

## Compositional Analyses of Oil Extracts of Microalgae Biomasses by NMR and Chromatographic Techniques

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### Abstract

The present study focuses on identification and compositional characterization of neutral (tri acyl glycerides, TAG; free fatty acids, FFA) and polar (glyco/glycerophospho) lipids, and fatty acid chains constituting these lipids in the cyclohexane, dichloromethane and methanol extracts or algal oils of microalgae biomasses. Algal oils were generated by step wise Accelerated Solvent and Soxhlet extraction methods from six microalgae biomasses (*Scenedesmus dimorph*, *Chlorella vulgaris*, *Spirulina*, *SD Flakes*, *Cyanothece* and *Chlamydomonas*) obtained from commercial sources. The analytical protocol developed based on NMR (300 & 400 MHz)(<sup>1</sup>H & <sup>13</sup>C), IR, GPC, GC-FID and GC-MS techniques has provided authentic quantitative information about the nature of neutral lipids, polar lipids, fatty acid profiles, oxylipins (oxo fatty acids) and hydrocarbons present in the extracts. The analyses of fractions generated by silica column chromatographic separation of solvent extracts have facilitated demarcation of <sup>1</sup>H NMR chemical shift regions of functional groups corresponding to neutral and polar lipids. Specifically,  $\alpha$  (n-3) and  $\gamma$  (n-6) C18:3 were differentiated in the *Spirulina* by critical examination of <sup>1</sup>H NMR spectral features of algal oils from various biomasses. The comparative results by different analytical techniques have shown that newly developed methods based on <sup>1</sup>H NMR techniques can provide direct and rapid quantitative results of neutral lipids components including poly unsaturated fatty acid/ester (PUFA/PUFE) in the algal oils without prior sample treatment. The neutral lipids results obtained by NMR and GPC show good agreement as compared by linear regression analyses. The results showed that nature and contents of neutral lipids, and fatty acids profile in the extracts were dependent upon the methods of extraction and the polarity index of solvent used for extraction, and specific to types of algae strains. The application of NMR and IR techniques enabled assessment of the solvent extraction efficiency towards extraction of neutral and polar lipids including types of fatty acids constituting these lipids.

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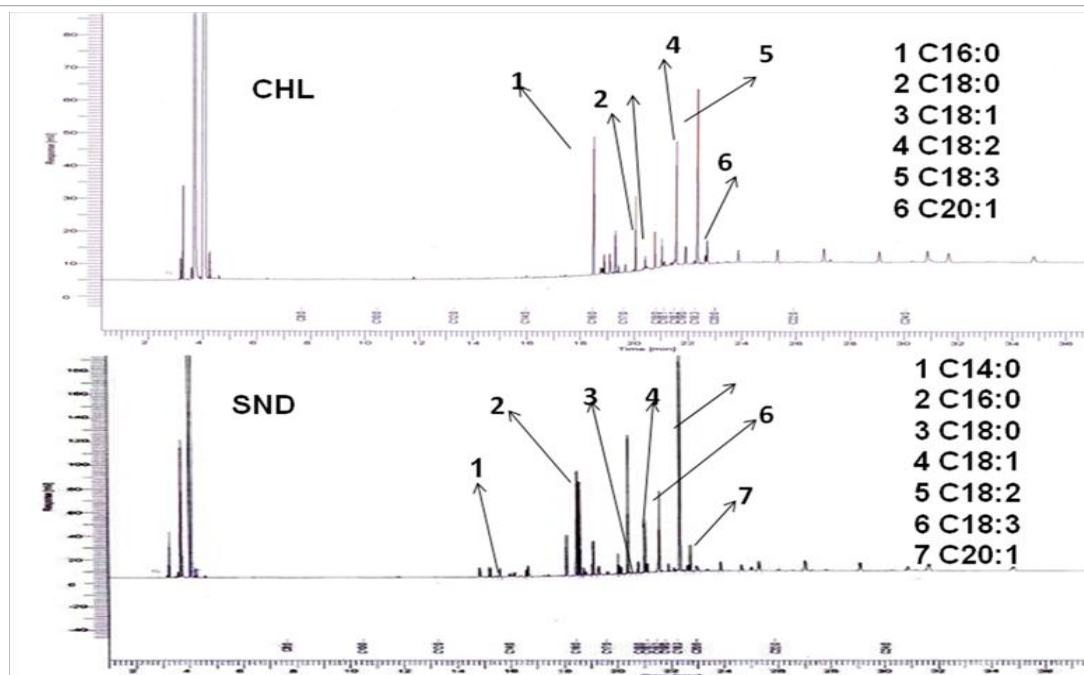
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## Introduction

The demand for alternative sources of fuels is increasing due to the rapid depletion of global petroleum reserves and instability in countries producing oils. Bio fuels produced from microalgae have the great promise as algae have potential to grow rapidly, synthesize and accumulate large amounts of lipids compared to regular oil crops. Practically, algae can yield several times larger quantities of oil from lipids (40 - 120 ton/ha year) compared to regular oil crops such as soybean and palm oils (0.2 - 6 ton/ha year)<sup>[1-6]</sup>. Oleaginous microbial oil is produced by the cultivation of algae and bacteria, which is promising sustainable sources of biofuels for the future<sup>[5-8]</sup>. Microalgae are sunlight-driven cell factories that convert carbon dioxide to biofuels. They can provide several different types of renewable biofuels such as biodiesel, methane produced by anaerobic digestion of the algae biomass and photo biologically produced biohydrogen<sup>[1]</sup> and green hydrocarbons biofuel made by catalytic deoxygenation<sup>[2]</sup>. Many microalgae such as *Chlorella*, *Baruni*, *Scenedesmus*, *Tetraselmis* etc., have the ability to produce substantial amounts of 20 – 50% of dry cell weight of TAG as a storage lipids on account of photo-syntheses<sup>[3-8]</sup>. Algae biomass is comprised of neutral lipids (TAG; mono, di, tri glycerides; FFA, free fatty acids), polar lipids (glyco/glycerophospho), proteins, alcohols, carbohydrates, vitamins, enzymes etc. The neutral lipids are important components of interest to energy sector because of their high potential to produce biodiesel<sup>[8]</sup>. Basically, the fatty acid composition of fat or glycerides has the same profile resembling edible/non-edible/fish oil (C12 to C20/24), therefore, they can be considered as potential substitutes for the fossil oils<sup>[2,7-9]</sup>. TAG from algae biomass is converted into fatty acid methyl ester (FAME) (biodiesel), usually by chemical hydrolysis or in vivo enzymatic transesterification<sup>[10,11]</sup>. Most of microalgae biomasses are rich source of  $\omega$ 3 and  $\omega$ 6 fatty acids including C18:3, C20:5 (EPA) and C22:6 (DHA) as indicated by very high iodine value of 90 to 140 g I2/100 g of solvent extracts which provide equivalent health benefits compared to PUFAs from fish oil<sup>[7,11]</sup>. Besides these complex polar components, hydrocarbons similar in nature to crude oil fractions are found to be present in certain strains of microalgae such as green colonial microalgae *Botryococcus brauni*<sup>[7,12,13]</sup>. The composition and nature of these components including fatty acids are dependent upon the types of species/strains, culture media and feed used for their cultivation<sup>[1-8]</sup>. Stressed culture conditions such as nitrogen limitation or nutrient imbalance as well as variation in temperature, pH, light, carbon source and salinity, can be applied during cultivation period for stimulating increase in biomass productivity for high biodiesel potential<sup>[14]</sup>. Improvements in molecular biology through genetic modification methods have resulted in enhancement of lipid yield and bi-products as well as improvement in their quality<sup>[5,6]</sup>. The type of photo bioreactor (open pond, closed or hybrid) used for the cultivation of algae also influence the yield and quality of algae biomass<sup>[1-6]</sup>.

The major challenge faced by the technology developer involved in the production of biodiesel and by-products from microalgal biomass is the extraction of oil, particularly neutral lipids (TAG and FFA), as microbial oil extraction processes are energy extensive and expensive<sup>[14-18]</sup>. A successful and economically viable algae-based biofuels industry depends on the rapid screening of algae strains for lipid content (glycerides) and improved extraction methods<sup>[2,3]</sup>. Oils from algae/yeast biomass are extracted from dried or wet mass by chemical and mechanical methods<sup>[7,8,19-24]</sup>. For extracting oils, various methods are available such as (1) chemical methods through solvent extraction, (2) sonication using hexane, chloroform, methanol, and isopropanol and their mixture, (3) supercritical fluid (SFC) extraction or (4) mechanical methods including milling, expelling, and extrusion (screw, expeller, piston) in the presence of solvents<sup>[4-8,19-24]</sup>. The extraction cost is further escalated depending on the processes that have been used for drying of the harvested biomass (5 - 15 % solid mass) such as sun drying, low-pressure shelf drying, spray drying, fluidized bed drying and freeze drying<sup>[15,21]</sup>. The Accelerated Solvent Extraction (ASE) process using heat or pressure, has been shown to improve extraction efficiency for lipid recovery besides having a reduction of process time, and recovery of solvent for re-use<sup>[19]</sup>. Soxhlet extraction is an extraction method that employs hexane, chloroform, and methanol or their mixture for the extraction of neutral and polar lipids selectively. This is normally used on a lab scale for the extraction of TAG or specialty chemicals as the process is truly time consuming. Oils from the algae are extracted through repeated washing, or percolation, with an organic solvent such as hexane or petroleum<sup>[19-21]</sup>.

Ultrasound-assisted methods are the most efficient, economic, eco-friendly, less time consuming, and highly reproducible as they generate less effluent compared to mechanical cell disruption methods. Ultrasound-assisted lipid extraction methods cause microalgae cell disruption due to cavitations and acoustic streaming resulting in cracking of cell walls and membranes. The method can be used for selective extraction of neutral and polar lipids by variation of polarity of solvents such as cyclohexane, chloroform, and methanol or their blends<sup>[7,8,25]</sup>. The use of ultrasound extraction method has been practiced by the Origin Oil Company, which has developed a technology that does not use organic solvents for algal oil extraction. Instead, it uses low-wattage, frequency-tuned microwave/ultrasound bursts that break the rigid and complex algae cell walls. Quantum fracturing is then applied to the now pre-cracked cells to complete the oil extraction with ease. Ultra-sonication and microwave-assisted extraction with appropriate solvents have many advantages such as reduced extraction time, reduced solvent consumption, and greater penetration of solvent into cellular materials causing high cell disruptive effects for quick release of oil<sup>[7,8,14,25]</sup>. Extractions using the subcritical or supercritical fluids (SFC), mainly supercritical CO<sub>2</sub>, are applied to extract lipids and are considered economically viable and green process as drying process is completely eliminated<sup>[23]</sup>. The SFC methods are efficient, particularly for the extraction of PUFAs and essential fatty acids<sup>[26]</sup>.



**Figure1:** GC-FID chromatograms of FAMES of combined cyclohexane, dichloromethane and methanol extracts of *Scenedesmus* (SND) and *Chlorella* (CHL) by Soxhlet extraction methods.

The extraction process using hexane/cyclohexane, and milling in combination with hexane are quite efficient methods and practiced by the algae-based biodiesel industry in the extraction of neutral lipids (TAG, FFA) from the dried algae biomass, which are easily esterified into biodiesel. The oil and cyclohexane are separated by means of distillation. These two stages, cold press and hexane solvent, together will be able to extract more than 95% of the total oil present in the algae biomass<sup>[13]</sup>. Hexane-based extraction method assisted with high speed homogenization has been demonstrated as the more convenient for using in lab-scale and potentially scalable for implementation in a microalgae-based biorefinery<sup>[15]</sup>. The much larger scale industrial method is based on the extraction of lipids from intact or lysed microorganisms in aqueous culture using a partially water-soluble co-solvent, with or without a second organic solvent, and/or pressurized CO<sub>2</sub> in the extraction method<sup>[27,28]</sup>.

The oil extracted from a biomass is not pure and besides neutral lipids, it contains glyceroglyco /phospholipids, pigments, proteins and carbohydrates. Detailed chemical composition of algae biomass is essential to understand the chemistry of neutral and polar lipids to explore their biodiesel and high value product potential as well as help in the prediction of quality, yield and physio-chemical properties of the algae derived fuels. Therefore, analytical methods based on NMR<sup>[7,8,14,24,29-39]</sup>, GC/GC-MS<sup>[7,8,40,41]</sup>, IR<sup>[24,42]</sup> and HPLC<sup>[43]</sup> techniques are used to characterize and determine the neutral lipids content and their fatty acids composition of the algal oils. The importance of solvent polarity in the extraction of lipids and their composition, fatty acid profile, and yield has been reported from critical analyses of extracted oils obtained by different solvents by GC-MS and gravimetric methods<sup>[21]</sup>. Other lipid measurement methods are Neil Red and BODIPY staining methods<sup>[44-47]</sup>, which are time consuming, involve multiple steps, and do not provide composition and nature of fatty acids. The problems faced in the use of analytical methods are associated with the large variations of results among methods due to complex matrices, non-availability of standards and also matrices differences among different algae<sup>[48]</sup>. NMR techniques have great potential in the characterization and direct estimation of components in algal oils, edible/non-edible oils, biomass oil etc., and their products<sup>[49]</sup>. The direct methods will be useful for quick monitoring the oil content of solvent extracts in the labs and commercial plants. The methods based on NMR have been applied for quantitative analyses and compositional characterization. These methods have multiple advantages such as direct determination without prior separation, requiring small amounts of sample (2 to 5 mg), being rapid and convenient, and having potential for online process monitoring of the production of biodiesel from algae as discussed in details in our earlier published work on algal oils from microalgae and vegetable oils<sup>[7,8,14,24,50]</sup>.

The present research work is a continuation of our endeavor and passion for unraveling and understanding the chemistry and various analytical aspects of oils and fats from vegetable oils, and algal oils extracted from different species/strains of microalgae, which possess biodiesel potential as investigated by high field NMR spectroscopic and other analytical techniques<sup>[7,8,14,24,49,50]</sup>. *Chlorella vulgaris*, *Spirulina* and *Scenedesmus dimorph* and other potential species/strains have been widely investigated for their biodiesel, PUFA, and co-product potential in fresh and waste water cultivation<sup>[2,6-8,14]</sup>. The present work highlights the importance of NMR, IR, GPC, GC and GC-MS techniques for the compositional characterization of algal oils obtained from these microalgae by Accelerated Solvent (AS) and Soxhlet (X) extraction methods. The main objectives are to characterize and monitor the extraction efficiency of solvents of different polarity on the extraction of neutral lipids and their fatty acids including the extraction of PUFAs by different analytical techniques, particularly NMR for the determination of the neutral lipid contents and differentiation of fatty acid profile in algal oils.

## Materials and Methods

### Materials

Microalgae samples of *Spirulina* (SP), *Scenedesmus dimorph* (SN), *Cyanothece* (CYN) and *SD Flakes* (FL) were obtained from Stellar Winds, Inc. (Doylestown Pennsylvania, USA). The Agricultural Biological Engineering (ABE) department of university of Illinois provided algae samples of *Chlorella vulgaris* (CHL) and *Chlamydomonas* (CHM). All samples were grinded in ball mill before extraction. The term 'algae' will be mentioned for microalgae throughout the text.

