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RESEARCH PAPER

Determination of lipid content of oleaginous microalgal biomass by NMR spectroscopic and GC–MS techniques

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Abstract Direct methods based on ^{1}H NMR spectroscopic techniques have been developed for the determination of neutral lipids (triglycerides and free fatty acids) and polar lipids (glyceroglycolipids/phospholipids) in the solvent extracts of oleaginous microalgal biomasses cultivated on a laboratory scale with two species in different media. The chemical shift assignments observed in the ^{1}H and ^{13}C NMR spectra corresponding to unsaturated (C18:*N*, *N*=1–3, C20:3, C20:5, C22:6, epoxy) and saturated (C14–C18) fatty acid ester components in a complex matrix involving overlapped resonances have been unambiguously confirmed by the application of 2D NMR spectroscopy (total correlation spectroscopy and heteronuclear single quantum coherence–total correlation spectroscopy). The study of the effect of a polar lipid matrix on the determination of neutral lipids by an internal reference blending process by a systematic designed experimental protocol has provided absolute quantification. The fatty acid composition of algal extracts was found to be similar to that of vegetable oils containing saturated (C16–C18:0) and unsaturated (C18:*N*, *N*=1–3, C20:*N*, *N*=3–4, C22:6) fatty acids as confirmed by NMR spectroscopy and gas chromatography–mass spectrometry analyses. The NMR methods developed offer great potential for rapid screening of algal strains for generation of algal biomass with the desired lipid content, quality, and potential for biodiesel and value-added polyunsaturated fatty acids in view of the cost economics of the overall cost of generation of the biomass.

Keywords ^{1}H and ^{13}C NMR · Algal biomass solvent extracts · Neutral lipids · Triglycerides · Biofuel · Fatty acids

Introduction

Algal biomass is associated with high biodiesel potential, and besides the presence of raw material for biodiesel production, mainly neutral lipids [triglycerides (TG) and free fatty acids (FFA)] and polar lipids (glyceroglycolipids and phospholipids), contains proteins, sugars, vitamins, etc. The neutral lipids are important components of interest to the energy sector because of their high potential to produce biodiesel with high growth rates of seven to 30 times those of traditional food crops. Many microalgae, such as *Chlorella vulgaris*, *Scenedesmus ecornis*, and *Dunaliella*, have the ability to produce a substantial amount of biomass with 20–50 % of dry cell weight of TG as storage lipids on account of photosynthesis [1–6]. Basically, the fatty acid composition of neutral lipids has the same profile as that of vegetable/fish oil (C12–C20/C24), including *n*−3 C18:3, C20:5 (eicosapentaenoic acid, EPA), and C22:6 (docosapentaenoic acid, DHA) [5–7]. The biomass and lipid productivity, composition, and nature of the components are dependent on the algal species, the cultivation media, the light flux, and the feed used for their cultivation. Stressed culture conditions such as nitrogen limitation or nutrient imbalance for stimulating an increase in the neutral lipid content in the microalgal cells are used for growth of algae of high biodiesel potential. The types of cultivation system (open

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pond, closed or hybrid bioreactor) for cultivation of algae also influence the yield and quality of the algal biomass [1, 5]. Lipids are extracted by chemical methods which involve extraction by solvents such as hexane/cyclohexane, chloroform, methanol, 2-propanol, and their mixtures aided by Soxhlet extraction, accelerated solvent extraction, sonication, and supercritical fluid chromatography methods [1, 3, 4, 8, 9].

Analytical techniques such as chromatography [gas chromatography (GC), high-pressure liquid chromatography (LC), gel permeation chromatography, supercritical fluid chromatography], spectroscopy [NMR spectroscopy, IR spectroscopy, mass spectrometry (MS), UV/fluorescence spectroscopy], and atomic microscopy (scanning electron microscopy, transmission electron microscopy) are being used extensively for lipid content and compositional analyses, including fatty acid profile, and monitoring the production and quality of feedstocks, and their conversion to biodiesel [7–36]. The determination of lipid content in algal biomass is done by methods based on solvent extraction followed by gravimetric lipid quantification [3, 4, 6, 9, 27], fat cellular staining by fluorescence microscopy using a lipid stain such as Nile red [19], solid-state time-domain NMR spectroscopy for direct estimation [15], liquid-state NMR spectroscopy [16, 23, 25, 26, 31], Fourier transform IR spectroscopy with statistical correlation [28], and fatty acid methyl ester (FAME) conversion determined by GC–MS [30, 34]. Specifically, high-pressure LC can be used to quantify different types of lipids in algal extracts, e.g., monoglycerides, diglycerides, and TG [21]. GC alone and coupled with MS (GC–MS) is used routinely to identify and quantify FAMEs from algal and yeast extracts [7, 17, 26, 30]. The review by Han et al. [28] presents a brief overview and description of the methods used for lipid quantification in microalgal biomass.

High-resolution NMR spectroscopic techniques because of their inherent sensitivity, high resolution, and multinuclear capability offer many advantages over existing analytical methods owing to the ease of direct, rapid, simultaneous, and nondestructive analyses in the multicomponent system of fat, algal oils, and biodiesel obtained from different sources, such as edible oil, nonedible oil, and algal and yeast biomass [8, 10–16, 18, 22–26]. More important is the diverse analytical capabilities of multinuclear one-dimensional (1D) and two-dimensional (2D) NMR ($^{1}H/^{13}C/^{31}P$) to provide detailed quantitative compositional analyses, such as lipid content, polyunsaturated fatty acid (PUFA) content, fatty acid profile, and iodine value, and production and quality monitoring of oil and biodiesel from different sources [8, 10–16, 18, 22–26, 31]. However, NMR spectroscopy lacks sensitivity when compared with LC–MS and GC–MS. Usually, the identification and quantification of the components in algal extracts from the NMR spectral interpretation is done by comparison of the generated with the literature chemical shift assignments and coupling constant data of standard compounds. Single-component standards such as triglyceride oleate (TGO), triglyceride stearate, or triglyceride palmitate have been used to study the fatty acid composition of algal extracts. The matrix effect has not been taken into account when single-component standards have been used for quantitative analyses of lipid content in algal extracts [16, 23, 31]. If matrix effects are not considered, the results may be misleading owing to overlapping chemical shift regions or resemblance with the matching structures in complex matrices containing a multicomponent system of TG, FFA, and polar lipids as observed in the present studies.

Knowledge of the detailed chemical composition of algal biomass is essential to understand the chemistry of neutral and polar lipids to explore their biodiesel and high-value-product potential as well as prediction of quality, yield, and physiochemical properties of fuels derived from algae. The accurate determination of neutral lipids in the algal biomass is a challenging task owing to the presence of a complex multicomponent matrix comprising glyceroglycolipids/phospholipids, proteins, carbohydrates, and minor constituents. Methods based on analytical techniques are not standardized, and have been found to be laboratory specific as well as the composition of biomass [30]. Thus, there is an essential requirement for standardization of methods in order to ensure reproducibility and accurate assessment of lipid yield and productivity of the produced biomass.

In the present investigation, the potentials of ^{1}H and ^{13}C NMR (1D and 2D) spectroscopic techniques have been explored to derive directly detailed compositional information on the fatty acid profile and to develop a direct method for the absolute determination of TG, FFA, and polar lipids in the solvent extracts of microalgal biomass samples generated on a laboratory scale while systematically addressing the matrix effect by internal and external reference standardization.

Materials and methods

Samples

The ten biomass samples of *S. ecornis* and *C. vulgaris* were cultivated in flasks and bottles, under white fluorescent light illumination of 135 and 300 μmol photon m^{-2} s^{-1}, respectively. Five different media were used: WC medium (WC; see [37] for the components of this medium); WC where the source of nitrogen was replaced by Chilean saltpeter (WCS) or urea (WCU); WC where the source of phosphorus was replaced by superphosphate (WCSP); and WC where the sources of phosphorus and nitrogen were replaced by superphosphate and Chilean saltpeter (WCSPS). The source of CO_2 was air which entered the cultivation media by agitation or by air pumping. The cellular suspensions were centrifuged and lyophilized. The biomasses lyophilized were frozen until

extractions were performed. The designation of the biomasses as mentioned in the text is given in Table 1. The freeze-dried samples were reduced to a powder in a mortar. The samples were extracted with a mixture of solvents. After extraction, the extracts were kept in a refrigerator at −4 °C to protect them from degradation.

Mixoil14 blend for reference

Mixoil14 blend containing 13 vegetable oils—*Jatropha* (from Brazil and from India), corn, sunflower, soybean, sesame, coconut, cotton, babassu (*Attalea speciosa*), palm kernel, canola, wild mango (*Mangifera indica*), and fish oil—and one biodiesel from soybean oil in different proportions was prepared for use as an internal and external reference for quantitative analyses of lipid content in different algal oils.

Extraction and analytical strategy

Single-step ultrasonic extraction

The extraction of neutral lipids (TG and FFA) and polar lipids from the algal biomasses of *S. ecornis* and *C. vulgaris* was done by single-step ultrasonic (200 W, 50 Hz) extraction methods using a mixture of chloroform, methanol, and water (2:1:0.3; ClMe for 60 min as per the procedure described by Sarpal et al. [26] and presented in scheme 1 in Fig. 1. The sample amount taken was 120–800 mg in 15–60 mL of the solvent or solvent mixture depending on the sample quantity available. Extracts were designated according to the species followed by the cultivation medium and by the kind of extract,

Table 1 Nomenclature of extracts of algal biomasses cultivated under different conditions/in different media

Algal extract	Species	Cultivation media/solvent used in extraction
SNDWCSPClMe	*Scenedesmus ecornis*	WC+superphosphate/ClMe
SNDWCSClMe	*S. ecornis*	WC+ Chilean saltpeter/ClMe
SNDWCUClMe	*S. ecornis*	WC+urea/ClMe
SNDWCSPSClMe	*S. ecornis*	WC+superphosphate+Chilean saltpeter/ClMe
SNDWCClMe	*S. ecornis*	WC/ClMe
CHLWCSPClMe	*Chlorella vulgaris*	WC+superphosphate/ClMe
CHLWCSClMe	*C. vulgaris*	WC+Chilean saltpeter/ClMe
CHLWCUClMe	*C. vulgaris*	WC+urea/ClMe
CHLWCSPSClMe	*C. vulgaris*	WC+superphosphate+Chilean saltpeter/ClMe
CHLWCClMe	*C. vulgaris*	WC/ClMe

The other extracts can be understood similarly: in case of the cyclohexane extract, there is "CH" at the end of the term instead of "ClMe" (for "chloroform–methanol").

