) growth stages

HPLC/MS showed that it was a complex mixture of molecules with chain lengths from 14 to 60 carbons (Fig. 6c). An NMR of this HC fraction (Fig. 6d) showed it to consist of saturated HC, as indicated by the chemical shifts occurring at 0.85–0.9 (–C**H**$_3$), 1.2–1.3 (–C**H**$_2$–), and 1.6 ppm (–C**H**(CH$_3$)$_2$). The absence of any downfield peaks indicates that this material does not contain appreciable amounts of acyl groups or double bonds.

A more detailed analysis of the HC fraction by GC/MS (chemical ionization) showed that it consisted of over 30 different chemical species ranging from 14 to 32 carbons. The most abundant peaks were seen for C17, C18, C19 and C20 *n*-alkanes and C15 and C20 branched-chain species. To illustrate, Fig. 6e shows the mass spectrum of a 19 carbon straight-chain HC whereas Fig. 6f shows the corresponding spectrum of a 17 carbon branched-chain molecule. For straight-chain alkanes, the $[M-1]^+$ ion is predominant and preceded by a series of low intensity fragments differing by 14 u (–CH$_2$) [23]. In addition the $[M-15]^+$ ion is approximately 1/10th the intensity of the $[M-1]^+$ ion. For branched HC, a series of fragments differing by 14 u were also detected but the intensities of the $[M-1]^+$ and $[M-15]^+$ ions, while roughly equal, are lower than the remaining fragments [22, 24, 25]. These