### Extraction procedure

The solvent extracts or algal oils of solid algae biomass samples for this study were generated using different solvents by successive stepwise solvent extraction methods including Soxhlet extraction (X) and Accelerated Solvent extraction (AS). Successive stepwise extractions were carried out with 150 to 200 ml of solvents, such as cyclohexane (CH), dichloromethane (DCM) and methanol (MeOH), with algae sample amounts of 3 to 5 g. The successive stepwise extraction procedure involves the extraction first by solvent CH. The solvent was removed after the extraction was completed as indicated by a colorless solution in the Soxhlet column. The residue in the thimble was then extracted with DCM; solvent was removed in the similar manner as above, and finally the residue left over in the thimble was extracted with MeOH. Similarly, the AS procedure was carried out twice by each solvent and the extracts were marked 1 and 2 (CH1, CH2, DCM1, DCM2, ME1 and ME2). The solvents in the extracts were evaporated under nitrogen stream. The AS extraction time was 30 minutes for each extraction. The extracts were also treated with acetone to remove traces of water. The samples were marked, for example, *Spirulina* extracts by Soxhlet as SPXCH (cyclohexane extract), SPXDCM (dichloromethane extract) and SPXME (methanol extracts) for stepwise extraction. For AS, the extracts were marked as SPASCH1 and SPASCH2, SPASDCM1, SPASDCM2, SPASME1 and SPASME2. In a similar way other samples were marked as indicated in the Table 1. The extraction results are given in Table 1. All the extracts were kept in the refrigerator at -4°C before subjecting these to analyses or further processing.

**Table1:** Accelerated Solvent extraction (AS) and Soxhlet extraction (X) results (%w/w)\*.

Sample	AS CH1	AS CH2	AS DCM1	AS DCM2	AS ME1	AS ME2	Total AS	X CH	X DCM	X ME	Total X
<i>Spirulina</i> (SP)	3.2	0.6	3.2	0.5	6.7	0.3	14.5	2.4	5.0	13.0	20.4
<i>Scenedesmus</i> (SN)	12.5	0.7	4.7	0.5	2.0	2.0	22.4	6.8	8.7	19.1	34.6
<i>Chlorella</i> (CHL)	2.6	0.2	7.6	1.3	10.0	0.2	21.9	2.0	8.3	7.3	17.6
<i>Chlamydomonas</i> (CHM)	4.5	1.5	2.8	0.2	1.8	0.4	11.2	4.7	4.7	8.5	17.9
<i>Cyanothece</i> (CYN)	3.1	0.5	1.4	0.3	6.0	0.5	11.8	1.8	1.8	4.0	7.6
<i>SD Flakes</i> (FL)	5.9	0.7	5.1	0.6	7.5	0.5	20.3	--	--	--	--

\*CH, CH1 or CH2 = Cyclohexane, 1 or 2; DCM, DCM1 or DCM2 = Dichloromethane, 1 or 2; ME, ME1 or ME2 = Methanol, 1 or 2, AS = Accelerated Solvent extraction, X = Soxhlet, -- not determined, Total AS or X = sum of Accelerated or Soxhlet solvent extracts (CH1 + CH2 + DCM1 + DCM2 + ME1 + ME2 or CH + DCM + ME).

### Hydrolyses for FAME conversion

The solvent extracts were hydrolysed by the KOH-MeOH (1N KOH in methanol) procedure as discussed previously<sup>[7,8,14]</sup>. A sample of extract of 0.1 gm was added in 10 ml of the KOH - MeOH solution and refluxed at 70°C. The hydrolyses time was 80 minutes including refluxing with BF<sub>3</sub>-MeOH. The diethyl ether-chloroform extracts were collected and evaporated to dryness and kept in the refrigerator for further characterization by GC, GC-MS, GPC, IR and NMR techniques.

### Instrumental methods

**Elemental analyses of solid algae biomass:** The CHNS analyses of solid algae samples were carried out on ANTEC instrument. Results are given in the Table 2.

**Table 2:** Carbon, Hydrogen, Nitrogen and Sulfur Content (%w/w) of solid algae Biomasses.

Algae	Nitrogen	Carbon	Sulfur	Hydrogen
<i>Scenedesmus</i>	9.2	52.3	0.61	8.3
<i>Spirulina</i>	10.0	45.6	0.73	7.5
<i>Chlorella</i>	9.4	50.6	0.90	6.6
<i>SD Flakes</i>	8.9	48.6	0.85	7.0
<i>Cyanothece</i>	11.0	41.9	0.70	7.2
<i>Chlamydomonas</i>	8.4	48.5	0.70	6.7



**GC analyses for fatty acid profile:** The fatty acid profile identification and composition of extracts dissolved in toluene were carried out on Perkin Elmer Claurus Gas Chromatograph (500GC) with FID detector. A capillary polar wax column (CP wax 52 CB, length 30 m, diameter 0.32 mm, film thickness 0.25  $\mu$ m) and helium as carrier gas was used for the separation of unsaturated and saturated fatty acid esters by the standard method EN14103. The GC condition such as split ratio 10:1, FID temperature 300°C, the injector split/split less temperature 300°C, the oven/column temperature 260°C and gas flow were used as mentioned in the method. The retention times of methyl esters (FAME) were confirmed by analyzing the Aldrich standards containing C14:0, C16:0, C18:0, C18:1, C18:2, C18:3 etc. and compared with those present in the FAMES of extracts. The ester components were separated at 80°C and then ramped to 260°C at an interval of 10°C. The area percentages of each peak in the chromatogram were measured for determination of individual FAME composition (Table 3 and 3a).

**Table 3:** Fatty acid composition (area %) of combined Soxhlet CH, DCM and MeOH extracts of algae biomasses analyzed by GC-FID.

S.N	Components	CHL	SN	SP	FL	CYN	CHM
1	C14:0	--	1.2	--	1.3	3.2	--
2	C16 :0	13.8	23.8	28.7	17.0	43.0	38.3
3	C16 :1	1.5	6.0	7.9	1.6	7.2	2.3
4	C17 :0	1.4	--	--	--	--	0.6
5	C18 :0	--	3.2	--	13.8	1.4	3.4
6	C18 :1	7.2	15.7	15.8	11.5	1.4	20.2
7	C18 :2	17.7	6.5	9.32	5.4	3.3	17.2
8	C18 :3	28.4	36.8	19.3*	33.0	24.9	14.4
9	C20 :0	0.8	--	--	2.6	1.2	0.6
10	C20 :1	1.5	--	--	--	0.7	--
11	C22 :0	1.8	--	--	--	1.7	0.4
12	C24 :0	2.3	1.5	--	--	1.9	--
13	Unidentified	25.0	5.3	19.1	13.8	10.1	2.6
14	Unsaturated	56.3	65.2	52.0	51.5	37.5	54.1
15	Saturated	18.7	29.5	28.9	34.7	52.4	43.3

\* $\gamma$ -C18:3, -- not detected, refer to Table 1 for algae short names, CHL, SN etc.

**Table 3a:** Fatty acid composition (area %) of cyclohexane (CH), dichloromethane (DCM) and methanol (Me) extracts by Accelerated Solvent extraction (AS) method of algae biomasses analysed by GC-FID.

SN	Components	SNAS CH	SNAS DCM	SNAS Me	CHLAS CH	CHLAS DCM
1	C14:0	1.4	--	1.4	3.5	--
2	C14 :1	1.6	--	1.6	2.9	--
3	C16 :0	37.1	19.4	30.2	44.3	20.6
4	C16 :1	6.8	3.3	5.5	9.6	8.1
5	C17 :0		--		1.7	13.5
6	C18 :0	3.2	--	3.5	4.9	3.6
7	C18 :1	15.7	7.7	15.0	8.1	3.3
8	C18 :2	6.5	19.4	10.1	12.8	20.2
9	C18 :3	13.6	41.4	14.1	4.9	29.8
10	C20 :0	--	8.8	--	0.6	--
11	C20 :1	--	--	2.3	--	--
12	C20 :2	4.3	--	2.3	--	--
13	C20 :4	--	--	3.2	--	--
14	C22 :0	1.8	--	1.2	1.6	--
15	C24 :0	--	--		0.4	0.9
16	Unidentified	8.0	--	9.6	4.7	--
17	Saturated	43.5	28.2	36.3	57.0	38.6
18	Unsaturated	48.5	71.8	54.1	38.3	61.4

SN = *Scenedesmus*, CHL = *Chlorella*, -- not detected

**GC-MS analyses:** Autospec Ultima High resolution mass spectrometer coupled with Agilent 6890N Gas Chromatograph has been used to carry out identification of components present in the FAMES of extracts. The mass spectrometer was tuned for 1000 resolution in the mass range of 50 to 400 Daltons. Per Fluoro Kerosene (PFK) was used as reference for calibration of masses. The magnet scan was set at 0.5 seconds/decade and interscan delay of 0.1 s. Ionization of samples in the mass spectrometer was carried out using electron of 70eV potential. The source was maintained at high vacuum of  $10^{-8}$  mbar and temperature of source unit was maintained at 225°C. Data acquisition and processing were done using OPUS 3.6V software, and the NIST mass spectral library was used for identification of components in the samples.

Capillary column DB5 (30 m x 0.25 mm id, 0.32  $\mu$ m film thickness) was used for the analysis. Helium gas (99.999% purity) was used as carrier gas. The GC conditions set for the analysis are as follows:

Oven Temperature (Initial) : 70°C (hold 3 min)  
 Oven Temperature (Final) : 300°C  
 Ramp rate : 4°C/min  
 Helium flow rate (split mode) : 1.2 ml/min

An amount of 0.2  $\mu$ L of solution was injected through the heated injector port of the gas chromatograph. Simultaneously, magnet scan and data acquisition were initiated.

The GC-MS chromatogram was generated for the FAMES of three samples of combined of DCM and ME extracts of different algae's (CHM, SN, CHL). The components separated in the GC were identified from their fragments pattern in EI mass spectrum (Table 4).

**Table 4:** GC-MS profile of fatty acids, hydrocarbons and oxoacids (%) in the combined solvent (CH, DCM, MeOH) extracts.

S.N.	Components	MF	M.WT	CHM	SN	CHL
1	ME oxoheptanoic acid	C <sub>8</sub> H <sub>14</sub> O <sub>3</sub>	158	1.9	1.2	11.2
2	ME8-oxo-octanoic acid	C <sub>9</sub> H <sub>16</sub> O <sub>3</sub>	172	2.5	2.1	3.8
3	ME 9-oxononanoic acid	C <sub>10</sub> H <sub>18</sub> O <sub>3</sub>	186	19.0	26.5	29.8
4	ME10-oxo decanoic acid	C <sub>11</sub> H <sub>20</sub> O <sub>3</sub>	200	0.0	0.0	0.5
	<b>Total</b>			<b>23.4</b>	<b>29.8</b>	<b>45.3</b>
5	ME octanoic acid	C <sub>9</sub> H <sub>18</sub> O <sub>2</sub>	158	2.9	2.1	4.2
6	ME nonanoic acid	C <sub>10</sub> H <sub>20</sub> O <sub>2</sub>	172	1.9	0.1	0.0
7	MME	C <sub>12</sub> H <sub>24</sub> O <sub>2</sub>	200	0.0	0.0	1.6
8	ME tetradecanoic acid	C <sub>15</sub> H <sub>30</sub> O <sub>2</sub>	242	0.5	1.0	0.4
9	ME pentadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256	0.0	0.5	0.0
10	ME hexadecenoic acid	C <sub>17</sub> H <sub>32</sub> O <sub>2</sub>	268	0.0	0.3	0.1
11	ME hexadecenoic acid	C <sub>17</sub> H <sub>32</sub> O <sub>2</sub>	268	1.1	3.5	0.9
12	ME hexadecanoic acid	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	270	54.6	47.7	30.7
13	ME octadecenoic acid	C <sub>19</sub> H <sub>36</sub> O <sub>2</sub>	296	0.5	0.3	0.6
14	ME hexadecanoic acid	C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>	298	0.2	0.1	0.0
15	ME methyltetradecanoic	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256	2.4	0.5	0.1
	<b>Total</b>			<b>64.5</b>	<b>53.1</b>	<b>38.6</b>
16	MME nonanedioic acid	C <sub>10</sub> H <sub>18</sub> O <sub>4</sub>	212	0.2	4.5	2.6
17	Methyl 8-(2-furyl)octanoate			2.4	2.0	3.7
18	nonanal	C <sub>9</sub> H <sub>18</sub> O	142	2.5	2.8	0.5
19	2-nonenal	C <sub>9</sub> H <sub>16</sub> O	140	--	--	--
20	Decanal	C <sub>10</sub> H <sub>18</sub> O	154	1.3	0.3	0.2
21	Undecenal	C <sub>11</sub> H <sub>22</sub> O	168	--	--	--
22	Trimethyl -2-pentadecanone	C <sub>18</sub> H <sub>36</sub> O	268	0.8	4.2	3.0
23	Tetradecanal	C <sub>14</sub> H <sub>28</sub> O	212	--	0.5	0.1
24	Hexadecane	C <sub>16</sub> H <sub>34</sub>	226	-	0.5	0.2
25	Branched C <sub>15</sub> H <sub>32</sub>		--	--	--	0.6
26	ME methylpentanoic acid			--	--	1.0
	<b>Grand Total</b>			<b>95.1</b>	<b>85.2</b>	<b>88.1</b>
	Unidentified			4.9	14.8	11.9

ME = methyl ester, MME = monomethyl ester, CHM = *Chlamydomonas*, CHL = *Chlorella*, SN = *Scenedesmus*, -- not detected

**NMR Spectra recordings:** The  $^1\text{H}$  NMR spectra of extracts were recorded on a Varian 400 MHz NMR spectrometer. The concentration of the sample used was 20 - 30 mg in  $\sim 0.7$  ml of deuterated chloroform ( $\text{CDCl}_3$ ), containing tetramethylsilane (TMS) as an internal reference as per the following experimental conditions:

Spectral width (SW) = 4800 Hz (0.0 – 12.0 ppm), spectral size = 16 K,  $90^\circ$  pulse = 12.5  $\mu\text{s}$ , relaxation delay = 10 s, and number of scans (NS) = 16 or 32 depending upon the concentration of a sample and presence of low intensity signals. Each spectrum was integrated thrice and the average of the three integrated areas was taken for calculation of structural parameters.

The  $^{13}\text{C}$  NMR spectra were recorded on a Bruker 300 MHz NMR Spectrometer equipped with a broad band probe (BBO) of  $\text{CDCl}_3$  solution of extracts (60 - 80 mg /0.7 ml of  $\text{CDCl}_3$ ) containing internal standard TMS. Relaxation reagent, 0.05M Cr (acac) $_3$ , was added to get the quantitative spectra. Overnight recordings ( $\sim 10,000$  scans) with a relaxation delay time of 5.0 sec. in CPD mode were carried out to achieve the reasonable S/N ratio.

**FTIR spectra recording:** The FTIR-ATR recordings were done on a Perkin Elmer FTIR instrument equipped with diamond ATR. The powdered solid (3 - 4 mg) or extracts of 2 - 3 mg in chloroform were properly spread on the plate so that the diamond tip touches the mass equally and homogeneously. Chloroform was evaporated completely before recording. Blank correction and 32 or 64 scans were given for each recording. The spectral processing and stacking of spectra were carried out using standard software provided with the instrument.