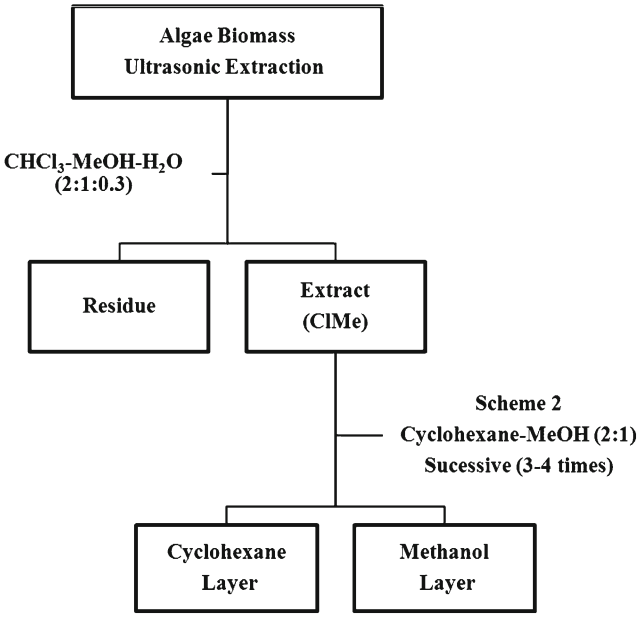


Fig. 1 Ultrasonic solvent extraction schemes 1(ClMe extraction) and 2 for the extraction of lipids from algal biomass. *ClMe* chloroform–methanol–water (2:1:0.3)

such as SNDWCSPSClMe and CHLWCSPSClMe. These mean *S. ecornis* (i.e., SND) or *C. vulgaris* (i.e., CHL) cultivated in WCSPS and extracted by the solvent mixture ClMe, respectively. Some of the extracts were further extracted successively three to four times with a cyclohexane–methanol (2:1) blend in order to preconcentrate the neutral lipids in the cyclohexane layer and the polar lipids in the methanol layer (scheme 2 in Fig. 1). These extracts are designated as SNDW CSPSCH, SNDWCCH, CHLWCSPSCH, or CHLWCCH. The nomenclature of the solvent extracts of algal biomass samples cultivated using different species and media is presented in Table 1. The extraction efficiency of lipids from the algal biomass was optimized and monitored by Fourier transform IR analyses as described by Sarpal et al. [26]. The amounts of extracts obtained are given in Table 6.

The repeatability of the standard deviation of the extraction procedure developed and the amount of extracts obtained was checked by doing the extraction three times for each algal biomass in the required solvent amount. The standard deviation in the 2.92–11.99 % range as given in Table 2 for five samples is self-evident of the excellent repeatability demonstrated by the ultrasonic procedure developed for the extraction of lipids in view of the number of parameters involved, such as temperature (30 °C), solvent ratio, and homogeneity of the cultivated biomass.

Transesterification for FAME production

Lipids from lyophilized biomasses were converted into FAMEs by adoption of the usual methanol–KOH in situ transesterification using the procedure described by Menzes

Table 2 Repeatability of solvent ($CHCl_3$–MeOH–H_2O) extraction results (% w/w)

Algal extract	Extract 1	Extract 2	Extract 3	Average	SD (%)
SNDWCSPS	28.81	30.52	29.50	29.60	2.90
SNDWC	21.53	19.32	22.96	21.27	8.62
CHLWCSPS	25.64	24.49	28.98	26.37	7.21
CHLWC	21.62	22.51	20.37	21.50	5.0
CHLWCU	17. 92	20.60	16.26.	18.26	11.99

Extracts 1, 2, and 3 were obtained by repeated ultrasonic chloroform–methanol–water extraction of algae.

SD standard deviation

et al. [32]. Lipids extracted from the biomasses using different mixtures of solvents were transesterified and transformed into FAMEs using this same method. The conversion efficiency was around 70–90 % depending on the amount of polar lipids present in the biomass or extract.

Instrumental analyses

NMR recordings

All the ^{1}H NMR spectra were recorded with a Bruker 500-MHz NMR spectrometer (AVANCE 111) equipped with broadband (BB) probe and a broadband inverse (BBI) probe. The solutions were prepared by dissolving approximately 5–10 mg of algal extracts, FAMEs, vegetable oils, and biodiesels in 0.7–0.8 mL of $CDCl_3$ containing the internal reference tetramethylsilane. Instrument parameters such as the relaxation delay and the receiver gain were optimized, and the 90° pulse width was calibrated in order to sufficiently relax the nuclei to obtain the quantitative spectra as described in our previous work [26]. The 90° pulse width was calibrated for each sample. Variation in the pulse width was observed in the 8.1–8.64-μs range depending on the concentration of the sample. To study the effect of relaxation delay on the quantitative analyses, the spectra of two extracts (SNDWCSPS, CHLW CSPS) and soybean oil were recorded under relaxation delays of 5, 10, and 15 s, and the integral areas of functional groups were measured. The results did not show any variation in the integral intensities of protons of functional groups of esters, unsaturations, methyls, etc., at relaxation delays of 10 and 15 s. Similar observations were reported in our earlier work on the analysis of yeast extracts [26] and also by Oromí-Farrús et al. [13] in their work on the analysis of biodiesels. The following parameters were used for each recording: relaxation delay 10 s; acquisition time 3.4 s, 90° pulse width 8.13–8.64 μs; number of scans 16 or 32, chemical shift range 0–12 ppm.

All the spectra were integrated three times after proper phase and baseline corrections, and average integral areas were taken for quantitative analyses. The ^{13}C NMR spectra were recorded with a Bruker 500-MHz NMR spectrometer using a BBI probe in a $CDCl_3$ solution of algal extracts (40–70 mg in 0.8 mL). The overnight recordings (approximately 10,000 scans) were performed with a relaxation delay of 10 or 15 s in composite pulse decoupling mode, a pulse width of 15 μs, and a sweep width of 0–240 ppm. However, 25 % w/v solutions of vegetable oil or biodiesel were used for ^{13}C NMR recordings, with a maximum number of scans of 2,500–5,000 under identical conditions.

Two-dimensional NMR recordings

The 2D NMR TOCSY experiments were conducted with a 15–20 mg of sample in 0.7 mL $CDCl_3$ using a Bruker AVANCE 111 500-MHz NMR spectrometer equipped with a BBI probe as per the pulse program described in [33] with 128 increments, eight transients, 1 s relaxation delay, and a sweep width of 7 ppm in both dimensions. A mixing time (d9) of 100 ms was found to be optimum for algal extracts. The data were processed with TopSpin (Bruker) using a 90°-shifted sine-squared window function for a total of 512×512 data points in the F2 and F1 dimensions. The HSQC–TOCSY spectra were recorded for a solution of 30–50 mg of sample in 0.7 mL $CDCl_3$ with 512 increments, 16 transients, 2 s relaxation delay, a mixing time of 100 ms, and a sweep width of 7 and 150 ppm for the ^{1}H and ^{13}C dimensions, respectively. The data were processed with TopSpin using a 90°-shifted sine-squared window function for a total of 1,024×512 data points in the F2 and F1 dimensions [33].

GC–MS analyses

FAMEs produced directly from the algal biomasses or from the extracts were analyzed by GC–MS with use of a Shimadzu gas chromatograph equipped with a quadrupole mass spectrometer using electron impact ionization. A capillary polar PEG wax column (30-m length, 0.25-mm diameter, 0.25-μm film thickness) was used for separation of FAMEs. The temperatures of the injector and column were maintained at 265 and 250 °C, respectively. Heptane solutions (3–5 mg in 1 mL) were injected in a split ratio of 150:1 under application of the following ramp: 70 °C for 3 min, 10 °C per minute from 70 to 240 °C, hold time 13 min, and finally 5 °C per minute up to 250 °C and held for 10 min. The identification of all types of saturated and unsaturated components, including DHA and EPA, in algae was done by comparison with the profile of NIST SRM 2772, PUFA standard, and FAMEs of fish oil.

Results and discussion

Fatty acid profile determined by GC–MS

The fatty acid profiles of extracts of different algal biomasses given in Table 3 indicate that the extracts were composed of both saturated (C14–C18) and unsaturated fatty acids, including higher carbon number unsaturated fatty acids such as C20:3, 20:4, and C22:6 compared with other biomasses. The fatty acid profiles of extracts of biomasses of SNDWCSPS, CHLWCSPS, SNDWC, and CHLWC contain C20:3 and C20:6 besides other fatty acids compared with other samples. The fatty acid profiles of algal biomasses grown in other media are similar except for the variation in the concentration of saturated and unsaturated fatty acids. These biomasses were found to contain a negligible amount of higher carbon number unsaturated fatty acids.