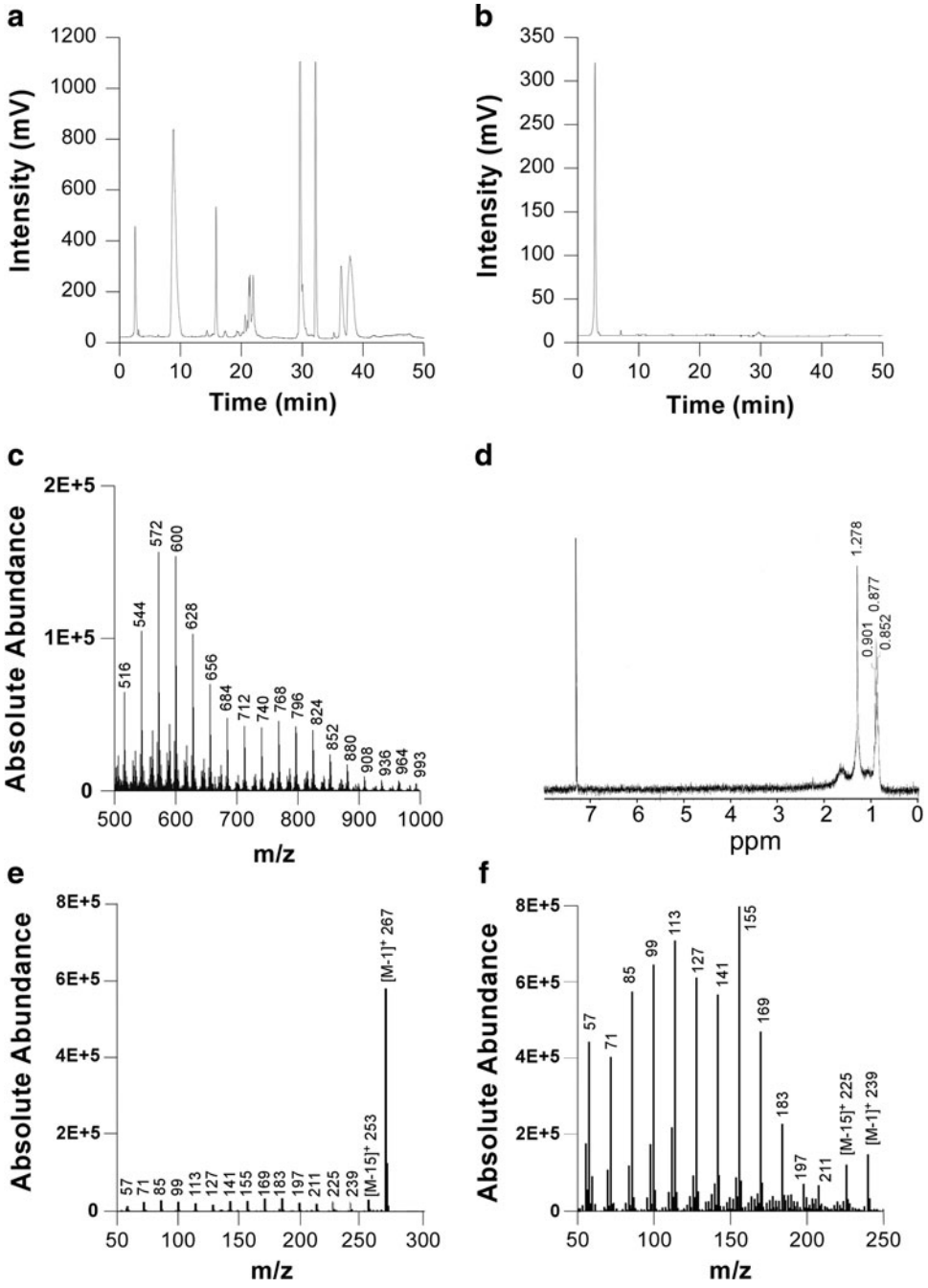

Fig. 6 Analysis of the hydrocarbon-like algal lipid. The HC fraction was isolated from the crude algal extract (**a**) and shown to be a single peak by HPLC/ELSD (**b**). HPLC/MS analysis of this material showed that it was a complex mixture of different carbon chain lengths ranging from 14 to greater than 60 carbons (**c**). 1H NMR confirmed that this fraction was comprised mainly of saturated HC (**d**). GC/MS analysis of the isolated material showed that it contained straight-chain (**e**) and branched-chain alkanes (**f**). For comparison, GC/MS data are presented for *n*-C$_{22}$ (a straight-chain alkane; Supplementary Data S6a) and pristane (a branched-chain alkane; Supplementary Data S6b) that were obtained during the same series of runs

features are illustrated in mass spectra of straight- and branched-chain standards obtained using the same instrument and operating conditions (Supplementary Data S6).

The detection of HC in algal lipid extracts has been reported previously [19, 26, 27], but for the most part they differ from what we report here. The various alkadienes and botryococcenes produced by *Botryococcus braunii* are unsaturated species, as are the 21:6 HC from diatoms. Cyanobacteria do produce saturated 17:0 species, but we did not detect any cyanobacterial contamination in our cultures. The HC material also differs from algaenans which contain a mixture of double bonds, acyl esters and ether linkages [28, 29]. On the other hand, Tornabene reported a mixture of HC in *Neochloris oleoabundans* extracts similar to those we report here, albeit at much lower levels [30].

The presence of saturated HC in algal extracts raises the question of where this material comes from and how it is made. One obvious concern is that it arises due to contamination from oil present in the environment, although contamination seems an unlikely explanation for several reasons. Firstly, the HC is observed in algae grown in a laboratory bioreactor using reagent-grade water and chemicals and an oil-free aquarium diaphragm pump as a source of air. Secondly, based on the data shown in Fig. 4e, the HC material shows a resistance to extraction that is hard to explain by mere contamination. Finally, this HC material can be present, under some conditions, in amounts that rival or exceed the amount of TAG produced by the algae.

Conclusion

We have developed methods for the extraction that provides superior recovery of total and neutral lipids. The direct analysis of this crude lipid extract by HPLC gives quantitative information on all lipid classes while minimizing losses in information and material associated with further derivatization or purification, as might be necessary for analysis by GC. Although normal-phase chromatography coupled to an ELSD will not resolve the sample to individual molecular species, simultaneous detection by the mass spectrometer allowed for the identification of particular molecules within a given peak.

Analysis of these lipid extracts revealed an unusually high percentage of HC composition whose composition most resembles that reported by Tornabene and colleagues [30]. This algal HC fraction has similarities to petroleum in its complexity and degree of saturation. In this respect it has several advantages over biodiesel in that it could feed directly into the existing petroleum infrastructure and it would be more resistant to oxidation or biodegradation. Studies aimed at understanding the synthesis and regulation of this HC-like material are ongoing.

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