**GPC analyses for triglycerides and free fatty acid:** The determination of mono, di and tri glycerides (MG, DG, TG) and FFA was determined using Gel Permeation Chromatography (GPC) method with Evaporative light scattering detector (Sedex 75 with nitrogen pressure of 2 bar at  $30^\circ\text{C}$ ). The solution was prepared by dissolving extracts in solution containing acetic acid (0.25% w/w) and toluene. The suspended impurities and chlorophyll was removed by Solid Phase Extraction prior to injection to GPC. Separation was done using isocratic elution with 0.25% acetic acid in toluene ( $1.0\text{ mL}\cdot\text{min}^{-1}$ ) as eluent, and a Phenogel column ( $5\text{ }\mu\text{m}$  100 Å,  $300 \times 7.8\text{ mm}$ ) with a Phenogel  $5\text{ }\mu\text{m}$  linear/mixed guard column.

## Results and Discussion

### Solvent extraction results

The results of the lipid extraction by various solvents as given in Table 1 show that the Soxhlet (X) method extracts a higher content of total (neutral and polar) lipids compared to the Accelerated Solvent (AS) extraction method in most of the microalgae biomasses. This is due to the much higher extraction time by the X (500 - 600 minutes) compared to the AS (180 minutes) methods. Interestingly, the content of cyclohexane extracts were higher for the AS method than the X method despite the lower total lipid content extracted by the former method.

### CHNS analyses of solid algae biomasses

The results of six solid algae biomasses samples as given in Table 2 show nitrogen in the range of 8.4 - 11.0 % w/w besides the usual C, H and S elements commonly found in a algae biomass. The source of nitrogen was proteins as revealed by a strong amide band at  $1645 - 1655\text{ cm}^{-1}$  characteristic of proteins in their respective IR (ATR) spectra. Some of the solvent extracts were also analyzed for CHNS content to know the extent of proteins extracted by various solvents in order to determine their interferences in the NMR spectral analyses and presence of the phospholipids. The analyses indicated nitrogen content in the range of 0.3 to 0.5 % w/w in the CH and DCM extracts respectively. This has been ascribed to the small amount of proteins extracted in these solvent extracts compared to quite high amount extracted in the methanol extracts (N,  $\sim 1.0\text{ }\%$  w/w), which was also authenticated by the IR analyses discussed in the succeeding section.

### GC analyses for fatty acid profile

The FAMES of Soxhlet combined CH, DCM and MeOH solvent extracts of various algae biomass samples were analyzed by GC-FID in order to determine the nature of fatty acids comprising algae biomasses. The GC chromatograms as given in the Figure 1 show major peaks corresponding to saturated (C14 - C18:0, C22:0) and unsaturated (C16:1, C18: N = 1-3, C20:1) fatty acids characteristics of algal and vegetable oils. The chromatograms also show minor peaks in between the major peaks, whose nature could not be identified due to their unusual characteristics and lack of standards. These unidentified minor peaks are comprised of 5.3 to 25.0% in different extracts and might be due to the presence of hydrocarbons and conjugated oxylipins as discussed in the GC-MS and NMR sections. The C18:3 was the predominant unsaturated fatty acid (24.9 - 36.8%), unlike vegetable oils such as soybean and radish oils which contained 7 to 9.3 %. The FAMES of *Spirulina* were found to contain  $\gamma$ -linolenic acid (n-6 or  $\omega$  6 C18:3) (Table 3). The C16:1 were present in significant amount similar to those found in algal and fish oils<sup>[7,8,19]</sup>. The Soxhlet CYN and FL extracts were found to be rich in saturated fatty acids (43.3 - 52.4%) compared to other algae samples (18.7 to 34.7%), but much higher than saturated fatty acids present in vegetable oils (12 - 16 %)<sup>[8,36]</sup>. The higher number fatty acids C20-24 were present in the range of 0.7 - 2.6%, which were also common in algae biomasses. The low intensity peaks appeared at higher RT might be due to the presence of low amount of C22: 6 (DHA) as confirmed by NMR analyses.

The GC results of the Accelerated Solvent extracts (AS) of *Scenedesmus dimorph* (SNASCH, SNASDCM, SNASMe) and *Chlorella vulgaris* (CHLASCH, CHASDCM) were compared to ascertain the extraction efficiency of these solvents towards extraction of fatty acid components (Table 3a). The saturated fatty acid components (C14:0 - C18:0) are extracted in large amounts

by CH (43.5 to 57 %) compared to DCM (28.2 - 38.6 %). This was expected based on the concept of the polarity index of solvent used for the extraction of neutral or polar lipids components. Cyclohexane, which is lowest on the polarity index scale with level of 0.2 compared to level of 3.1 for dichloromethane, will extract more of the glycerides enriched with saturated fatty acids (14:0 - C20:0). It is interesting to note that the polarity index concept has not prevailed in the case of methanol extracts (polarity index 5.5) as indicated by its fatty acid profile given in the Table 3a. This has been ascribed to the low hydrolyses efficiency of phospholipids, which has been found to be present in large amount in methanol extracts, as revealed by NMR and IR analyses discussed in the following sections.

Thus, the extraction efficiency towards extraction of types of lipids constituting saturated or unsaturated fatty acids was found to be dependent on the polarity of the solvent used for extraction of biomass. The extraction of PUFAs (C18:3 or higher) was also found to be specific to the types of microalgae strains used for the cultivation of biomasses. This was evident in case of Soxhlet extracts of biomasses of SN and FL, which were found to be enriched with PUFAs (33 - 36.8 %) compared to biomasses of CHL, CHM and CYN (Table 3). Similar results have been achieved in case of AS extracts as mentioned in Table 3a.

### GC-MS analyses for complete component analyses

The GC-MS analyses of FAMES of combined Accelerated Solvent CH, DCM and MeOH extracts of three algae biomass samples, CHM, SN and CHL, have been carried out with the objective to find out the nature of the polar components, particularly conjugated/ un conjugated olefinic oxy fatty acid or esters known as oxylipin<sup>[51-54]</sup>. The GC-MS results were interesting, particularly the profile of oxo acids and minor hydrocarbon components (Table 4). The oxo acids were found to be present in the carbon range of C7 to 10 with oxo group i.e. aldehyde at the other end of the carbon chain of acids. The extracts of CHL contained the highest amounts of oxo acids (45.3 % w/w) compared to CHM (23.4% w/w) and SN (29.8 % w/w) algae biomasses. The GC results also indicated higher amount of unidentified in CHL as shown in the Table 3. The NMR spectral analyses have clearly shown aldehyde groups at downfield in the chemical shift range of 8.0 - 11.1 ppm, characteristic of oxo acids with saturated and unsaturated acid chain, as covered in the following section of NMR analyses. The GC-MS analyses also showed both saturated and unsaturated long alkyl chains hydrocarbons in addition to the presence of common fatty acids. The lower carbon number acids (C8 - C10) and branched acids were found to be present in small amount.

The NMR analyses also indicated conjugated unsaturated oxo acids containing CH=O groups adjacent to double bonds. However, GC-MS analyses showed only the presence of saturated oxo acids. The conjugated unsaturated oxo acids might not have been eluted or identified. The oxo acids are generated by enzymatic cleavage of oxidized unsaturated fatty acids such as C18:3, C20:5 and C22:6. These are precursors for the formation of pheromones, the C11 conjugated hydrocarbons<sup>[51-54]</sup>.

### GPC analyses of algal extracts

The GPC analyses of solvent extracts obtained by Soxhlet and Accelerated solvent extraction methods were carried out to determine the distribution of mono (MG), di (DG) and tri (TG) glycerides and free fatty acids (FFA) (Table 5). The results showed that in most of the extracts of SN, CHL and SP obtained by either Soxhlet (X) or Accelerated Solvent (AS) extraction methods, cyclohexane extracts (SNXCH or SNASCH1; CHLXCH or CHLASCH1 etc.) were found enriched with FFA (23.3 - 50.0 % w/w) compared to DCM extracts, which contained both glycerides and FFA (1 - 16.7 % w/w) in lower amount. The lower amounts of neutral lipids (TAG, FFA) in the DCM and DCM1 extracts of these biomasses were due to the presence of higher amount of polar lipids (amides, glyco, glyceroglycophospho lipids) as confirmed by their NMR and IR analyses discussed in the following sections. The TG contents were higher than DG and MG (Table 5). The MG content has been found to be the lowest in the range of 1 - 2.8 % w/w. The methanol extracts were found to contain very small amount of FFA and TAG; also indicated by their NMR and IR analyses discussed in the following sections. There was no trend, higher or lower, observed between Soxhlet and Accelerated Solvent extraction methods for the extraction of TAG or FFA as shown in the Table 5. However, higher amounts of TG were extracted by CH<sub>2</sub> and DCM2 extracts of SN, CHL and SP biomasses. The Soxhlet extraction method provided higher amount of neutral lipids (TAG+FFA) compared to the Accelerated Solvent extraction method. However, the solvent extraction method has the advantage of time of extraction, which was 180 minutes in case of the Accelerated Solvent extraction compared to approximately 500 - 600 minutes for the Soxhlet extraction.

The analyses of extracts of CYN, CHM and FL biomasses indicated FFA were the main components being in the range of 75.4-92.4 % w/w, independent of the method of extraction.

**Table 5:** Triacylglycerides profile and structural parameters determined by GPC and NMR (%w/w).

S.N	Solvent extracts	GPC TG	GPC DG	GPC MG	GPC FFA	GPC TOTAL	FFA NMR	NMR TG	ICHO* %	IV	PUFA
1	SNASCH1	12.2	10.4	< 1.0	34.0	56.6	35.1	11.1	0.46	185.1	48.8#
3	SNASCH2	2.71	3.56	< 1.0	7.38	14.7	9.2	9.2	0.6	161.0	55.3#
4	SNASDCM1	1.63	1.1	1.0	< 1.0	4.7	< 5	< 5.0	1.5	135.4	39.4#
6	SNASDCM2	60.5	< 1.0	< 1.0	< 1.0	60.5	< 5	63.5	0.9	148.2	39.8#
7	SNXH	20.4	13.0	< 1.0	23.3	57.7	25.1	20.4	0.5	173.6	42.7#
8	SNXDCM	3.0	2.2	2.8	6.9	14.9	8.5	4.0	0.6	161.0	41.6#
9	CHLASCH1	10.4	5.0	< 1.0	50.0	65.4	51.7	10.7	1.04	147.8	35.8



10	CHLASCH2	3.5	8.3	2.5	5.8	20.1	< 5	5.1	0.72	143.9	35.6
11	CHLASDCM1	3.8	6.8	1.0	16.7	28.3	16.3	6.2	1.5	137.0	32.6
12	CHLASDCM2	32.8	< 1	< 1	< 1	32.8	< 5	34.3	1.6	140.7	31.9
13	CHLXCH	10.4	16.7	< 1	43.8	71.9	45.2	9.8	0.71	111.2	24.3
14	CHLXDCM	9.3	28.2	< 1	30.0	67.5	33.1	8.6	0.72	144.6	37.2
15	SPASH1	25.4	25.5	< 1	< 1	50.9	< 5	27.2	0.53	88.06	11.4*
16	SPXH	20.6	21.8	< 1	< 1	42.4	< 5	19.4	nd	69.8	14.6*
17	SPDCM	4.7	1.4	2.0	48.5	56.6	59.2	5.2	0.42	110.6	16.6*
18	SPASDCM2	60.3	1	1	10.5	73.8	11.6	64.2	0.31	94.7	2.3*
19	SPASDCM1	5.2	1.4	2.3	50.3	59.2	52.4	5.9	0.24	112.7	17.1*
20	CYNASCH1	8.6	2.9	1.0	85.9	98.4	84.9	7.7	nd	109.8	38.6
21	CYNASDCM1	2.7	1.6	2.3	6.1	12.7	6.3	4.0	0.5	104.9	30.6
22	CYNXCH	4.8	3.9	1.0	76.0	85.7	80.2	5.7	nd	109.5	32.7
23	CYNXDCM	2.8	2.1	1.0	67.0	72.8	75.4	< 5	0.61	106.1	31.2
24	CHMXCH						87.3	< 5	nd	138.4	26.3#
25	CHMASH1						92.4	< 5	nd	141.2	26.8#
26	CHMXDCM						86.4	< 5	0.7	141.5	30.5#
27	FLASCH1						81.6	< 5	0.43	144.7	49.8#
28	FLASDCM1						80.2	6.2	2.45	117.7	37.2#
29	FLXCH						82.5	7.2	0.50	139.1	36.9#
30	FLDCM						84.7	6.2	1.94	119.3	

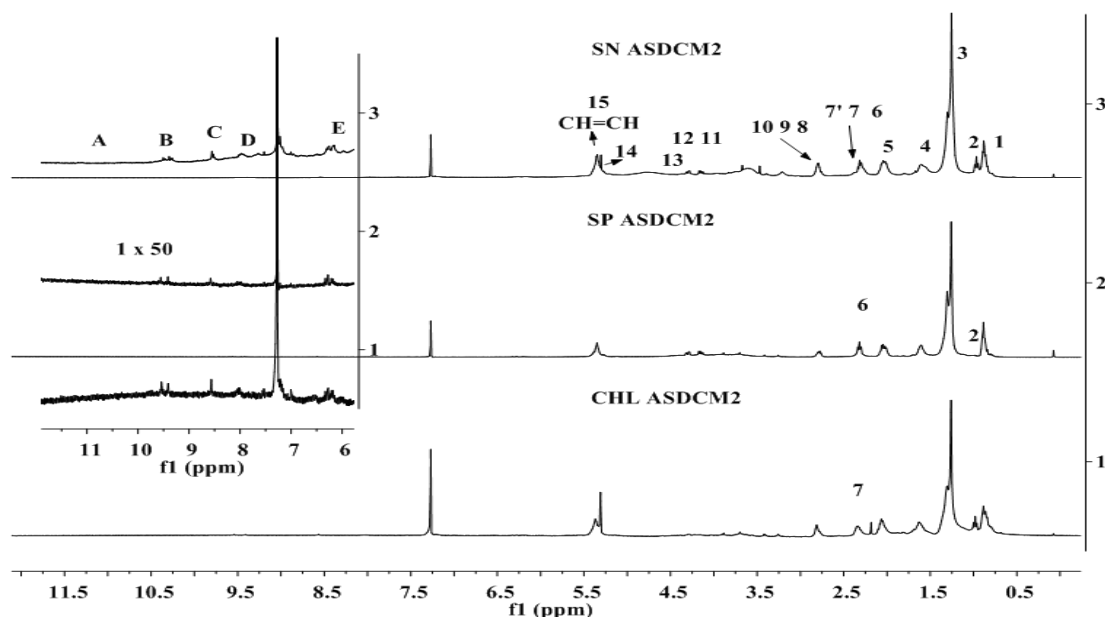
IV = Iodine value; \* $\gamma$ -C18:3, # C18:3 + C22:6; ICHO % = percentage integrated intensities of aldehyde and conjugated olefinic hydrogens from 6.0 - 11.3 ppm (A to E, Figure 5); MG, DG or TG = mono, di or tri glycerides, AS=Accelerated Solvent extraction, X = Soxhlet extraction, FFA = Free fatty acid, SN = *Scenedesmus*, CHL = *Chlorella*, CYN = *Cyanobacteria*, CHM = *Chlamydomonas*, FL = *SD Flakes*, SP = *Spirulina*.

### NMR spectral analyses of extracts

The NMR ( $^1\text{H}$  &  $^{13}\text{C}$ ) analyses were conducted to find out nature and content of TAG, and fatty acids comprising of TAG, FFA and polar components as discussed below.