^{1}H NMR spectral features of algal extracts

The spectral features of algal extracts were described in detail in our earlier work [25]. However, to correlate them with the present objectives, the ^{1}H NMR features are briefly described. The algal extracts of algal biomasses generated by extraction with various solvents using ultrasonic methods from different species cultivated in different media as described in Table 1 were characterized for the nature of neutral lipids (TG and FFA) and polar lipids and their fatty acid profile. The ^{1}H NMR spectra of ClMe and cyclohexane extracts of *S. cornis* algal biomass (SNDSPSClMe, SNDSPSCH), fish oils, and soybean oils are given in Fig. 2. The algal extracts clearly indicate signals at 4.05–4.38 ppm (OCH_2; *sn*-1, *sn*-3), 5.25 ppm (OCH; *sn*-2), 5.27–5.5 ppm (CH=CH), 2.6–2.9 ppm (bisallylic, CH=CH–**CH_2**–CH=CH), 2.1–2.5 ppm (CH_2C=O), 2.1–1.9 ppm (allylic, **CH_2**–CH=CH–), 1.61 ppm (**CH_2**-CH_2C=O), 1.24 and 1.30 ppm [long alkyl chain $(CH_2)_n$], 0.88 ppm (terminal CH_3 groups of saturated C14–C18, etc., or $n-6/n-9$ types of unsaturated components C18:1, C18:2), and 0.98 ppm (terminal CH_3 of $n-3$ types of three or more than three double bonds) characteristic of TG comprising both saturated and unsaturated fatty acid chains. The spectra also indicate less intense signals at 2.40 and 2.42 ppm (**CH_2**C=O), which have been specifically assigned to C22:6 (DHA). The spectra shows the presence of **CH_2C**=O signals at 2.35 ppm due to FFA, which slightly overlap with the ester signals (**CH_2C**=O) of fats at 2.30–2.31 ppm. The characteristic signals due to different unsaturated fatty acids/esters in the 2.6–3.0-ppm region, such as C18:2 (2.77 ppm), C18:3 (2.81 ppm), C22:6 (2.84 ppm), and FFA, are well marked in the spectra, as shown in the expanded part of the spectra of different algal extracts (Fig. 3).

The low-intensity signals in the regions from 4.38 to 5.2 ppm and from 3.0 to 4.05 ppm have been assigned to OCH_2 and OCH ester groups, and **CH**OH and **CH_2**OH

Table 3 Fatty acid composition of algal and fish oils determined by gas chromatography–mass spectrometry (GC–MS) (area %)

CN	SNDWC	CHLWC	SNDWCSPS	CHLWCSPS	SNDWCSP	CHLWCSP	SNDWCU	CHLWCU	SNDWCS	Mixoil14	Fish oil
C14:0	0.50	0.95	0.29	0.35						2.46	7.28
C16:0	31.34	30.78	30.56	31.82	77.0	37.0	63.0	46.0	39.0	10.86	17.91
C16:1	12.34	3.53	1.91	2.16		2.0			2.4	1.04	10.35
C16:2	1.72	2.02	1.64	1.81		1.2			1.2		1.30
C16:3	1.20	1.88	1.51	2.13		1.0			1.1		1.41
C17:1	0.08	0.23	-	0.17							
UI	1.01	0.71	0.83	0.54							2.21
UI	4.86	4.21	4.76	3.85		2.3					
C18:0	1.20	1.39	1.37	1.5	4.5	2.0	4.7	6.2	1.9	3.62	3.91
C18:1cis	14.06	13.99	17.34	17.33	5.5	19.0	14.0	19.0	21.0	26.61	9.13
C18:1trans	12.21	11.40	10.51	9.74	5.1	10.6	11.0	15.0	10.0	1.44	3.69
C18:2	13.53	14.77	12.90	14.71	4.1	13.0	6.5	10.0	11.0	37.64	1.32
C18:3	4.86	4.71	4.30	4.2	1.4	3.2	0.8	1.5	3.3	2.75	0.71
C20:0										0.47	0.80
C20:1										0.48	0.28
C20:3	9.61	8.37	10.05	8.14							
C20:4					2.8	8.6		2.3	6.4		0.96
C20:5										1.87	19.10
C20:6	1.48	1.06	1.17	1.74						1.18	12.29
UI						0.1			4.2	2.2	

CN carbon number, *UI* unidentified

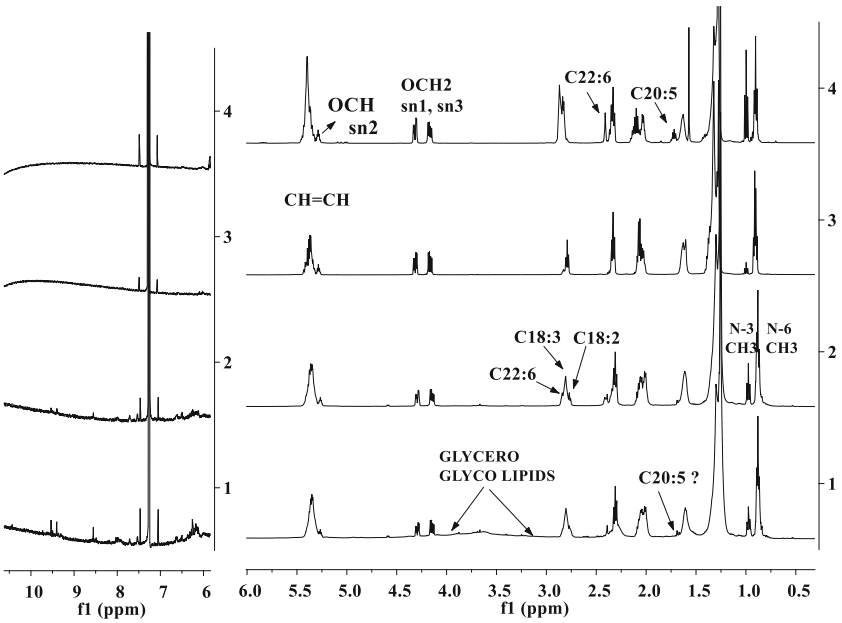


Fig. 2 500-MHz ^{1}H NMR spectra of algal extracts. *1 Scenedesmus ecornis* (SNDSPSClMe; see Table 1 for an explanation of the nomenclature), *2* SNDSPSCH, *3* soybean oil, and *4* fish oil

groups from the glycerol part of glyceroglycolipids and phospholipids (polar lipids), as shown in Fig. 2. This region may also contain a contribution from diglycerides and TG, if they are present in a sample. The presence of phospholipids in the extracts was confirmed by ^{31}P NMR analyses, which showed the presence of a very small amount of phosphorus components in the region from −5.0 to 6.0 ppm [25]. The spectra also show a signal of very weak intensity at 3.67 ppm, resembling signals due to phospholipids. This might be due to the OCH_3 ester signal of biodiesel, and it is a unique revelation as the presence of naturally occurring FAMEs in biomass is rarely reported. The ^{1}H NMR spectra of algal extracts also show very weak intensity signals due to olefinic protons (**CH=CH** or –C=**CH**–) in the 5.8–6.8-ppm region and aldehyde protons in the 8–11-ppm region (8.0, 8.55, 8.69, 9.40, 9.50–9.53, and 10.4 ppm), as shown in the expanded part in Fig. 2. These signals have been assigned to conjugated/nonconjugated unsaturated protons of oxylipins (lipoxygenase-derived oxygenated acid products) of PUFAs (polyunsaturated fatty esters) such as DHA and EPA [29].

The cyclohexane extract (SNDSPSCH) (Fig. 2) shows spectral features similar to those observed for the ClMe extract, except the variation in the integral intensities, which are particularly very low in the 3–4 ppm region assigned to glyceroglycolipids/phospholipids compared with the ClMe extract. Similar spectral features are demonstrated by the different solvent extracts of *C. vulgaris and S. ecornis* biomasses cultivated in different media, as shown in Fig. 4. The

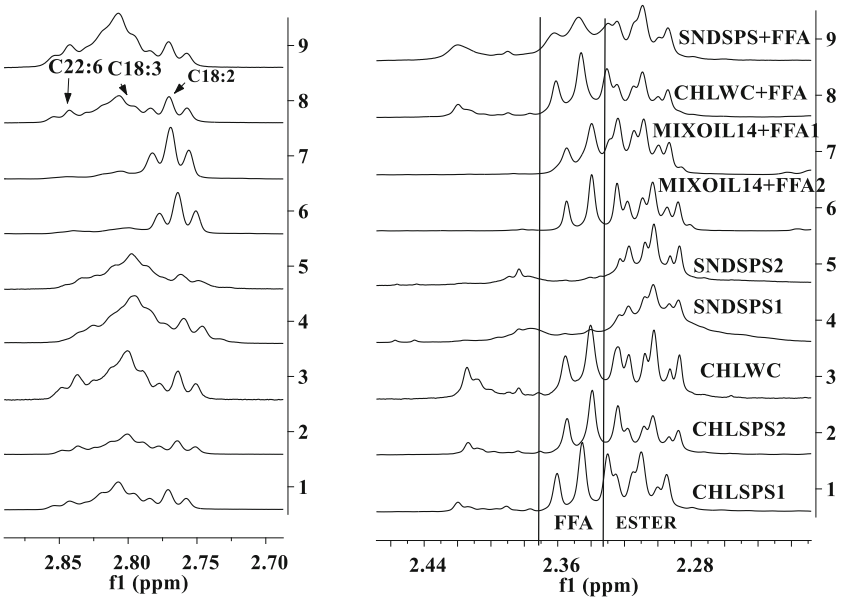


Fig. 3 Expanded view of 500-MHz ^{1}H NMR spectra of algal extracts of *S. ecornis* and *Chlorella vulgaris* cultivated in different media: WC medium [37], superphosphate and saltpeter, and blends of free fatty acids (*FFA*) in Mixoil14, and algal extracts showing demarcation of CH_2C=O of FFA (2.35 ppm) and triglyceride (TG) (2.25–2.45 ppm), and bisallylic (2.70–2.9 ppm) regions. *SPS1* and *SPS2* are extracts from repeated extractions, and *FFA1* and *FFA2* are free fatty acids with different amounts

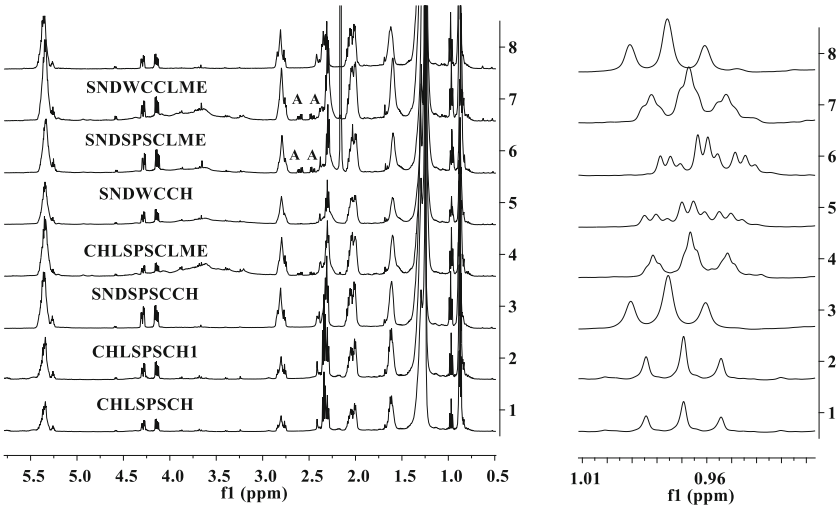


Fig. 4 Partial 500-MHz ^{1}H NMR spectra of cyclohexane and ClMe extracts of *S. ecornis* and *C. vulgaris* biomasses cultivated in different media. The partial spectra in the 0.925–1.01-ppm region are due to terminal methyls

expanded view of the region of the terminal CH_3 of $n-3$ fatty acid components at 0.98 ppm (0.93–1.0 ppm) in the spectra of ClMe extracts shows interesting features compared with the spectra of cyclohexane extracts (Fig. 4). Each singlet of a triplet is split into a doublet with the same coupling constant as shown in spectra 4–6 in Fig. 4. This might be due to either the presence of different types of CH_3 groups or polarization of these methyls by the presence of epoxy fatty acid components, as revealed later.

The identities of the signals observed at 2.42–2.43 ppm, symmetrical multiplets at 2.45–2.6 ppm (marked "A A" in Fig. 4), and signals in the 1.64–1.8-ppm region (marked "C20:5 ?" in Fig. 2) in the extracts could not be revealed. The spectra show a triplet at 1.69 ppm matching the features of C20:5. However, its presence could not be confirmed owing to it overlapping with other signals in the same region. The nature of these signals was characterized by ^{13}C and 2D NMR spectral analyses as discussed in succeeding sections.

TG constitute more than 95 % of the total monoglycerides, diglycerides and TG, as evident from the expanded part of the spectra in the 4–5-ppm region as shown in Fig. S1. The chemical shift assignments corresponding to various components as discussed above was done by comparison with spectral features of soybean oil, fish oil (Fig. 2), and standards of FAMEs. The chemical shifts of characteristic signals due to different components of various algal extracts described in Figs. 2, 3, and 4 are summarized in Table 4.

^{13}C NMR analyses

The ^{13}C NMR spectra of the ClMe extract of *S. ecornis* biomass (SNDSPSClMe) as depicted in Fig. 5 show signals characteristic of glycerides due to the carbonyl group of saturated and unsaturated fatty esters between 172 and 174.6 ppm, unsaturated carbons (CH=CH) between 126 and 133 ppm, OCH_2 (*sn*-1, *sn*-3) and OCH (*sn*-2) of TG at 62.12 and 68.96 ppm, $CH_2C{=}O$ at 34.05–34.3 ppm, and long alkyl saturated and unsaturated fatty chains at 31.95 ppm (γ-CH_2), 22.7 ppm (β-CH_2), 14.1–14.3 ppm (α-CH_3), 29–30 ppm $(CH_2)_n$, and 23–28 ppm $(CH_2)_n$. The expanded part of the spectral regions corresponding to different functional groups clearly shows the characteristic signals corresponding to unsaturated fatty acid, particularly for C18:*N*, *N*=1–3, and C22:6 (14.29, 132.04, and 172.17 ppm). The signals due to C20:5 (14.29, 128.0, 128.98, 173.04, and 173.11 ppm) were absent, as revealed by comparison with the ^{13}C NMR spectra of fish oil. The confirmation regarding the absence of C20:5 was provided by the 2D NMR HSQC–TOCSY analyses as described in the next section. The carbonyl signal characteristic of FFA is indicated at 178.9 ppm. The ^{13}C NMR spectra of ClMe extracts of algal biomasses SNDSPS and CHLSPS cultivated under different conditions indicate signals at 40.93, 67.69, and 169.17 ppm, which are uncommon to glycerides. The multiplicity of these signals was revealed to be OCH, OCH_2, and C=O, respectively, by analyses by means of distortionless enhancement by polarization transfer with a 135° angle (spectra not given), and the nature was confirmed by 2D NMR analyses.

The signals of very weak intensity between the intense signals, particularly between 38 and 72 ppm, are due to CH_2O, CH_2OH, CHO, and CHOH groups of glycolipids, and may also be due to monoglycerides and diglycerides. These signals were more intense in ClMe extracts than in cyclohexane extracts. The presence of these signals was also indicated by the ^{1}H NMR spectral analyses of these extracts [16, 20, 25, 35]. The region from 170 to 220 ppm did not show signals due to aldehyde (CH=O) and keto (C=O) groups, although signals of very weak intensity were clearly observed in the 7–11-ppm region in the ^{1}H NMR spectra. This is due to the long relaxation times of carbonyl carbons which inhibited the appearance of these very weak intensity signals with the acquisition parameters applied (relaxation delay 10 s). The

Table 4 ^{1}H NMR chemical shifts (ppm) of functional groups of algal extracts and oils

Sample	Mixoil14	Soybean oil	*S. ecornis* extracts	*C. vulgaris* extracts	Fish oil
CH=CH	5.05–5.52	5.0–5.7	5.0–5.7	5.0–5.7	5.0–5.7
OCH (*sn*-2)	5.26	5.25	5.25-5.26	5.26	5.26
OCH_2 (*sn*-1,*sn*-3)	4.05–4.38	4.05–4.38	4.05–4.38	4.05–4.38	4.05–4.38
OCH_3, BD			3.66	3.66	
$(CH=CH)_n\mathbf{CH_2}$, C18:2	2.77	2.77	2.77	2.77	2.77
$(CH=CH)_n\mathbf{CH_2}$, C18:3	2.81	2.81	2.80–2.81	2.80–2.81	2.81
$(CH=CH)_n\mathbf{CH_2}$, C22:6	2.84–2.85		2.84	2.84–2.85	2.84–2.85
$CH_2C=O$ ester	2.31	2.31	2.30–2.31	2.30–2.31	2.30–2.31
CH_2CO acid	2.35	2.35	2.35	2.35	2.34–2.35
CH_2CO (C22:6)	2.38		2.38, 2.40	2.38–2.40	2.38
$\mathbf{CH_2}CH=CH$	1.91–2.14	1.89–2.15	1.90–2.17	1.90–2.17	1.90–2.18
$\mathbf{CH_2}CH_2C=O$	1.61	1.61	1.61	1.62	1.62
$(CH_2)_n$	1.25 1.31	1.25 1.30	1.26 1.30	1.26 1.30	1.26 1.30
CH_3, PUFA	0.98	0.98	0.98	0.98	0.98
CH_3[a]	0.88	0.88	0.88	0.88	0.88
CH_3 sterols			0.53, 0.55	0.53, 0.55	
Epoxy ester			2.44, 2.66	2.44, 2.66	
Glycero/phospho, OCH, OCH_2,CHOH, CH_2OH, CH_2OP			3.2–4.05, 4.5–5.2	3.2–4.05, 4.5–5.2	

Mixoil14 is a blend of 13 different oils and one biodiesel (*BD*).

PUFA polyunsaturated fatty acid

[a] Terminal methyls of saturated (C14–C18) and unsaturated (C18:1, C18:2) fatty components

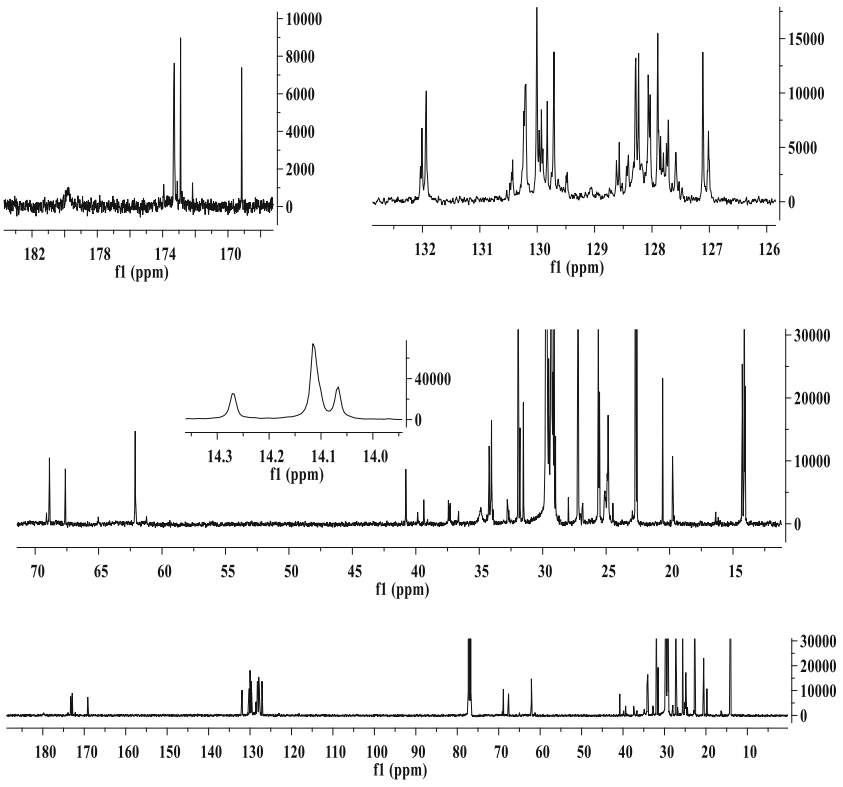


Fig. 5 Full 500-MHz ^{13}C NMR spectra (5–185 ppm) and expanded regions of terminal CH_3 (13.9–14.4 ppm); OCH_2/OCH esters (60–70 ppm), fatty acid/ester chain (5–50 ppm), carbonyl ester and acid (169–182 ppm); and unsaturated carbons (126–133 ppm) of ClMe extracts of *S. ecornis* cultivated in superphosphate and Chilean saltpeter. Refer to Table 5 for peak assignment

^{13}C NMR chemical shifts of the characteristic signals due to different components of various algal extracts are summarized in Table 5.