### $^1\text{H}$ NMR analyses of extracts

**Identification of TAG in the AS extracts of *Scenedesmus*, *Chlorella* and *Spirulina*:** The  $^1\text{H}$  NMR spectral features of solvent extracts of different microalgae biomasses with regard to the identification of neutral and polar lipids have been systematically presented in details in our earlier work<sup>[7,8,14]</sup>. However, the spectral features of the extracts being studied have been presented in order to apprise the readers about the wealth of useful information that can be obtained from NMR spectroscopic techniques.  $^1\text{H}$  NMR spectra of successive stepwise DCM2 extracts of SN, SP and CHL microalgae biomasses obtained by Accelerated Solvent extraction (AS) method as shown in Figures 2 to 4 show signals characteristics of triglycerides comprised of both saturated and unsaturated fatty acid alkyl chain. The identities of signals marked 1 to 15 are described in the Table 6. The extracts clearly indicate characteristic symmetrical multiplet signals due to  $\text{OCH}_2$  (sn1, sn3) groups of acyl group of triglycerides at 4.08 to 4.38 ppm (signals 11 and 12). The multiples at 4.05 - 4.22 ppm (signal 11) are due to the  $\text{OCH}_2$  groups of mono and di glycerides besides containing contributions from triglycerides (sn1,  $\text{OCH}_2$ ). The  $\text{OCH}$  (sn2) signal marked 14 of glycerol group is indicated at 5.26 ppm, partly merged with proton signals of  $\text{CH}=\text{CH}$  (signal 15) of unsaturated fatty acid chain. The identities of these signals were confirmed by comparing them with the identical signals of a blend of mono (MG), di (DG), and tri (TG) glycerides clearly depicted in the Figure 3 (signals 11, 12, 14, 16, 17) and described in the Table 6. The comparison of the spectral features of DCM2 extracts of the three microalgae biomasses with the spectral features of the blend also revealed the absence of MG and DG (signals 11, 16, 17) in these extracts as evident from the spectral features shown in Figures 2 and 3. The identification of MG, DG and TG in these extracts are in line with the results obtained by GPC analyses (Table 5).

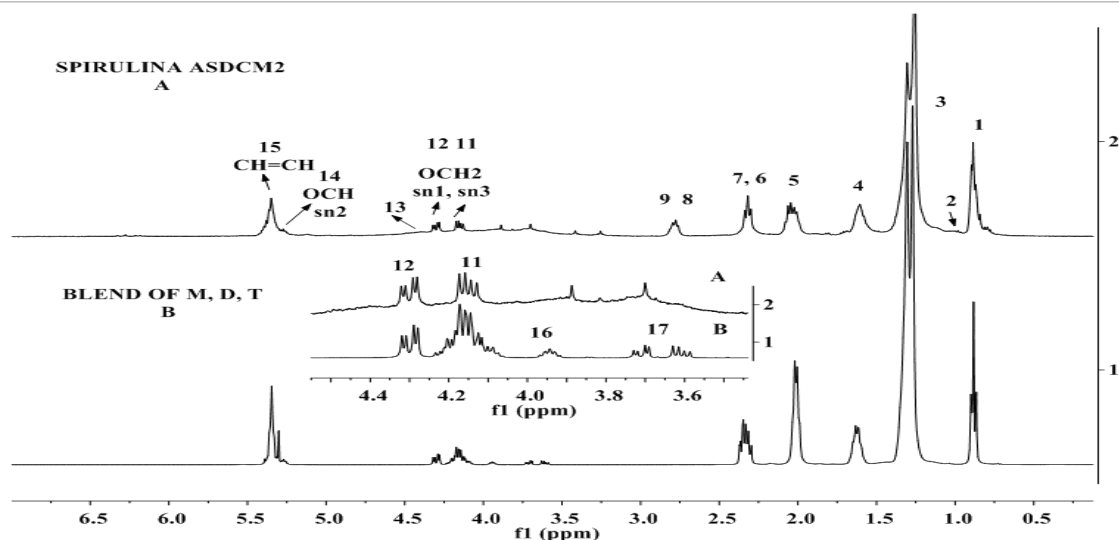


**Figure 2:**  $^1\text{H}$  NMR part spectra of dichloromethane (DCM<sub>2</sub>) extract of biomass of *Spirulina*, *Scenedesmus* and *Chlorella* by accelerated solvent (AS) extraction method. Signals marked A,B,C,D are aldehyde protons and E denotes protons of conjugated olefins, different from unsaturated protons of fatty acid chains (signal 15). The identities of signals marked 1 - 15 are described in Table 6.

**Table 6:**  $^1\text{H}$  NMR chemical shift regions of functional groups of glycerides, phospho, glyceroglyco lipids.

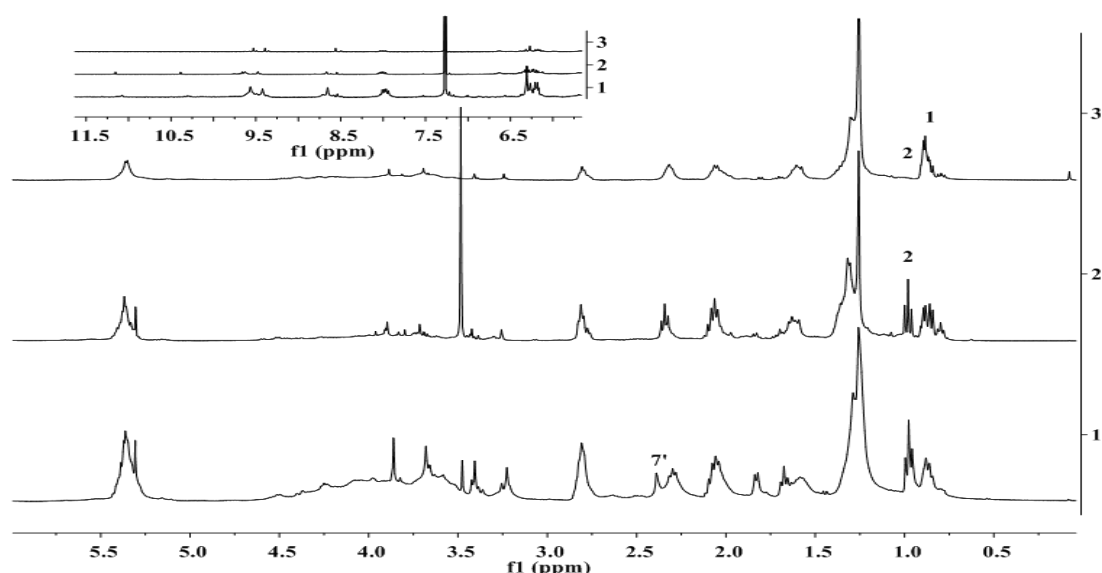
S.N	Assignments	Chemical shift Region (ppm)	Components
1.	Terminal methyls ( $\text{CH}_3$ ) of fatty acids chain ( $\omega$ , n-6, n-9)	0.88 (0.66 - 0.93)	C14:0-C18:4, C18:1, C18:2
2.	Terminal methyls ( $\text{CH}_3$ ) of fatty acids chain ( $\omega$ , n-3)	0.98 (0.93 - 1.04)	C18:3, C20:5, C22:6
3.	$-(\text{CH}_2)_n$ of long alkyl chain, saturated and unsaturated	1.26, 1.29, 1.30	C14:0-C18:0, C18:N=1-3, C20:5, C22:6
4.	$\alpha\text{-CH}_2$ of fatty acid chain	1.61 or 1.62	All fatty acids/esters, glycerides
5.	$\text{CH}_2\text{-(CH=CH-CH}_2)_n$ -, vinylic	2.06 (2.20 - 1.86)	All fatty acids/esters, glycerides
6.	$\text{CH}_2\text{C=O}$ of ester	2.30 or 2.31 (2.26 - 2.35)	All fatty esters, glycerides
7.	$\text{CH}_2\text{C=O}$ of FFA	2.34 or 2.35 (2.34 - 2.39)	All fatty acids
7'	$\text{CH}_2\text{C=O}$ of C22:6	2.39 or 2.40	$\omega$ n-3C22:6
8.	$(\text{CH}_2\text{-CH=CH})_n$ , bis-allylic	2.77	C18:2, n=2
9.	$(\text{CH}_2\text{-CH=CH})_n$ , bis-allylic	2.81	C18:3, n=3
10.	$(\text{CH}_2\text{-CH=CH})_n$ , bis-allylic	2.84 or 2.85	C20:5 or C20:6
11.	$\text{OCH}_2$ , sn1, acyl group	4.16 (4.05 - 4.22)	Tri glycerides (TG)
11.	$\text{OCH}_2$ , $\text{HOCH}_2$	4.02 - 4.22	MG, DG, TG
12.	$\text{OCH}_2$ , sn3, acyl group	4.29 (4.22 - 4.37)	Triglycerides( TG)
13.	$\text{OCH}_2$	4.44(4.39 - 4.5)	Phospholipids
14.	$\text{OCH}$ , sn2, acyl group	5.26	Triglycerides (TG)
15.	$\text{CH=CH}$	5.26 (5.05 - 5.7)	Unsaturated fatty acids chain, C18:N = 1-3 etc.
16.	$\text{HOCH}_2$	3.94 (4.02 - 3.89)	1- MG
17.	$\text{HOCH}_2$	3.70 (3.79 - 3.66), 3.60 (3.66 - 3.52)	2-DG

S.N = Signal number marked in the  $^1\text{H}$  NMR spectra, ( ) = chemical shift region; without bracket = peak maxima; MG, DG, TG = mono, di, tri glycerides



**Figure 3:** (A) <sup>1</sup>H NMR part spectra of dichloromethane (DCM<sub>2</sub>) extract of biomass of *Spirulina* (A) by Accelerated Solvent extraction (AS) method, and (B) a blend containing mono (M), di (D), tri (T) glycerides and free fatty acid (FFA).

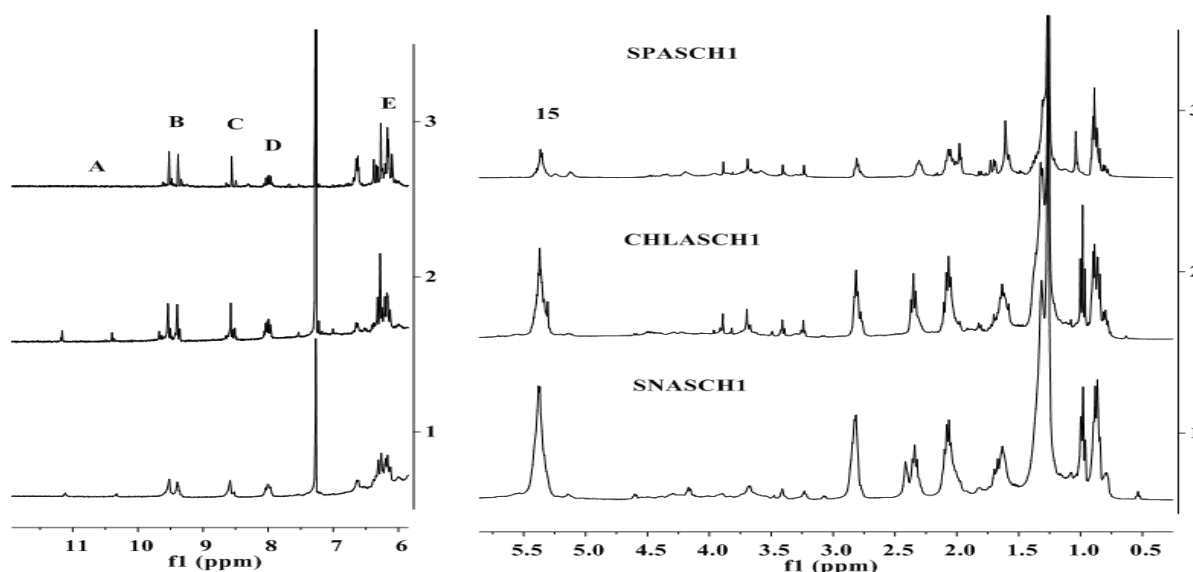
The signals due to CH<sub>2</sub>OH and CHOH of glyceroglyco/phospho lipids were observed spread over in the region of 3.0 to 4.0 ppm. The broad signals at 2.6 - 2.9 ppm were due to bis-allylic protons of (CH<sub>2</sub>-CH=CH)<sub>n</sub> corresponding to lenoleic (C18: 2) (signal 8, 2.77 ppm), lenolenic (C18:3) (signal 9, 2.81 ppm) and C22:6 (signal 10, 2.84 - 2.85 ppm) fatty acid chain. These signals were clearly distinguishable on expansion and amplification of the chemical shift region of 2.6 - 2.9 ppm as discussed in the next section (Figures 10 - 11). The spectra of CHL and SN clearly showed triplets at 0.98 ppm (signal 2) due to terminal methyl protons of ω n-3 fatty acid chain of PUFE/PUFA (C18:3, C22:6), which were quite separated from the signals of terminal methyls of n-6 & n-9 ω C18:2 and 18:1, and saturated fatty acid chain of C14:0 - C18:0 that appeared at 0.88 ppm (signal 1) (Figure 2). The shoulder appeared at 1.3 ppm besides signal 3 was due to the presence of (CH<sub>2</sub>)<sub>n</sub> of saturated fatty acid components such as C14: 0 to C18:0. The relative higher intensities of signals 9 due to γ or α-linolenic acid in the spectra of the extracts compared to signals of C18:2 (signal 8) show their higher amount present in the oil from these microalgae species. The presence of C18: N =1-3 and saturated fatty acid components of TAG were confirmed by GC-FID analyses (Table 3). The signals marked A to E in the expanded chemical shift region of 6 - 12 ppm are assigned to aldehyde and conjugated olefinic hydrogens of oxy fatty acid or esters known as oxylipins as discussed in details in the section of identification of oxylipins (Figures 2, 4, 5).



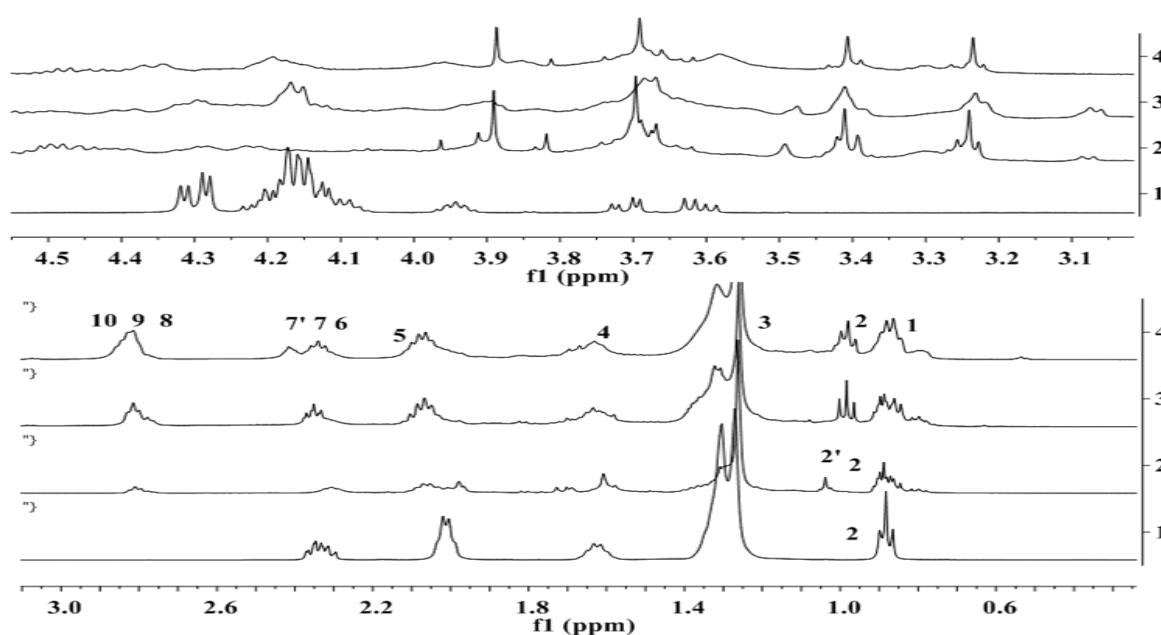
**Figure 4:** <sup>1</sup>H NMR expanded view of part spectra of dichloromethane (DCM<sub>1</sub>) extracts of biomasses of *Scenedesmus* (spectrum 1), *Chlorella* (Spectrum 2), and *Spirulina* (spectrum 3) by Accelerated Solvent extraction (AS) method. Signals 1= terminal methyls of C18:1, C18:2 and saturated fatty acids; 2 = terminal methyls of ω 3 (n-3) PUFA and 7' = CH<sub>2</sub>C = O group of C22:6 (DHA). Chemical shift region of 6-11.7 ppm is due to hydrogen attached to conjugated and non conjugated unsaturated hydrogens and aldehyde hydrogens.