Two-dimensional NMR analyses

Two-dimensional (2D) NMR (HSQC–TOCSY and TOCSY) analyses were conducted with the following two objectives:

1. To confirm the ^{1}H and ^{13}C chemical shift assignments made corresponding to various fatty acid components.
2. To resolve several overlapping signals, especially in the regions from 1.4 to 3.0 ppm and from 5 to 5.6 ppm assigned beta to $CH_2C=O$, allylic, bisallylic, and olefinic protons in the ^{1}H NMR spectra.

It is evident that each class of fatty acid components exhibited characteristic signals that could be selected as NMR fingerprints for their identification in a mixture such as algal extracts as demonstrated in the next section. The 2D NMR techniques were used to resolve signals corresponding to various fatty acids, particularly unsaturated fatty acids such as C18:*N* (*N*=1–3), 20:3, C20:5, and C22:6 in order to confirm unambiguously their assignments. The identities of the signals at 1.65–1.71, 2.42, and 2.5–2.7 ppm, which were observed in ClMe extracts, were also revealed by 2D NMR TOCSY and HSQC–TOCSY contour analyses. The ^{1}H–^{1}H TOCSY analyses are well described in Fig. S2. Although the TOCSY plots clearly resolved the several overlapping peaks, especially in the 1.4–3.0-ppm region, the identities of the signals at 2.42 and 2.5–2.65 ppm (marked "A A" in Fig. 4) remained a mystery. The HSQC–TOCSY analyses resolved and identified these signals, as discussed in the next section.

HSQC–TOCSY analyses

The ^{1}H–^{13}C HSQC–TOCSY experiment combines ^{1}H–^{13}C HSQC and ^{1}H TOCSY experiments to give through-bond correlations between a ^{13}C-attached ^{1}H and all other coupled ^{1}H. The coupled ^{1}H nuclei can be seen along a line at the same ^{13}C chemical shift from the carbon atom attached to the primary ^{1}H. These NMR techniques are powerful analytical tools for structural elucidation of complex molecules where overlapping resonances are difficult to resolve by the TOCSY spectrum alone [33]. In the present studies, combining conventional structure determination by 1D NMR spectral techniques with HSQC–TOCSY experiments, we achieved unambiguously the ^{1}H and ^{13}C assignments in the overlapped regions, and applied these assignments to the identification of unsaturated and saturated fatty acid chains of glycerides in the algal extracts (Fig. 6) and fish oil (Fig. S3). The signals at 2.42 and 2.39 ppm in the ^{1}H NMR spectrum due to $CH_2C=O$ of DHA (C22:6) are very close to each other. The HSQC–TOCSY contour plot shows a cross peak indicating connectivity to the unsaturated carbons in the regions of PUFA types of components (line a). The symmetrical signals in the 2.5–2.65-ppm region in the ^{1}H NMR spectra show direct connectivities to signals at 5.26 ppm (^{1}H NMR) and 67.64 and 127–128 ppm

Table 5 ^{13}C NMR characteristic chemical shifts (ppm) for algal extracts and oils

Group	Soybean oil	Mixoil14	Fish oil	*S. ecornis* extracts	*C. vulgaris* extracts
OCH_2	68.89	68.95	68.90–69.14	68.90–69.13	68.89–69.15
OCH	62.06	62.04–62.30	62.03–62.27	62.06–62.20	62.07–62.13
CH_3					
C18:S+	14.09	14.14	14.14	14.13	14.13
C18:1					
C18:2	14.05	14.11	14.09	14.08	14.09
C18:3	14.26	14.32	14.30	14.29	14.28
C20:5		14.32	14.30	14.29	14.28
C22:6		14.32	14.30	14.29	14.28
CH=CH	127.0–132.1	127.5–132.5	127.45–132.05	127.47–132.10	127.50–132.10
C18:3	127.10, 131.89	127.13, 131.90	127.14, 131.90	127.12, 131.94	127.13, 131.96
C20:5		128.78, 128.98	128.80, 128.98		
C22:6		127.03, 131.97	127.05, 131.98	127.03, 132.03	127.02, 132.04
C=O (ester)	172.6–173.3	172–173.5	172–173.3	172–174.5	172–174.5
C20:5		173.05, 173.12	173.04, 173.11		
C22:6		172.16, 172.64	172.17, 172.64	172.17, 172.9	172.16, 172.89
C=O (acid)				179.75	179.89
Epoxy ester				169.18	169.18

(^{13}C NMR) in Fig. 6a and b (lines b and h). Thus, these signals are assigned to epoxy types of components of higher unsaturated fatty esters. The GC–MS analyses also revealed a FAME component at a retention time higher than that of C20:5 and C22:6. The lines indicating connectivities corresponding to saturated and unsaturated components are marked in the spectra of the algal extract and fish oil, and are described in the captions for Figs. 6 and S3, respectively. The contour plots show the absence of C20:5, as marked by line q in Fig. 6b in comparison with the contour plot of fish oil shown in Fig. S3 (lines O, P, and q).

From the detailed interpretation of the TOCSY and HSQC–TOCSY spectra above, it is evident that 2D NMR spectral analyses resolved the chemical shifts and unambiguously assigned them to the saturated and unsaturated fatty acid components, particularly C22:6, C20:5, C18:3/C20:3, and epoxy fatty acid, present in a complex matrix comprising multiple components of TG, FFA, and glyceroglycolipids/phospholipids.

From the detailed NMR spectral interpretation of solvent extracts of algal biomasses and GC–MS analyses, we summarize that algal extracts are composed of the following components:

1. Neutral lipids
   - (a) Fats (more than 95 % as TG) (major)
   - (b) FFA (low to medium)
   - (c) Saturated fats (as C14–C18)
   - (d) Unsaturated fats [C16:*N*, *N*=1–3, C18:*N*, *N*=1–3, C20:3, C22:6 (DHA), epoxy esters]
   - (e) Biodiesel (minor)
   - (e) Oxylipins (minor)
2. Polar lipids: glyceroglycolipids/phospholipids

Quantitative analyses of neutral (TG, FFA) and polar lipids

As a general practice reported in the literature, the fat or glyceride content in algal or vegetable oils is determined by use of a single-component external reference such as TGO, triglyceride stearate, or triglyceride palmitate [16, 23, 31]. This approach might not be appropriate in the case of algal oils for the following reasons:

1. The fatty acid composition comprises both saturated and unsaturated C12–C24, including EPA and DHA, as evident from the NMR and GC–MS analyses (Table 3). Therefore, whether it is a appropriate to determine the composition against an external single-component reference needs to be investigated.
2. The algal oils comprise a complex matrix of neutral and polar lipids with structures quite different from the components of vegetable oils, which are mainly composed of TG.
3. Overlapping of signals of glyceroglycolipids/phospholipids with the signals of glycerides, particularly in the region of interest from 4.0 to 4.40 ppm.

Fig. 6 **a** Full 500-MHz ^{1}H–^{13}C 2D heteronuclear single quantum coherence (HSQC)–total correlation spectroscopy (TOCSY) contour plot of algal extract SNDSPSClMe. *a*, *b* C18:3, C22:6; *c*, *e* epoxy ester; *n* C22:6; *q* absence of C20:5; *f*, g OCH_2 (*sn*-1, *sn*-3); *h* OCH (*sn*-2). **b** Partial 500-MHz ^{1}H–^{13}C 2D HSQC–TOCSY contour plot of algal extract SNDSPSClMe. Connectivities are shown as *lines*: *a* C18:3 and C22:6; *b*, *h* epoxy ester; *c* 18:3, *e* C22:6; *g* 18:1; *i*, *f*, *j*, *k*, *l* CS; *n* C12–C18. Lines P, *q*, and *n* (C20:5) are missing in SNDSPSClMe compared with fish oil (Fig. S3). **c** Partial 500-MHz ^{1}H–^{13}C 2D HSQC–TOCSY contour plot of algal extract SNDSPSClMe. Connectivities are shown as *lines*: *l* epoxy ester; *m*, *n* glycerides

In the present studies, the effect of these factors on the quantitative determination was investigated in detail by both external reference and internal reference blended processes.

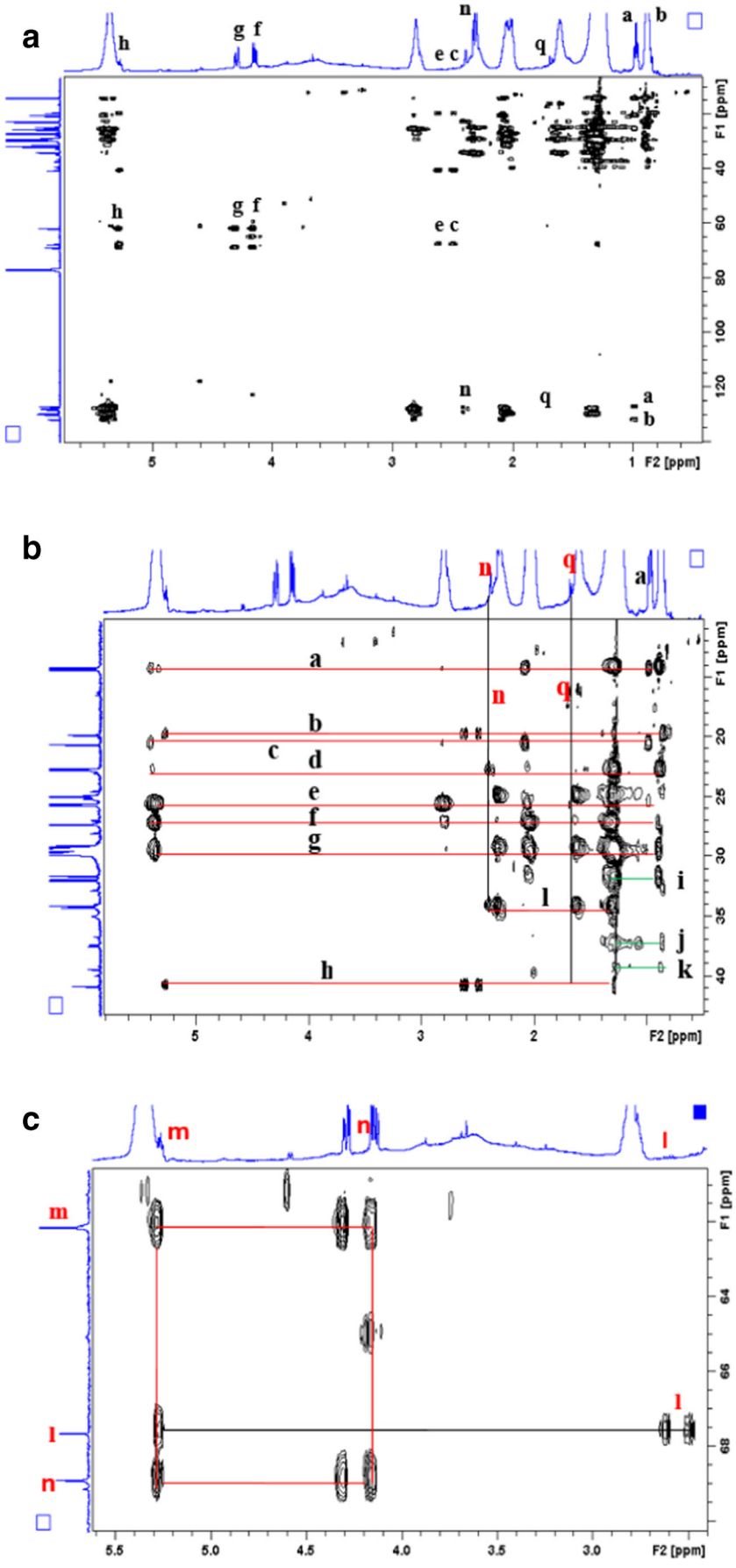
Internal reference standardization by blending processes

In NMR investigations, internal standard calibration involves the addition of standard organic compounds to the solution containing the target analytes, and comparing the integral areas of the protons of resonances originating from the standard compound with the integral areas of the protons of resonances originating from the analytes. Since the areas of the resonances are proportional to the number of protons present, the purity or concentration of target compounds can be measured quantitatively in mole or weight percentages. The standards are either certified reference materials or matching standards of target analytes. The internal reference standardization takes care of the matrix effect as matching standards with nature similar to that of the analytes are used, in contrast to external standards, where integral areas are measured separately for a standard and the analytes [34–36]. In the present studies, the matching internal standard of Mixoil14 was used for quantitative determination of the lipid content as per the approaches described later.

The following equation was developed for the determination of the TG content:

$$\text{TG content} = K_{\text{tg}} \times 2I_{\text{tg}}(4.05{-}4.35\text{ppm}) \quad (1)$$

where K_{tg} is a proportionality constant, and I_{tg} is the integral intensity in the 4.25–4.35-ppm region in the ^{1}H NMR spectra. The approaches adopted for the development of Eq. 1 are described in the following sections.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Polar lipid component matrix interference

Generation of polar lipid matrix

To distinguish the ^{1}H NMR spectral features of polar lipids from the features of neutral lipids, the polar lipids were separated from the ClMe extracts of SNDWCSPS, SNDWC, CHLWCSPS, and other biomasses by successive extractions with the cyclohexane–methanol (2:1) blends (Fig. 1, scheme 2). The ^{1}H NMR spectra of the methanol layers (polar lipids) as shown in Fig. 7 indicate practical absence of TG as evident from the structural features in the region from 3.0 to 5.0 ppm and at 5.26 ppm. The spectral features resemble the features of glyceroglycolipids/phospholipids owing to the appearance of intense signals in the 3–5-ppm region [25]. The fatty acid chain mostly comprises unsaturated carbons as evident from the higher iodine values compared with those for the cyclohexane layer. Since the polar lipid region largely extends into

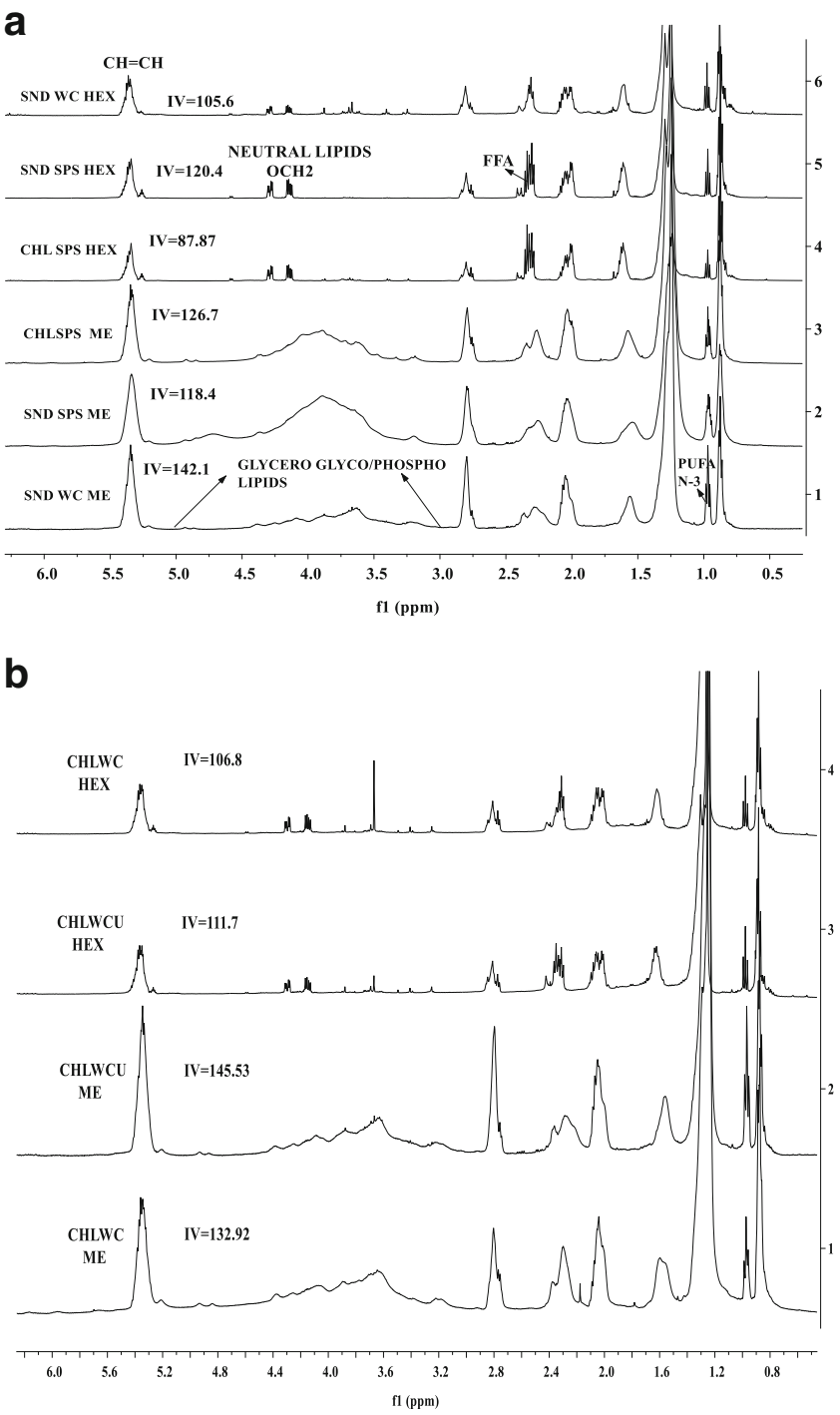

Fig. 7 **a.** 500-MHz ^{1}H NMR spectra of fractions of algal extracts (scheme 2). Spectra *1*, *2*, and *3* are for polar lipids (methanol layer, *ME*), and spectra *4*, *5*, and *6* are for neutral lipids (cyclohexane layer, *HEX*) of *S. ecornis* and *C. vulgaris*. **b** 500-MHz ^{1}H NMR spectra of fractions of algal extracts (scheme 2). Spectra *1* and *2* are for polar lipids (methanol layer), and spectra 3 and 4 are for neutral lipids (cyclohexane layer) of *C. vulgaris*. *PUFA* polyunsaturated fatty acid

the TG region (4–4.4 ppm), this will result in a larger integral area for TG owing to the signal overlapping effect. Therefore, it was considered essential to minimize or eliminate the overlapping effect in order to accurately quantify the lipid content.

To study the matrix effect, Mixoil14 and FFA were blended in different proportions in the polar lipids (methanol layer) in order to generate blends with large variation of polar and neutral lipids. Mixoil14 was used as an internal reference in order to generate a fatty acid composition matching that of the algal extracts (Table 3). In such a blending process, 60 blends were generated, each containing different proportions of TG, FFA, and polar lipids. The amount of polar lipids in the blends was judged from the integral intensity in the 3–4.0-ppm region. The plot of the integral intensities of blended TG, i.e., theoretical, I_{tg} (Thr), versus the experimentally determined intensities of the TG, i.e., I_{tg} (Exp), in the 4.05–4.40-ppm region (*sn*-1, *sn*-3; OCH_2) was obtained as shown in Fig. 8 (top left). The regression coefficient R^2=0.674 is indicative of the large variation on the order of 20–40 % w/w in the experimentally determined TG content compared with the blended TG content. This is ascribed to a signal overlapping effect as discussed in the preceding paragraph.