The <sup>1</sup>H NMR spectra of DCM<sub>1</sub> and CH<sub>1</sub> extracts of SN, SP and CHL biomasses obtained by AS method as shown in the Figures 4 - 7, exhibit similar spectral features characteristic of MG, DG and TG, and their fatty acid profiles as marked by signals 1 - 15. The signals due to oxylipins marked A-E are also visible in their spectra (Figures 4 and 5). The SNASDCM<sub>1</sub> extracts were

found mainly rich in polar lipids as evident from the appearance of intense peaks in the region of 3 - 4.0 ppm compared to the spectral features of SPASDCM1 and CHLASDCM1 in the same region. FFA is practically absent as no signal is observed at 2.34 ppm in case of SNASDCM1. The SPASDCM1 and CHLASDCM1 were found to contain both TAG and FFA as intense signals were clearly visible in the region of 4.05 - 4.35 ppm ( $\text{OCH}_2$ ), and at 2.31 ppm ( $\text{CH}_2\text{C}=\text{O}$ ) (TAG) and 2.34 ppm ( $\text{CH}_2\text{C}=\text{O}$ ) (FFA). However, the spectrum of SPASDCM1 shows an intense signal at 2.34 ppm due to  $\text{CH}_2\text{C}=\text{O}$  group of FFA, and very weak signals in the regions of 4.35 - 4.05 ppm and 3 - 4.0 ppm, thus confirming the presence of mostly FFA (Figure 4, spectra 3). The NMR results are supported by GPC results with regard to the distribution of TAG and FFA as given the Table 5. The appearance of a signal at 2.39 - 2.4 ppm (signal 7') in the spectrum of SNASDCM1 indicates protons due to  $\text{CH}_2\text{C}=\text{O}$  of C22:6 (DHA) fatty acid chain as reported previously (Figure 4)<sup>[7,8]</sup>. The spectra of CH1 extracts shown in Figures 5 - 7 indicate mostly FFA in addition to the presence of triglycerides and polar lipids as the signals marked 7 at 2.35 ppm are quite intense compared to signal marked 6 at 2.31 ppm (Figure 6). In order to confirm the presence of TAG, particularly triglycerides (TG), the spectral features of SNASCH1 were compared with those of a blend containing MG, DG, TG and FFA as shown in Figure 7. The signal marked 13 (4.38 - 4.5 ppm), which can be mistaken for TG (4.22 - 4.37 ppm), has been assigned to the hydrogens of  $\text{OCH}_2$  groups of glycerol ( $\text{CH}_2\text{O}-\text{CHO}-\text{CH}_2\text{O}$ ) chain of glycerophosphates lipids, the important components of polar lipids. Similarly, the extracts of SPASCH1 and CHASCH1 were found to be rich in FFA besides containing TAG and polar lipids (Figures 5 and 6).

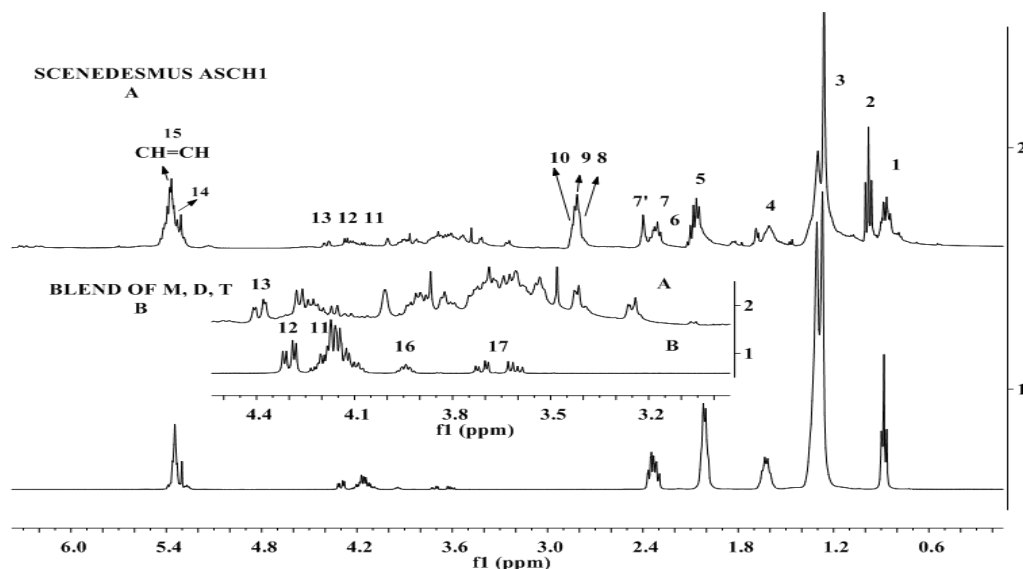


**Figure 5:**  $^1\text{H}$  NMR expanded view of part spectra of cyclohexane extracts (CH1) of biomasses of *Spirulina* (SPASCH1), *Chlorella* (CHLASCH1), and *Scenedesmus* (SNASCH1) by Accelerated Solvent extraction (AS) method. Signals marked A to D are aldehyde hydrogens and E denotes hydrogens of conjugated olefins, different from unsaturated protons of fatty acid chains (signal 15).



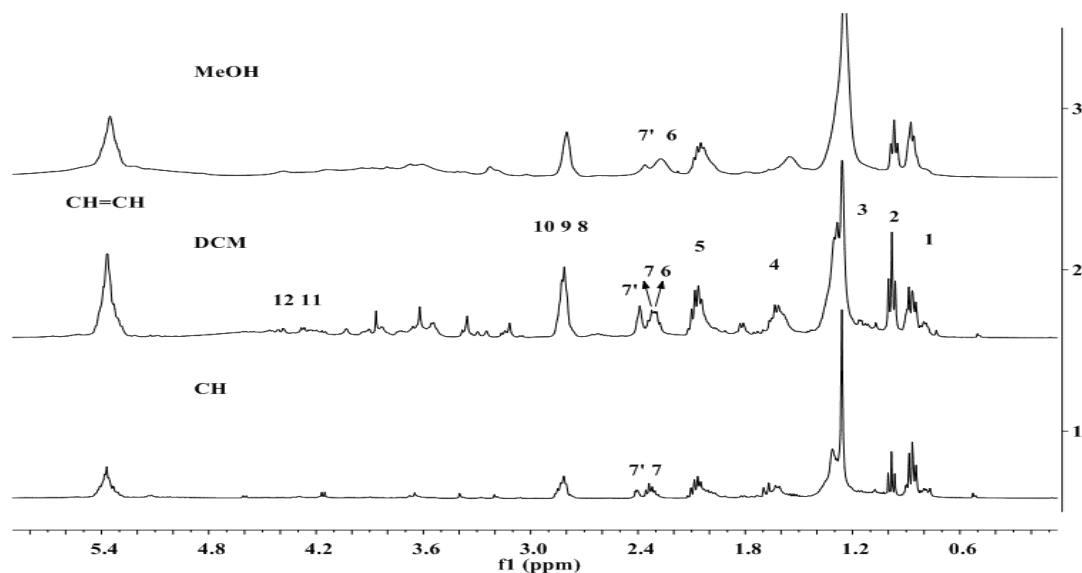
**Figure 6:**  $^1\text{H}$  NMR expanded view of part spectra of a blend containing mono, di, tri (T) glycerides and free fatty acid (FFA) (spectra 1) and cyclohexane extracts (CH1) of biomasses of *Spirulina* (spectra 2), *Chlorella* (Spectra 3), and *Scenedesmus* (spectra 4) by accelerated solvent extraction (AS) method. For designated signals marked as 1 to 10, refer to Table 6.





**Figure 7:**  $^1\text{H}$  NMR part spectra of cyclohexane extract of *Scenedesmus* by accelerated solvent extraction (ASCH1) (spectra A) method and a blend containing mono (M), di (D), tri (T) and free fatty acid (FFA) (spectra B). For designated signals marked 1 to 17, refer to Table 6.

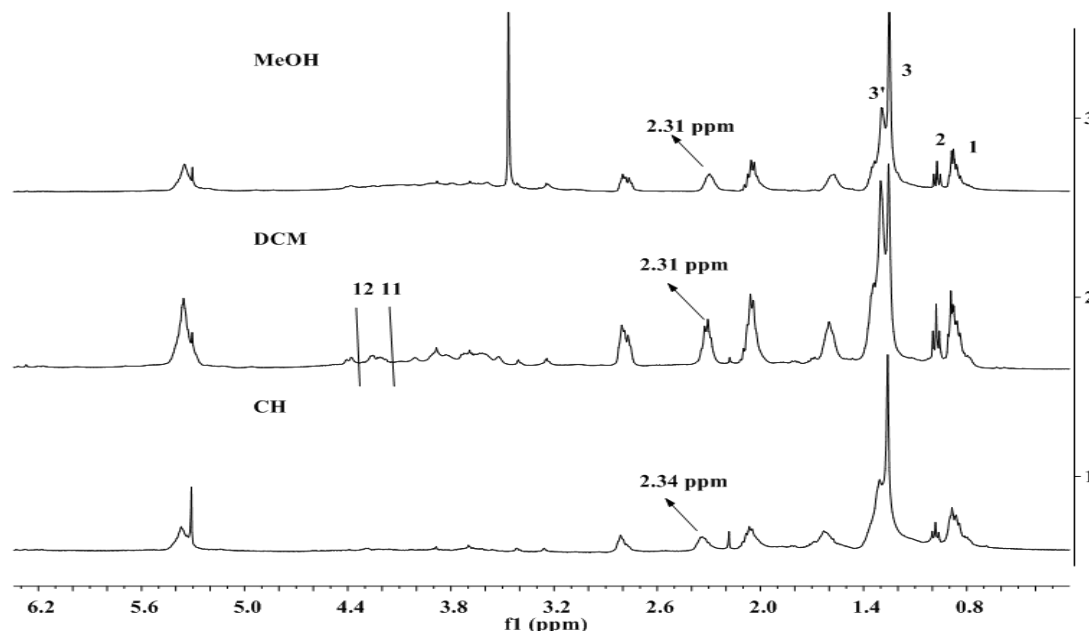
The appearance of signals at 2.81 ppm (signal 9) and absence of signals at 0.98 ppm (signal 2), confirm the presence of  $\gamma$ -C18:3 as discussed in the following section (Figure 6, spectra 2)<sup>[7]</sup>. The spectrum show a sharp signal at 1.03 ppm marked 2', which could possibly be assigned to  $\text{C}-(\text{CH}_3)_3$  types of signals. Such types of signals generally appear in the spectra of isoprene types of hydrocarbons<sup>[56]</sup>. The signal 7' observed in SNASCH1 is assigned to  $\text{CH}_2\text{C}=\text{O}$  group of C22:6 as discussed in case of SNASDCM1. Its presence is also supported by the appearance of signals at 2.84 ppm due to bis-allylic groups (Figure 6). As shown in the Figures 10 and 11, the region corresponding to C18:1 (signal 1), C18:2 (signal 8), C18:3 (signal 9) and C22:6 (signal 10) are marked to distinguish these unsaturated fatty acids in the overlapped regions.



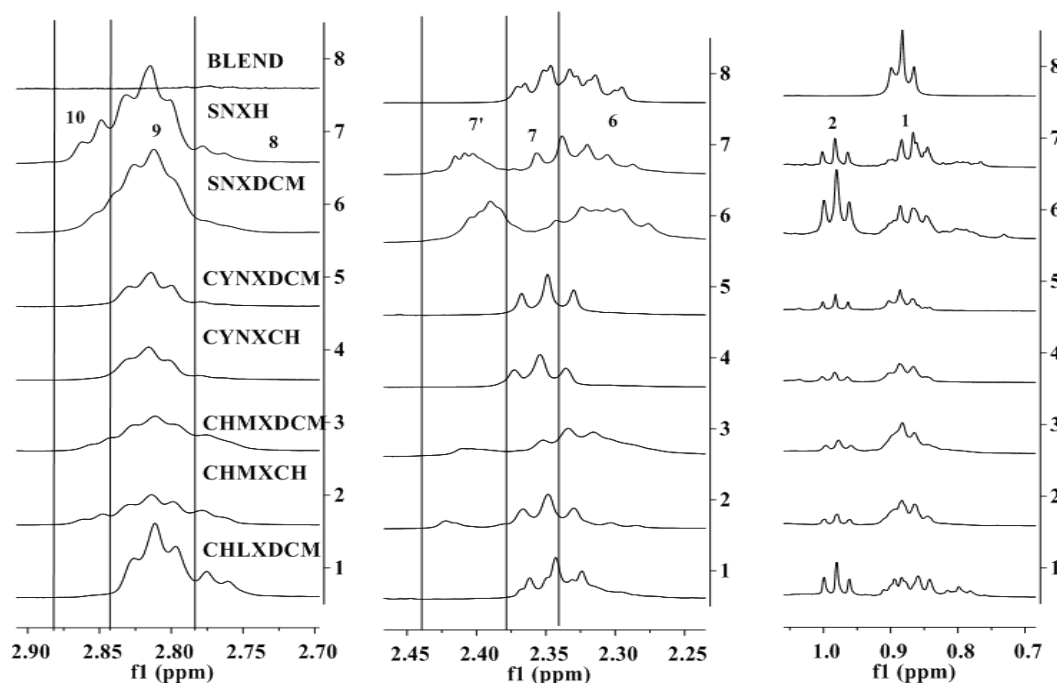
**Figure 8:**  $^1\text{H}$  NMR part spectra of cyclohexane (CH), dichloromethane (DCM) and methanol (MeOH) extracts of *Scenedesmus* by Soxhlet extraction method. For designated signals marked 1 to 12, refer to Table 6.