The overlapping effect was minimized or practically eliminated by considering one part of the OCH_2 signals (*sn*-1) as demonstrated in the plot of the integral intensities I_{tg} (Thr) and I_{tg} (Exp) in the 4.25–4.35-ppm region (*sn*-1, OCH_2) as shown in Fig. S1. R^2=0.983 indicates a reasonably good agreement between both determinations as shown in Fig. 8 (top right). I_{tg} is twice the integral intensity in the 4.25–4.35-ppm region. Consequently, a plot of blended TG content (% w/w) versus I_{tg} (%) using Eq. 1 resulted in R^2=0.984 with the following equation (Fig. 8, bottom left):

$$\text{TG content}(\%w/w) = 26.06I_{tg} - 0.62 \quad (2)$$

To determine directly the TG content by Eq. 2, I_{tg} should be calculated as twice the integral intensity in the 4.25–4.35-ppm region.

External reference method

To evaluate the effect of a single-component and a multicomponent external reference in the present studies, TGO, soybean, *Jatropha*, and Mixoil14 oils were used as external standards to determine K_{tg} by Eq. 1. The values of K_{tg} were determined as 26.0, 26.6, 26.3, and 26.9 for TGO, soybean, *Jatropha*, and Mixoil14 oils, respectively. The constants were calculated by dividing the TG content (100 %) by theoretical integral intensities of OCH_2 groups in the 4.05–4.38-ppm region. The TG content for algal extracts determined by applying these constants using Eqs. 3 and 4 shows insignificant variation in the range from 1.5 to3.5 % w/w irrespective of the nature of the external reference as demonstrated for TGO and Mixoil14 (Table 6):

$$\text{TG content}(\text{TGO}) = 26.0I_{tg}, \quad (3)$$

$$\text{TG content}(\text{Mixoi14}) = 26.88I_{tg}. \quad (4)$$

The results for different algal extracts obtained with Eqs. 2, 3, and 4 are given in Table 6.

Determination of FFA in the algal extracts

The ^{1}H and ^{13}C NMR analyses of the different solvent extracts of algal biomass samples confirmed the presence of FFA. There is an overlapping of the FFA signal at 2.35 ppm with the ester signal at 2.30 ppm, as shown in the expanded part of the spectra in Fig. 3. The 2.34–2.38-ppm region as marked in the spectra was selected for the

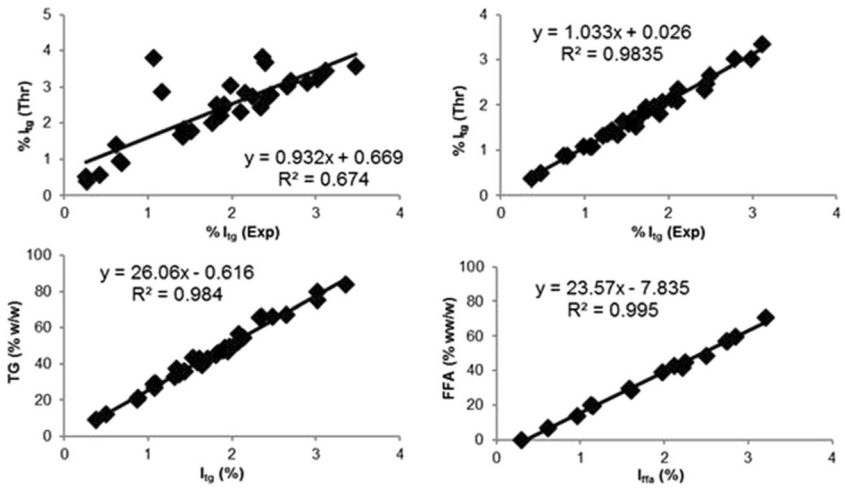

Fig. 8 Correlation curves for the NMR integral intensities in the 4.05–4.38-ppm region of blended (theoretical, *Thr*) TG versus determined (experimental, *Exp*) TG in the polar lipid matrix (*top left*) and for twice the integral intensity in the 4.25–4.35-ppm region (*top right*), and for the blended TG content versus twice the NMR integral intensities (I_{tg}) in the 4.25–4.35-ppm region in the polar lipid matrix (*bottom left*) (Eq. 2) and for the blended FFA content versus the NMR integral intensities (I_{ffa}) in the 2.34–2.40-ppm region in the polar lipid matrix (*bottom right*) (Eq. 5)

Table 6 ^{1}H NMR composition (% w/w) of $CHCl_3$–MeOH–H_2O extracts of algal biomasses (*S. cornis* and *C. vulgaris*) cultivated under different conditions obtained by application of Eqs. 1, 2, 3, 4, and 5 (extraction scheme 1)

Sample	TG[a] Eq. 2	FFA[a] Eq. 5	Total extract	TG[c]	FFA[c] Eq.5	NL[c] Eq.2	PL[c] Eq. 2	TG[a] Eq. 3	TG[a] Eq. 4	FFA[a] Eq. 6
SNDWCSP	40.53	8.48	17.69	7.17	1.50	8.67	9.02	41.06	42.44	1.67
SNDWCS	7.07[b]	8.68	14.86	1.05	1.29	2.34	12.52	7.61	7.93	1.43
SNDWCU	21.06 5.24[b] 15.77	10.67	21.37	1.12	2.28	3.40	17.97	5.84	6.04	2.53
SNDWCSPS	56.41	9.73	29.60	16.6	2.87	19.47	10.13	56.91	58.82	3.19
SNDWC	46.20	17.71	21.27	9.83	3.77	13.6	7.67	46.72	48.29	4.18
CHLWCSP	16.32	59.38	17.08	2.79	10.15	12.94	4.14	16.90	17.47	11.3
CHLWCS	20.74	30.22	16.05	3.33	4.85	8.18	7.87	21.31	22.03	5.38
CHLWCU	27.73	23.67	18.26	5.06	4.32	9.38	8.88	28.29	29.24	4.66
CHLWCSPS	33.35	16.42	26.37	8.80	4.33	13.13	13.24	33.90	35.05	4.80
CHLWC	24.88	16.42	21.5	5.35	3.53	8.88	12.62	25.45	26.30	3.89

Polar lipids (PL) in extracts SNDWCS=100–15.75 [triglycerides (*TG*) and free fatty acids (*FFA*)]=84.25 % w/w, and SNDWCU=(100–16.07) (TG and FFA)=83.93 % w/w.

NL neutral lipids (TG and FFA)

[a] In the extract

[b] By extraction scheme 2

[c] In the biomass

derivation of Eqs. 5 and 6. The blends prepared for the derivation of Eq. 1 for TG in a polar matrix were used for the derivation of Eq. 5 (Fig. 8, bottom right) and the blends of FFA in Mixoil14 were used for the derivation of Eq. 6 (curve not shown). The results obtained with these equations for algal extracts show standard deviations in the 0.15–0.8 range (Table 6) (Fig. 8, bottom right), which is insignificant in view of the large differences in the matrices between the two approaches. Equation 5, which takes care of the matrix effect, was used for the determination of FFA in the algal extracts (Table 6):

$$\text{FFA content}(\%w/w) = 23.57I_{\text{ffa}} - 7.84, \quad (5)$$

$$\text{FFA content}\,(\%w/w) = 24.74I_{\text{ffa}} - 7.28. \quad (6)$$

Comparison of internal and external methods of quantification

The standard deviation graphs among the results for the TG content obtained with Eqs. 2, 3, and 4 (Table 6) show standard deviation in the 0.22–1.7 range (Fig. 9a). The standard deviation indicating variation in results may be insignificant in view of the large matrix difference between algal extracts and vegetable oils. However, the higher standard deviation between the externally applied reference Mixoil14 (Eq. 4) and the internally applied Mixoil14 (Eq. 2) for algal extracts certainly demonstrates some degree of matrix effect. This can be significant when real-time productivity is compared and measured while screening the algal species or strains for neutral lipid enhancement efficiency. In such an experimental design, application of Eq. 2 would be an appropriate choice.

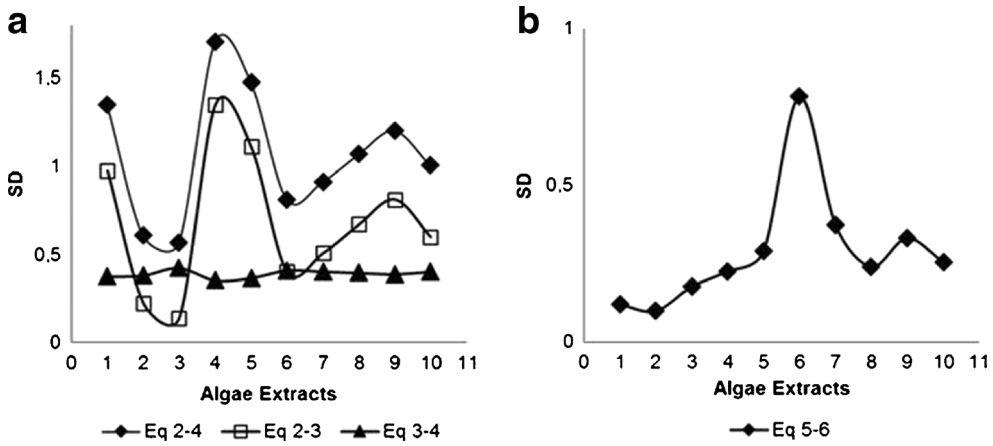

Fig. 9 Standard deviation (*SD*) curves for TG (**a**) and FFA (**b**) for *C. vulgaris* and *S. ecornis* algal extracts from different media obtained with Eqs. 2, 3, 4, 5, and 6

Similarly, higher standard deviation was observed between TGO and soybean oil when they were used internally and externally in the algal matrix. The standard deviation curves for determination of FFA in the algal matrix (Eq. 5) and without the algal matrix (Eq. 6) show similar results, with insignificant variation, as seen in Table 6 and Fig. 9b.

From these systematic designed experimental protocols, it is evident that the matrix has a signal overlapping effect. Once this has been eliminated by the proper demarcation of the chemical shift region (4.25–4.35 ppm), the external and internal single-component or multicomponent reference approaches both demonstrate an insignificant matrix effect on variation in the results.

Limitation of Eqs. 2 and 5 for lipid content determination

Equations 2 and 5 were applied to the determination of TG and FFA in the ten solvent extracts (ClMe) from ten algal biomasses from two species cultivated in different media (Table 6). The results reported in Table 6 show large compositional differences in the content of TG (5.24–56.4%w/w), FFA (8.48–59.38 % w/w), and polar lipids (23.30–84.32 % w/w) in the solvent extracts. This trend is quite supportive and indicative of the flexibility of the methods developed covering wide ranges of TG, FFA, and polar lipids in the algal biomasses grown under different conditions and with different species. The determinations by Eqs. 2 and 5 are independent of the composition and type of neutral and polar lipids of an algal biomass and, therefore, can be applied directly for the determination of TG and FFA in the solvent extracts.

Equation 2 estimates a higher TG content when the polar lipid content in the extracts is more than 80 % w/w, as shown in Table 6 for extracts of SNDWCS (84.25 % w/w) and SNDWCU (83.93 % w/w). This is due to the intense polar lipid signal overlapping effect as shown in the spectra in Fig. S1 and in Table 6. In such cases, it is recommended to further preconcentrate the ClMe extract for neutral lipids by the successive extraction with a cyclohexane–methanol (2:1) blend (Fig. 1, scheme 2). This is evident from the ^{1}H NMR spectral features of cyclohexane layers shown in Fig. 7. The cyclohexane layers contained 80–90 % of the neutral lipids. Since the methanol layer comprises polar lipids, extraction scheme 2 will allow polar lipids to be determined gravimetrically. The results obtained with schemes 1 and 2, and gravimetric extractions show good agreement (Table 7).

Validation of results obtained by Eqs. 2 and 5

1. The plot of I_{tg} (Exp) (determined) versus I_{tg} (Thr) (blended) shows a good correlation, with R^2=0.983 (Fig. 8, top right).
2. The average alkyl chain of the fatty acid components was C18.2:1.2 as determined from the fatty acid profile by GC–MS (Table 3). The value of K=25.7 (102.8/4) determined from the average fatty acid alkyl chain is very close to the values determined by Eq. 2.
3. The amounts of FAMEs, neutral lipids, and polar lipids of five algal biomasses and a fish oil determined by gravimetric extractions and GC–MS were compared with the amounts calculated by Eq. 7:

$$\begin{aligned}\text{NMR FAME content}\,(\%w/w) = {} & \text{TG content} \times 1.005 \\ & + \text{FFA content} \times 1.05 \\ & + \text{polar lipid content} \times 0.67.\end{aligned} \tag{7}$$

The results are in good agreement, as shown in Table 7. The factors in Eq. 7 were calculated on the basis of the structural determination of neutral and polar lipids by NMR spectroscopy taking the average alkyl chain as C18:1 [34].

Table 7 Comparison of TG and FFA (% w/w) content from application of scheme 1 and scheme 2 determined by NMR spectroscopy and fatty acid methyl ester (*FAME*) content determined by NMR spectroscopy and GC–MS

Sample	TG scheme 2	TG scheme 1	FFA scheme 2	FFA scheme 1	FAME GC–MS	FAME NMR
SNDWC	8.91	9.83	3.41	3.77	17.75	18.25
SNDWCSPS	13.03	16.64	6.06	2.87	20.15	26.35
CHLWC	5.62	5.35	3.96	3.53	19.1	20.52
CHLWCSPS	8.18	8.8	4.65	4.33	21.03	19.1
CHLWCU	4.29	5.06	4.57	4.32	16.82	15.43
SNDWCU	1.12	3.42	2.53	2.28	16.73	15.51
SNDWCS	1.05	3.16	1.41	1.29	9.43	10.78
Fish oil	99.72	99.72	ND	ND	–	–
Blend1	–	14.46	<5	<5	18.20	18.99

Blend1 blend of Mixoil14 and algal extract, *ND* not determined

Precision of method

The repeatability of the standard deviation for TG and FFA determination by Eqs. 2 and 5 was demonstrated on nine algal extracts obtained from different algal biomasses generated by *S. ecornis* and *C. vulgaris* in different media and in a blend. The NMR spectra of each sample were recorded three times, each spectrum was integrated three times, and the average integral intensities were used to determine the standard deviation (Table 8). The standard deviation was in the range from 0.11 to 3.79 % and from 0.67 to 12 % for TG and FFA, respectively. The higher standard deviation in the case of FFA is due to the overlapping of signals with the ester signal in the 2.33–2.38-ppm region.

Table 8 Precision data for *S. ecornis* (extracts 1–4), *C. vulgaris* (extracts 5–9), and the blend

II	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5	Extract 6	Extract 7	Extract 8	Extract 9	Blend
$I_{tg}1$[a]	1.800	2.362	2.352	1.788	1.170	0.908	1.102	0.631	2.294	2.836
$I_{tg}2$[a]	1.820	2.354	2.374	1.864	1.184	0.936	1.144	0.628	2.304	2.890
$I_{tg}3$[a]	1.750	2.360	2.350	1.840	1.158	0.933	1.120	0.619	2.292	3.050
Average	1.790	2.359	2.359	1.831	1.171	0.926	1.122	0.626	2.297	2.925
SD	0.036	0.004	0.010	0.038	0.013	0.015	0.021	0.006	0.0064	0.111
%SD	2.01	0.17	0.423	2.075	1.11	1.62	1.872	0.958	0.279	3.794
$I_{ffa}1$[a]	0.31	1.150	1.213	2.397	1.658	0.976	1.173	2.178	1.487	0.260
$I_{ffa}2$[a]	0.34	1.214	1.255	2.303	1.653	1.010	1.225	2.265	1.469	0.31
$I_{ffa}3$[a]	0.37	1.227	1.295	2.352	1.674	0.956	1.247	2.186	1.501	0.33
Average	0.340	1.197	1.254	2.351	1.662	0.981	1.215	2.209	1.486	0.300
SD	0.030	0.041	0.041	0.047	0.011	0.027	0.038	0.048	0.016	0.036
%SD	8.823	3.425	3.269	1.999	0.662	2.752	3.127	2.126	1.07	12.00

II integral intensity, *SD* standard deviation

[a] The repeated integral intensities of ester (OCH_2) and FFA ($CH_2C{=}O$) groups in the chemical shift regions from 4.05 to 4.38 ppm and from 2.34 to 2.38 ppm, respectively

Precision of the extraction process for the nature of the components

To study the precision of the extraction process with regard to the nature of the components extracted, each of three samples of algae was extracted three times under identical conditions by the ultrasonic method developed. These biomasses were generated in sufficient amount (approximately 1 g) to allow a component nature extraction repeatability study. The ^{1}H NMR spectral interpretation of functional groups corresponding to different components of the extracts such as fatty esters and acids comprising unsaturated and saturated fatty acids revealed practically no variation in the nature and concentration of these components present in three extracts of each algal biomass. The integral areas and the repeatability of the standard deviation of different functional groups in the range from 0.81 to 7.48 is self-evident of the presence of similar types of components in the solvent extracts obtained by repeated extraction of the same algal biomasses (Table 9). This exercise proved the excellent precision of both the solvent extraction process and identification and interpretation skill as demonstrated by the NMR methods developed. The results presented in Tables 2, 6, 7, 8, and 9 show that internal reference standardization provides absolute quantification of lipid contents.

Table 9 Repeatability of the standard deviation of the ultrasonic extraction procedure for algal biomasses

Sample	OCH_2	CH=CH	$CH_2C{=}O$	CH_3
SNDWCSPS extract 1	0.98	7.95	5.88	10.27
SNDWCSPS extract2	0.85	7.86	5.93	10.20
SNDWCSPS extract 3	0.89	7.93	6.07	10.20
%SD	7.48	0.59	1.66	5.88
SNDWC extract 1	0.71	7.59	7.02	10.37
SNDWC extract 2	0.76	7.24	6.76	9.93
SNDWC extract 3	0.72	7.76	7.04	10.30
%SD	3.69	3.4	2.22	2.32
CHLWCSPS extract 1	0.59	7.35	6.24	9.09
CHLWCSPS extract 2	0.51	7.23	6.40	9.49
CHLWCSPS extract 3	0.55	7.28	6.54	9.62
%SD	7.22	0.81	2.34	2.93

Displayed are the integral intensities of the chemical shift regions: OCH_2 (4.28–4.35 ppm), CH=CH (5.0–5.6 ppm), $CH_2C{=}O$, acid and ester (2.2–2.42 ppm), CH_3 (0.5–1.0 ppm). Extracts 1, 2, and 3 are from repeated ultrasonic chloroform–methanol–water extractions of algal biomass in different media.

Conclusions

NMR spectroscopic techniques provide direct, rapid, and convenient methods for the determination of glycerides, FFA, and polar lipids in the solvent extracts of algal biomass without any sample pretreatment and prior separation. The detailed 1D and 2D NMR spectral interpretation has provided unambiguous assignments of various chemical shift regions corresponding to the profiles of unsaturated (C18:*N*, *N*=1–3, C20:5, C22:6) and saturated fatty acids/esters, including identification of epoxy fatty acid esters in a way similar to that of those determined by GC/GC–MS analyses. The systematic designed experimental protocol for the study of internal and external reference standardization made it possible to derive Eqs. 2 and 5 for absolute quantification. The matrix effect of polar lipids is due only to the signals overlapping, and it was practically eliminated by an appropriate demarcation of chemical shift regions due to TG (4.25–4.32 ppm) and FFA

(2.35 ppm). Since the extracts of biomass of algae are generated in small quantities on a laboratory scale, the analytical protocol developed based on NMR spectroscopy and GC–MS can be applied to monitor rapidly the extraction efficiency of various solvents, the content of neutral lipids, and quality aspects with regard to the nature of fatty acids. The results will facilitate optimization of cultivation parameters for the enhancement of neutral lipid efficiency with regard to the fatty acid profile. The NMR methods offer great potential for analyses of a large number of samples requiring approximately 10 mg of an extract for rapid screening and comparison of algal strains for generation of algal biomasses with the desired neutral lipid content in view of the cost economics of the overall cost of generation of the biomass.

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