**Analyses of extracts from Soxhlet extraction method:** The  $^1\text{H}$  NMR spectra of extracts or algal oils of SN and CHL biomasses obtained by Soxhlet (X) extraction method using CH, DCM and MeOH solvents as shown in Figures 8 and 9 exhibit spectral features similar to those present in the spectra of the extracts obtained by their respective Accelerated Solvent (AS) extraction method presented in Figures 2 - 7. The signals characteristics of glycerides and polars including their fatty acid profile and PUFAs (C18:3, C22:6) were observed at their respective chemical shift regions. The CH extracts showed intense signals at 2.34 ppm (signal 7) corresponding to hydrogens of  $\text{CH}_2\text{C}=\text{O}$  of FFA in contrast to DCM extracts, which show signals due to both ester (signal 6, 11, 12) and FFA (signal 7). The presence of C22:6 were indicated at 2.4 ppm (signal 7') in the all extracts of SN. The signals corresponding to terminal  $\text{CH}_3$ s of n-3  $\omega$  PUFAs (C18:3, C22:6) are shown at 0.98 ppm (signal 2) with variation in their signal intensities depending on the polarity index of the solvent used for extraction (Figure 8). It is quite evident that DCM extracted higher amount of PUFAs (C18:3, C22:6: signals 2, 7, 10) compared to CH as shown in both the spectra of SN and CHL (Figures 8 and 9). The PUFAs

in the case of CHL extracts were comprised of only C18:3 because signals due to C22:6 were not observed (Figure 9). The spectral features of methanol extracts showed the presence of mostly polar lipids as indicated by the intense signals in the region of 3 - 5 ppm, characteristics of glyco/glycophospho lipids. Similar spectral features characteristics of both neutral and polar lipids were found to be observed in the extracts of SPX, SPXDCM and SPXMeOH (spectra not given). The signals due to n-6  $\gamma$ -C18:3 were indicated at 2.81 ppm without the appearance of a signal at 0.98 ppm in the extracts of SP<sup>[7]</sup>. The presence of  $\gamma$ -C18:3 in the SP extracts were also confirmed by GC-FID analyses (Tables 3 and 3a).



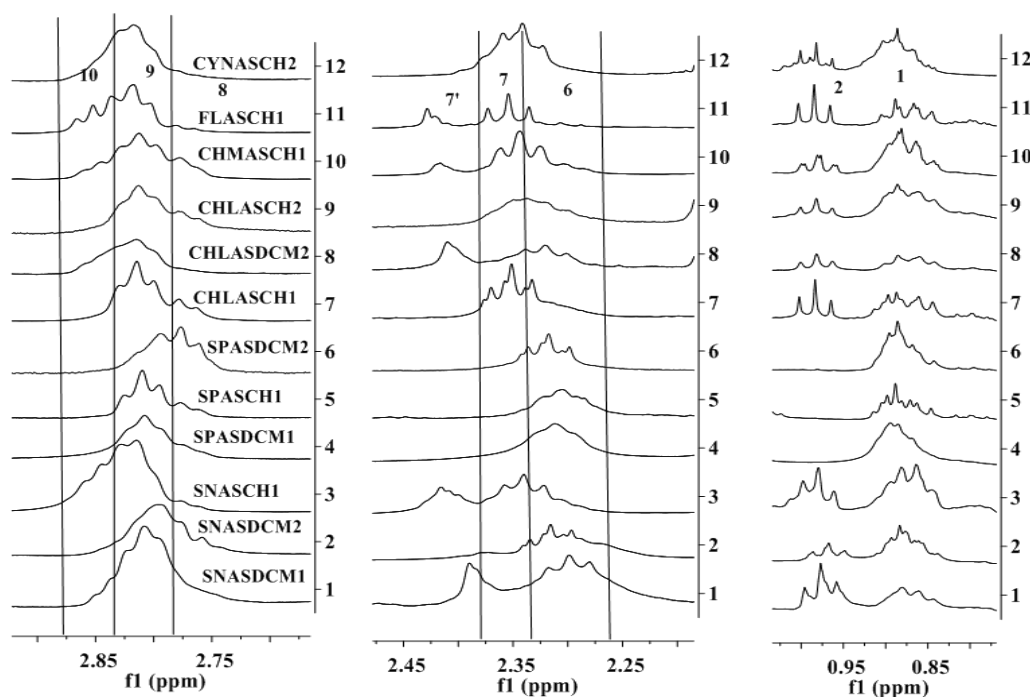
**Figure 9:**  $^1\text{H}$  NMR part spectra of cyclohexane (CH), dichloromethane (DCM) and methanol (MeOH) extracts of *Chlorella* by Soxhlet extraction method. Signals at 2.31 ppm = ester  $\text{CH}_2\text{C}=\text{O}$  group and 2.34 ppm = free fatty acid  $\text{CH}_2\text{C}=\text{O}$  group. Signals 11 and 12 =  $\text{OCH}_2$  of triglycerides.



**Figure 10:**  $^1\text{H}$  NMR expanded view of part spectra of solvent extracts (CH, DCM) of various algae biomasses in the different chemical shift regions demarcated for bis-allylic group (2.7 - 2.90 ppm) for signals marked as 8 = C18:2, 9 = C18:3 and 10 = C22:6;  $\text{CH}_2\text{C}=\text{O}$  (2.35 - 2.45 ppm) for signals 6 = ester group of glycerides, 7 = free fatty acid (FFA)  $\text{CH}_2\text{C}=\text{O}$  group and 7' = C22:6; and terminal methyls (0.7 - 1.05 ppm) signals 1 = C18:1, C18:2, CN:S = saturated and signal 2 = C18:3 of Soxhlet extracts (X).

### Identification of free fatty acids (FFA) by $^1\text{H}$ NMR spectral analyses

The GPC analyses of AS and X solvent extracts confirmed the presence of FFA, particularly in high amounts in the CH extracts (Table 5). As discussed in the preceding section, the signals due to  $\text{CH}_2\text{C}=\text{O}$  groups corresponding to ester of TAG and FFA could be distinguished in the overlapped region of 2.15 - 2.5 ppm. The chemical shift regions corresponding to each have been described in details in our earlier work on the compositional characterization of vegetable oils and microalgal oil generated by ultrasonic extraction method by the application of high magnetic fields (500 - 800 MHz)<sup>[7,8,14]</sup>. In order to identify the presence of FFA in the algal oils by 400 MHz  $^1\text{H}$  NMR spectral analyses, the chemical shift region 2.2 - 2.5 ppm was expanded and amplified to enable demarcation of regions of ester and FFA as presented in the Figures 10 and 11 for different extracts obtained by the AS and X extraction methods. The peak maxima of multiplet signals at 2.34 ppm (signal 7) and 2.31 ppm (signal 6) respectively, were demarcated for FFA and ester in comparison to the signals of a blend containing TAG and FFA (Figure 10)<sup>[8]</sup>. Although, these signals overlapped, the FFA can definitely be identified in the algal oil under study as shown in the Figures 10 and 11. The peak maxima has never been observed extended beyond 2.34 - 2.35 ppm irrespective of the nature of the saturated and unsaturated fatty acids<sup>[8]</sup>. The FFAs were further confirmed by  $^{13}\text{C}$  NMR analyses of solvent extracts as discussed in the following section.



**Figure 11:**  $^1\text{H}$  NMR expanded view of part spectra of solvent extracts (CH, DCM) of various algae biomasses in the different chemical shift regions demarcated for bis-allylic group (2.7 - 2.90 ppm) for signals marked: 8 = C18:2, 9 = C18:3 and 10 = C22:6;  $\text{CH}_2\text{C}=\text{O}$  (2.35 - 2.45 ppm) for signals 6 = ester group of glycerides, 7 = free fatty acid (FFA)  $\text{CH}_2\text{C}=\text{O}$  group and 7' =  $\text{CH}_2\text{C}=\text{O}$  of C22:6; and terminal methyls (0.7 - 1.05 ppm) signals 1 = C18:1, C18:2, CN:S (S = saturated) and signal 2 = C18:3 of extracts by Accelerated Solvent extraction (AS) method.

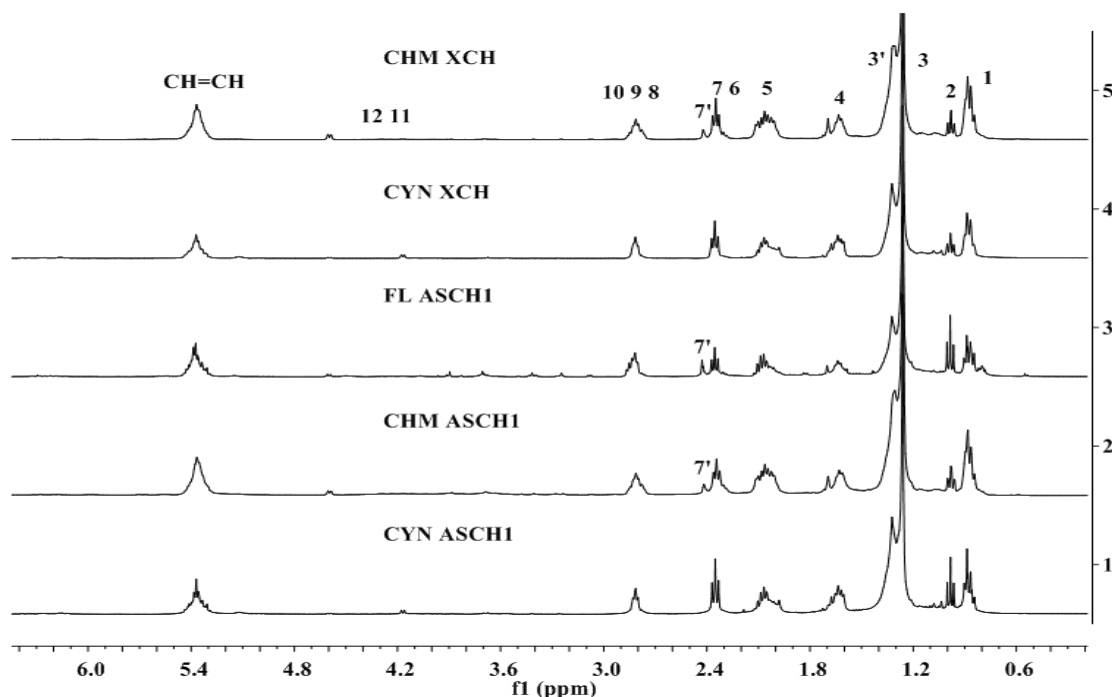
### Identification of mono, di and tri glycerides

As discussed in the preceding section, the  $^1\text{H}$  NMR analyses of different solvent extracts have shown the presence of mono, di and tri glycerides as revealed by their characteristic signals in the chemical shift regions of 3.4 - 4.8 ppm. The identities of signals corresponding to mono, di and tri acyl glycerides were further confirmed from the comparison of  $^1\text{H}$  NMR spectra of reference compounds of mono, di and trioleate with the spectra of solvent extracts as shown in the Figure 3. It is evident from the features of spectra that the first symmetrical multiplets of TG at 4.22 - 4.35 ppm due to  $\text{OCH}_2$  protons did not overlapped with the corresponding  $\text{OCH}_2$  groups of mono and di glycerides, which appeared at 4.0 - 4.22 ppm along with the second multiplet of TG. However, it was not possible to make clear distinction among mono and diglycerides due to interferences of signals of glyco and glycerophosphates in the same regions. The quantitative  $^{13}\text{C}$  NMR spectral analyses of extracts may provide direct analyses as discussed in the section of  $^{13}\text{C}$  NMR analyses.

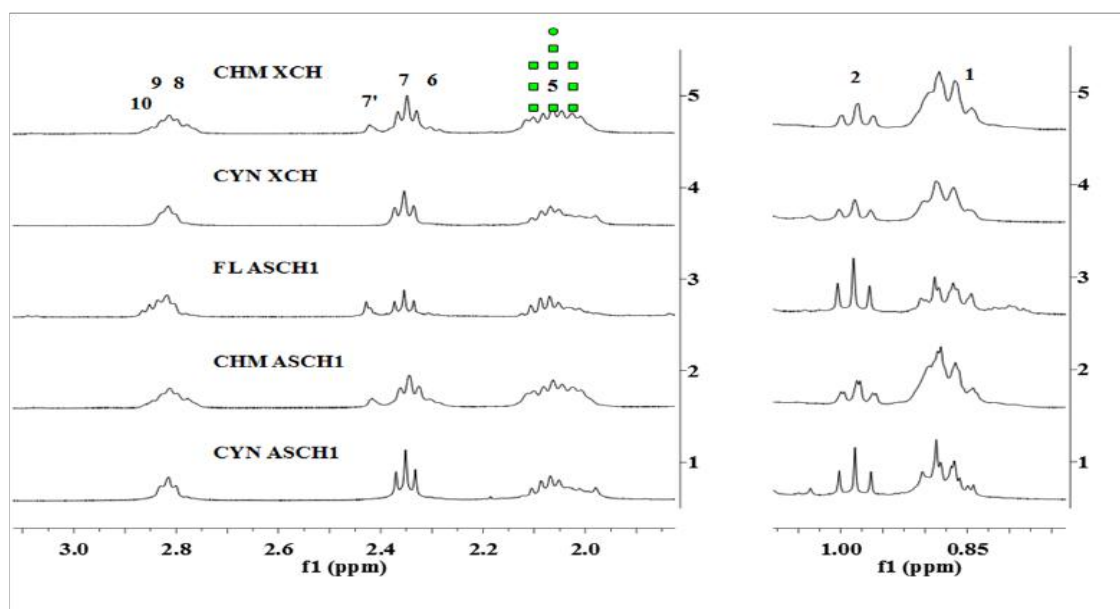
### NMR Analyses of extracts of *Cyanothecae*, *SD Flakes* and *Chlamydomonas*

The  $^1\text{H}$  NMR spectra of Accelerated Solvent and Soxhlet cyclohexane extracts (ASCH1, XCH) of these samples showed spectral features characteristic of long alkyl chain saturated and unsaturated fatty acids such as signals due to hydrogens of  $\text{CH}_2\text{C}=\text{O}$  (2.34 ppm), bis-allylic  $\text{CH}_2$  of C18:2 (2.77 ppm) and C18:3 (2.81 ppm),  $\text{CH}_2\text{-CH=CH-}$  (1.95 - 2.04 ppm),  $\text{CH=CH}$  (5.34 ppm), long alkyl chain  $(\text{CH}_2)_n$  (1.26 ppm, 1.30 ppm), terminal  $\omega$ -n-3 methyl of PUFA (0.98 ppm) and  $\omega$ -n-6/ n-9 unsaturated and saturated terminal methyls (0.88 ppm). Since the characteristics signals due to glycerides were observed of very weak intensities in the region of 4 - 4.4 ppm, this indicate their presence in very low concentration compared to FFA in these extracts (Figures 12 and 13). The spectra showed signals of very weak intensities due to hydrogen of  $\text{CH}_2\text{O}$  and  $\text{CHO}$  functional groups of glycerol/glyco phosphates in the regions of 3 - 5.1 ppm. The spectra showed signals due to PUFA (C18:3) at 0.98 ppm and 2.81 ppm, and their intensities were observed

to be higher for FL compared to CHM and CYN extracts. The spectra also depict very weak intensity signals in the regions of 6 - 7 ppm and 8 - 11 ppm due to oxo unsaturated fatty acids (oxylipins) in a pattern similar to those observed for SN and CHL types of extracts. The spectra of DCM and MeOH extracts show much higher amounts of polar lipids (glycero/glyco phosphates) compared to CH extracts. The signals of very weak intensities indicated at 2.39 ppm (signal 7') for CHM and FL extracts were assigned to  $\text{CH}_2\text{C}=\text{O}$  of C22:6. The characteristics signals due to unsaturated fatty acid chains, C18: N = 1-3, were clearly visible and marked accordingly in the expanded part of their respective spectra in the expanded region of 2.6 - 3.0 ppm (Figure 13). Similarly, DCM and MeOH extracts obtained by AS and X methods, show spectral features matching those of TAG and polar lipids. The signals due to polar lipids were quite intense compared to neutral lipids.



**Figure 12:**  $^1\text{H}$  NMR spectra of cyclohexane extracts (CH, CH1) of *Chlamydomonas* (CHM), *Cyanothece* (CYN), and *SD Flakes* (FL) by Accelerated Solvent (AS) extraction and Soxhlet (X) extraction methods. For designated signals marked 1 to 12, refer to Table 6.



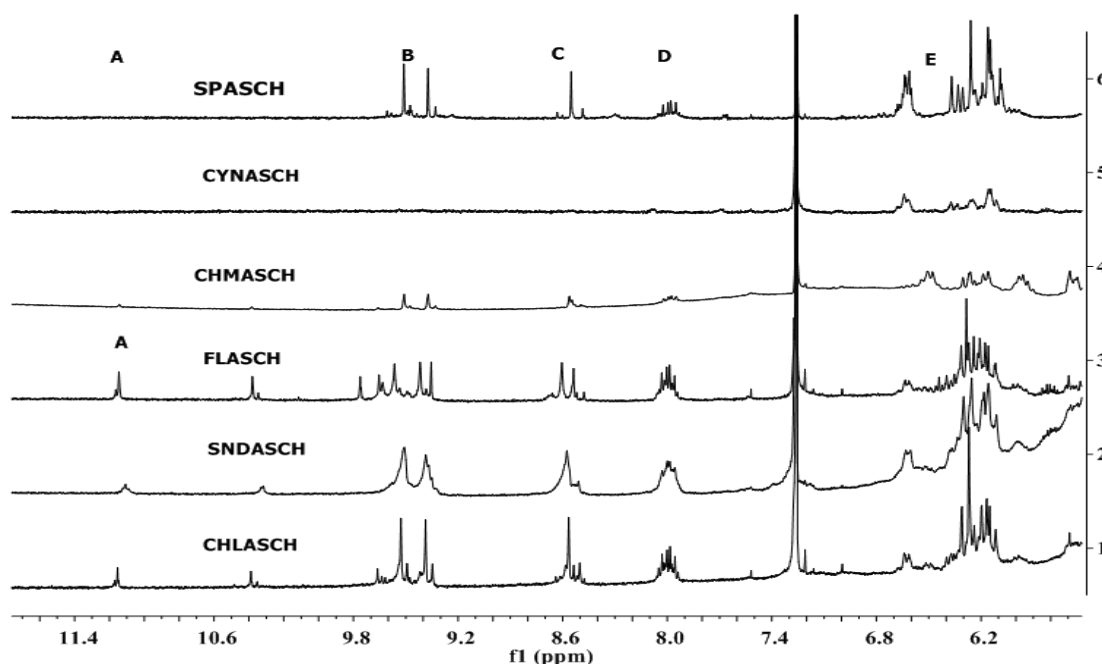
**Figure 13:**  $^1\text{H}$  NMR expanded view of spectra of cyclohexane extracts (CH, CH1) of *Chlamydomonas* (CHM), *Cyanothece* (CYN), and *SD Flakes* (FL) by Accelerated Solvent (AS) and Soxhlet (X) extraction methods. For designated signals marked 1 to 12, refer to Table 6.

### Identification of oxylipins in the algal oils

As discussed in the preceding sections, besides signals due to neutral and polar lipids observed in the  $^1\text{H}$  NMR spectra of AS and X extracts, the spectra showed signals of weak intensities in the regions of 5.5 - 6.8 ppm (marked E) and 7.7 - 11.2 ppm (marked A to D) in Figures 2-5. As shown in the Figure 14, the features and multiplicities were found to be different from the unsaturated protons ( $\text{CH}=\text{CH}$ ) of fatty acid chain of C18: N = 3 for extracts under study. These signals have been assigned to conju-



gated/unconjugated olefinic protons of oxy fatty acid or esters known as oxylipin. These are lipoxygenase-derived oxygenated acid products of PUFA such as C18:3, C22:6 (DHA), C20:5 (EPA) etc., which are naturally occurring in algae, particularly in marine algae<sup>[52-55]</sup>. These components contain aldehyde or keto groups in the fatty acid/ester chain. These are precursors for the formation of pheromones formed by oxidation and subsequent cleavage of long alkyl chain poly unsaturated fatty acids/esters. The identities of these signals will be discussed in details. As shown in the Figure 14, the expanded part of the spectrum in the chemical shift region of 7.5 - 11.5 ppm clearly showed sharp resonances at 8.0 ppm, 8.55 ppm, 9.4 ppm, 9.52 ppm, 10 - 10.3 ppm and 11.25 ppm due to protons of aldehyde groups. It has been observed that there exists a relationship between the integrated intensities of unsaturated protons at 5.7 - 6.8 ppm and aldehyde protons at 8 - 11.25 ppm as mentioned in the Table 7. The appearance of these signals confirmed the presence of oxylipins in the samples. The integrated intensities of chemical shift regions 5.6 - 6.8 ppm and 8 - 11.3 ppm in the NMR spectra of all the extracts were determined in the range of 0.5 - 3.2% and 0.24 - 2.0%, respectively, thereby indicating appreciable amount of oxylipins in the samples. The <sup>13</sup>C NMR and GC-MS analyses have also confirmed the presence of oxylipin in the FAMES of their corresponding extracts as shown in the Table 4. The chemical shift region 5.7 - 6.8 may also contain contribution of unsaturated protons of terpenoids or long alkyl chain normal or cyclic hydrocarbons<sup>[56]</sup>.

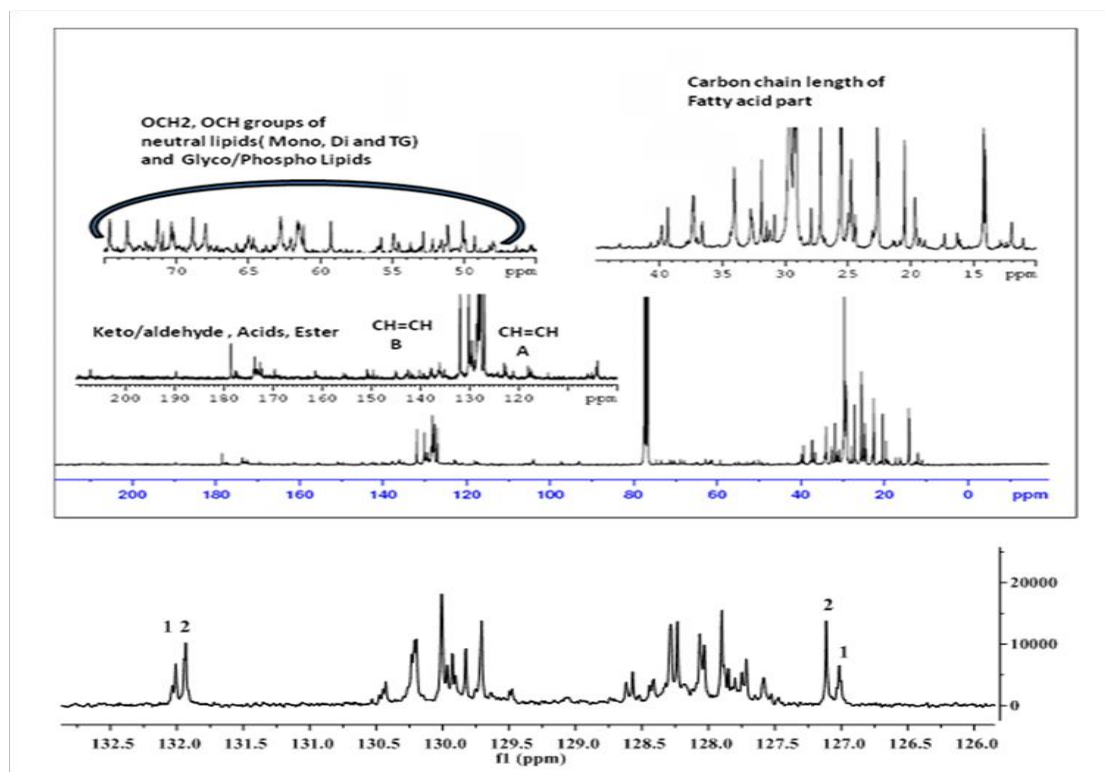


**Figure 14:** <sup>1</sup>H NMR part spectra of cyclohexane (CH) extracts of algae biomasses of *Chlorella* (CHL), *Scenedesmus* (SN), *Cyanothece* (CYN), *SD Flakes* (FL), *Chlamydomonas* (CHM), and *Spirulina* (SP) by Accelerated Solvent (AS) extraction method. Signals marked A to D are aldehyde protons and E denotes conjugated olefinic protons.

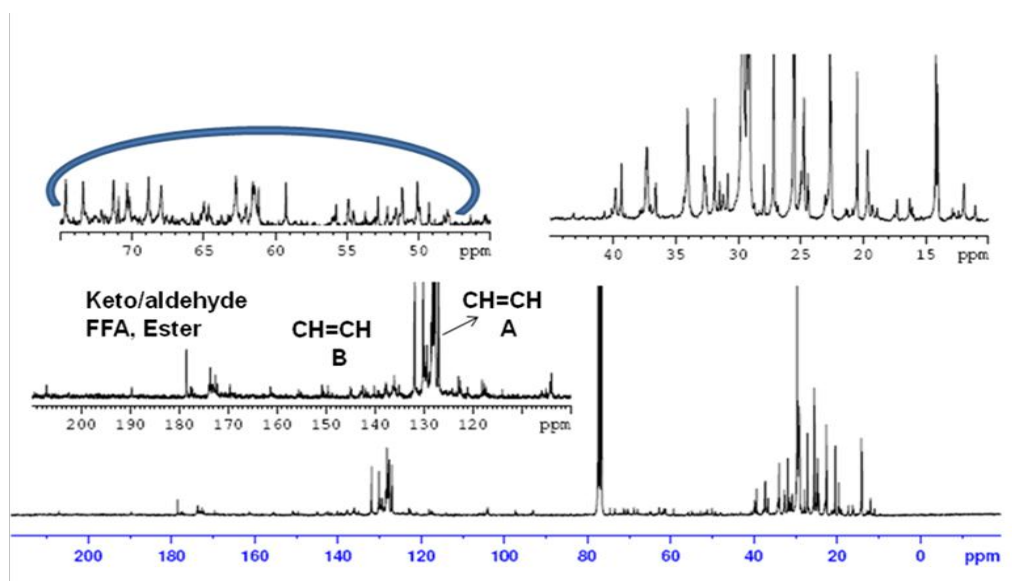
### <sup>13</sup>C NMR analyses of solvent extracts

The <sup>13</sup>C NMR spectral analyses of extracts of various algae samples have been carried out in order to confirm the results obtained by <sup>1</sup>H NMR spectral analyses, and also to extract information directly regarding the presence of free fatty acids; mono, di & triglycerides; oxylipins; and hydrocarbons. The 300 MHz <sup>13</sup>C NMR full and part spectra of different chemical shift regions of combined CH and DCM extracts of SN and CHL algae biomasses are shown in the Figures 15 and 16. The signals that are characteristic of glycerides and their fatty acid chains such as carbonyl group between 172 - 174 ppm; unsaturated carbons (CH = CH) of C18:N (N=1-3) and higher carbon chain between 125 - 132 ppm (intense); 112 - 125 ppm and 137 - 160 ppm (weak) are indicated at their respective regions. The OCH<sub>2</sub> (sn1, 3) and OCH (sn2) of glycerol back bone of triglycerides (TG) are visible at 61.94 and 69.0 ppm, in addition to appearance of signals of long alkyl fatty acid chain such as CH<sub>2</sub>C=O at 34.0 ppm; 32.02 ppm (γ-CH<sub>2</sub>); 22.7 ppm (β-CH<sub>2</sub>); 14.1 - 14.35 ppm (α-CH<sub>3</sub>); 29 - 30 ppm (CH<sub>2</sub>)<sub>n</sub>; and 23 - 28 ppm (CH<sub>2</sub>). The expanded part of the spectrum of regions 45 - 75 ppm clearly showed multiple signals due to phospholipids at 50 - 55 ppm (NCH<sub>3</sub>) and glyceroglyco lipids between 55 - 75 ppm. The spectra showed distinct and well separated carbonyl signals due to esters and free fatty acids at 170 - 174 ppm and 178.9 ppm (sharp), respectively. The appearances of multiple signals at 170 - 174 ppm due to carbonyl carbons are indicative of both saturated and unsaturated fatty acid chain of glycerides and polar lipids. The signals observed at 127.12 ppm and 132.06 (signal 2), and 127.02 ppm and 132.0 ppm (signal 1) in the expanded part of region of CH = CH (126-133 ppm) (recorded at 500 MHz NMR) in case of extract of SN have been assigned to ω (n-3) C18:3 and C22:6 respectively<sup>[7,8]</sup> (Figure 15). However, the CHL combined extract showed signals at 132.06 and 127.12 ppm indicative of the presence of only C18:3 (Figure 16). The appearance of signals due to CH = CH carbons at 129.92 ppm and 130.25 ppm (symmetrical) and terminal methyls along with saturated carbons at 14.15 ppm confirm the presence of oleic fatty acid. The signals of weak intensities between 132 - 145 ppm and between 10 - 40 ppm are due to hydrocarbons similar to those present in petroleum fractions and terpenoids<sup>[30]</sup>. The presence of hydrocarbons was also confirmed from the <sup>1</sup>H NMR spectral analyses of column fractions of some of the extracts as described in the next section.

The signals of very low intensities observed in the expanded part of the spectrum in the chemical shift region of 110 - 210 ppm revealed important information regarding both minor and major components containing keto, aldehyde and unsaturated hydrogen functional groups. The signals of weak intensities in the regions of 145 - 160 ppm (marked B), which are quite different in nature from the signals of long alkyl fatty acid/ester chain (marked A), and those of 190 - 210 ppm are assigned to components containing conjugated double bonds and keto/aldehyde carbonyl carbons. These are lipoxygenase-derived oxygenated acids products of poly unsaturated fatty acids/esters such as DHA and EPA known as oxylipins<sup>[52-55]</sup>. The <sup>1</sup>H NMR spectra of all extracts showed similar types of weak signals in the regions of 5.6 - 6.8 ppm and at 8.0 ppm, 8.55 ppm, 9.40 ppm, and 9.53 ppm due to conjugated unsaturated protons and aldehyde protons, respectively as discussed in the preceding section.



**Figure 15:** <sup>13</sup>C NMR Full and expanded view of part spectra of combined cyclohexane and dichloromethane extracts of *Scenedesmus*. Chemical shift regions: A = 125 - 133 ppm (unsaturated hydrogen (CH = CH) of fatty acid chain; B = unsaturated protons of conjugated double bonds of ox-ylipins; 132 - 170 ppm = amides; region = 170 - 210 ppm Keto/aldehyde/acids/ester; 12 - 45 ppm = long alkyl chain of fatty acid chain and 40 - 75 ppm = glycerides and polar lipids. The signals 1 and 2 in the expanded region of unsaturated hydrogens (126 - 132.5 ppm) are due to C22:6 and C18:3 respectively.



**Figure 16:** <sup>13</sup>C NMR Full and expanded view of part spectra of combined cyclohexane and dichloromethane extracts of *Chlorella*. Chemical shift regions: A = 125 - 133 ppm (unsaturated hydrogen (CH = CH) of fatty acid chain; B = unsaturated protons of conjugated double bonds of oxylipins; 132 - 170 ppm = amides; region = 170 - 210 ppm = Keto/aldehyde/acids/ester; 12 - 45 ppm = long alkyl chain of fatty acid chain and 40 - 75 ppm = glycerides and polar lipids.

### FTIR analyses of solid biomasses and extracts

In order to study the composition of microalgae biomasses and their extracts obtained by Soxhlet and Accelerated Solvent extraction methods for the presence of neutral and polar lipids, these samples were analyzed by FTIR using ATR accessory. The spectra of solid biomasses of SN and CHL in Figure 17, showed general characteristic bands due to ester (1740.81, 1743.33  $\text{cm}^{-1}$ ), amides (1651.68, 1647  $\text{cm}^{-1}$ ) and glycolipids/sugars (1151.75, 1151.39  $\text{cm}^{-1}$ ) besides strong bands due to hydroxyl (3321.50, 3321.85  $\text{cm}^{-1}$ ) and long alkyl fatty acid chain (2851.05, 2851.44  $\text{cm}^{-1}$ ) indicating the presence of glycerides, proteins and glycolipids/sugars. The bands at 1245.09 and 1244.75  $\text{cm}^{-1}$  may also contain contribution from phospholipids (POC) and ester (COC) linkages. The relative peak areas of ester bands of SN, CHL and SP biomasses have been calculated to be 0.445, 0.385 and 0.231, respectively, indicating relative amount of glycerides and glycerophospho lipids components in these samples. The NMR and GPC results given in the Table 5 also showed contents of TG in these samples in same order, thereby confirmed the results by IR analyses. It was difficult to determine peak area specific to glycerides (TAG) as the ester carbonyl bands at 1740.43  $\text{cm}^{-1}$  also contained contributions from ester groups of glyco/glycerophospho lipids. The NMR analyses of extracts provided the actual content of glycerides in each of the extracts (Table 5). The IR spectra of samples of other algae biomasses as given in the Table 1 show similar spectral features except variation in the intensities of bands corresponding to glycerides, proteins and glyco/glycerophospholipids.

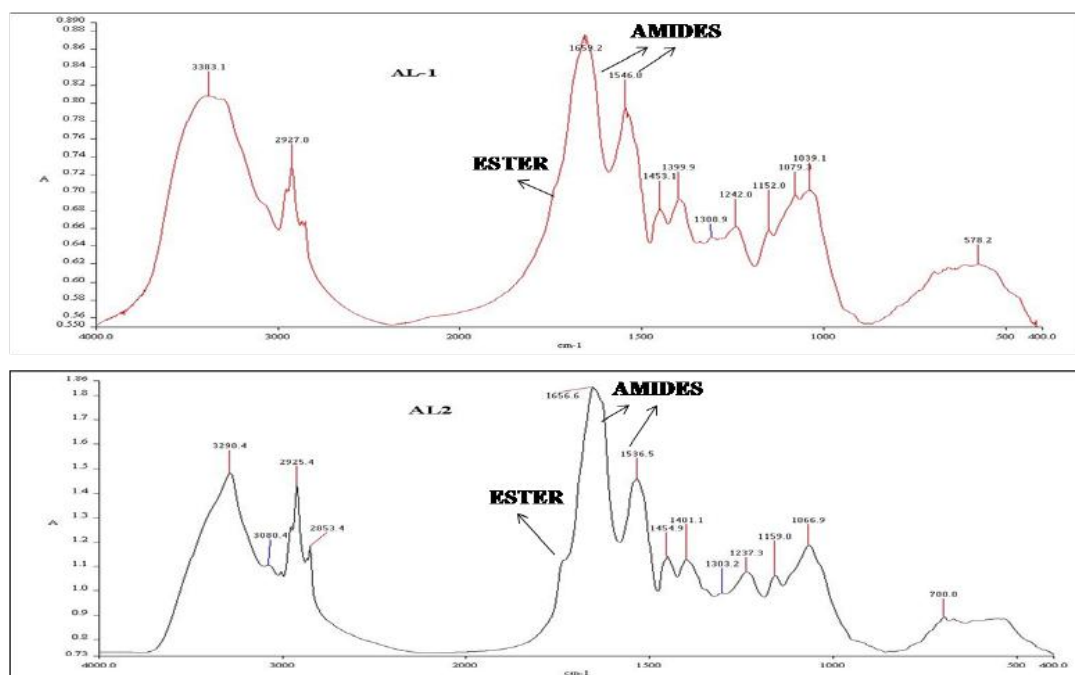


Figure 17: FTIR (ATR) spectra of solid biomasses of *Scenedesmus* (AL2) and *Chlorella* (AL1).

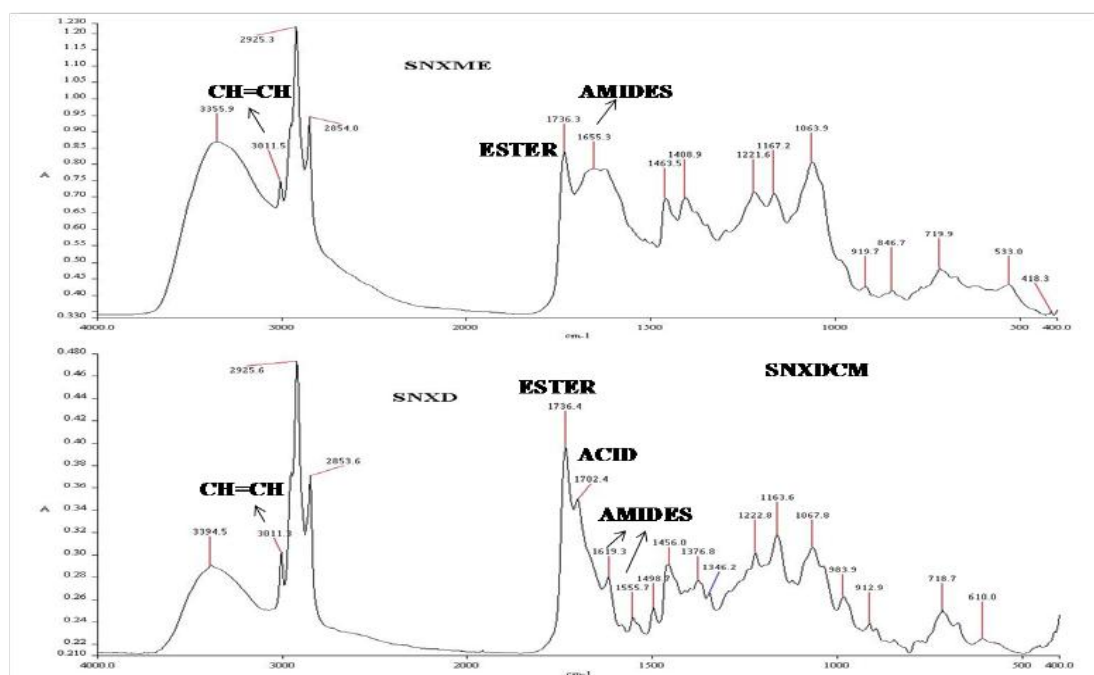
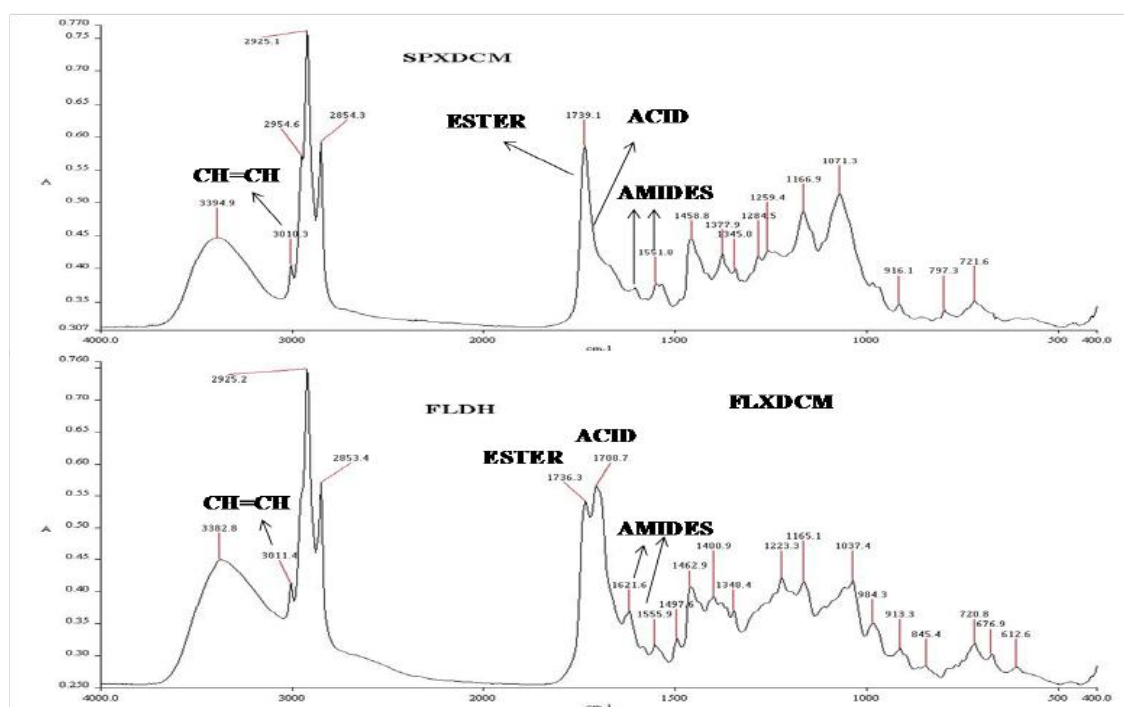


Figure 18: FTIR (ATR) spectra of dichloromethane (SNXDCM) and methanol (SNXME) extracts of *Scenedesmus* by Soxhlet extraction (X) method.

The IR spectra of DCM and MeOH extracts of biomass of SN obtained by Soxhlet extraction method as given in the Figure 18 showed both ester and acid bands at  $1736\text{ cm}^{-1}$  and  $\sim 1710\text{ cm}^{-1}$  respectively. The appearance of bands at  $1560 - 1630\text{ cm}^{-1}$  were indicative of the presence of amides in both the extracts besides appearance of bands of weak intensity due to glycolipids/sugars. The higher intensity of bands due to amides in case MeOH extract compared to DCM extract was indicative of the presence of higher amounts of amide components in the MeOH extract. However, free acids (FFA) were extracted in quite higher amount in DCM extract compared to MeOH extract as indicated by their relative intensities of acid bands in their respective spectra. The appearance of strong  $\text{P}=\text{O}$  bands at  $1063 - 1067\text{ cm}^{-1}$  and  $900 - 1200\text{ cm}^{-1}$  in both the spectra showed the presence of phospholipids, glycolipids and carbohydrates. Similarly, IR spectra of DCM extracts of SP as shown in Figure 19 showed bands characteristic of components rich in ester/fat rather than FFA and amides. The IR spectral features of the DCM extract of FL biomass showed the presence of large amounts of FFA compared to ester (fat) components as evident from the intensities of their respective bands. The spectrum also indicated very weak bands corresponding to amides, phospholipids, glyco lipids and carbohydrates compared to those noticed in the extracts of SN and CHL. Similarly, solvent extracts of other biomasses obtained by Soxhlet and Accelerated Solvent extraction methods were analyzed to monitor extraction of various components, particularly neutral lipids, by different solvents. These results are in line with those obtained by NMR analyses of these extracts.



**Figure 19:** FTIR (ATR) spectra of dichloromethane of extracts of biomasses of *Spirulina* (SPXDCM) and *SD Flakes* (FLXDCM) by Soxhlet extraction method.

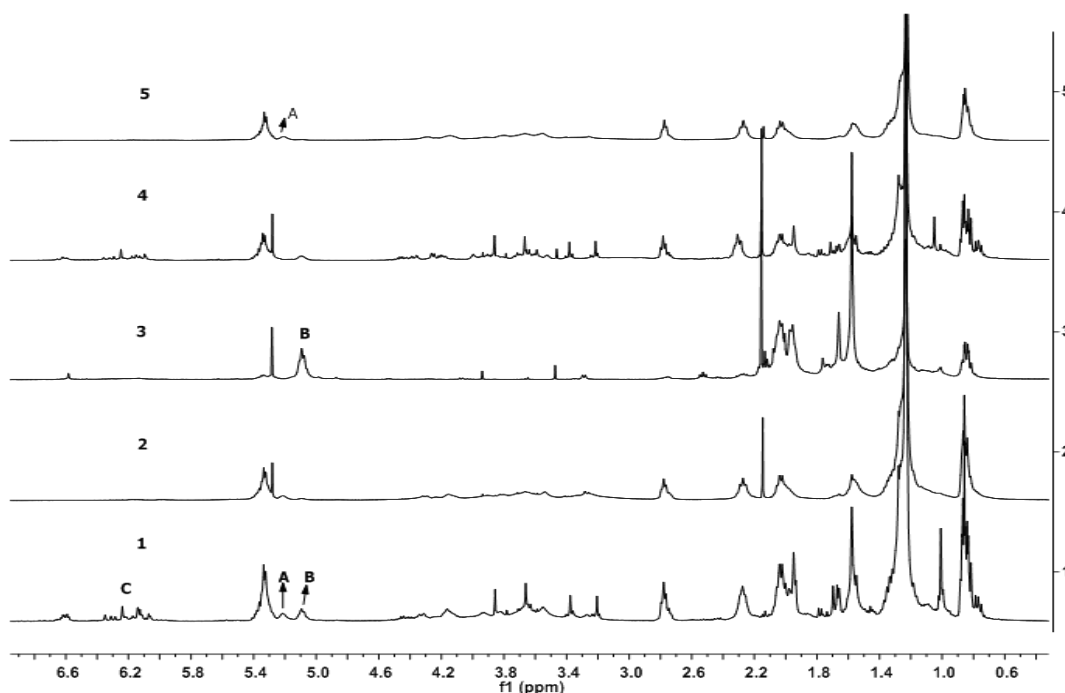
### Analyses of silica column fractions of cyclohexane extracts by NMR

In order to describe and demark the NMR spectral features specifically for glycerides, free fatty acids, glycerophospholipids, oxoacids/esters, and hydrocarbons in the extracts comprised of complex mixture of different components, the separation of these components were achieved on a silica column using solvents of varying polarities. Since the CH extracts of SN and SP biomasses were found to be quite enriched with these components, especially hydrocarbons and oxoacids/esters, their column fractions were critically examined by  $^1\text{H}$  NMR spectral analyses. The  $^1\text{H}$  NMR spectra of different fractions along with parent CH extract of SP are given in Figures 20 - 22. The hexane fraction 1 (spectrum 2) is found to contain more FFA than triglycerides as evident from their characteristic intense signals at 5.32 ppm, 5.26 ppm, 2.77 - 2.81 ppm, 2.32 ppm, 2.0 ppm etc. The hexane fraction 2 (spectrum marked 3) shows signal at 5.08 ppm (B, CH=CH),  $\text{CH}_2\text{-CH=CH}$  (1.95 - 2.0 ppm), CH (1.4 - 1.9 ppm),  $(\text{CH}_2)_n$ , and terminal  $\text{CH}_3$  (0.88 ppm) characteristic of long normal alkyl and cyclic saturated, and unsaturated hydrocarbons similar to petroleum fractions. The signal marked B in the spectrum of the parent extract (spectrum 1) has been enhanced in the hexane 2 fraction due to enrichment of hydrocarbons as shown in spectrum 3. The spectrum did not show any signals in the regions of 3 - 5 ppm, thus confirming absence of neutral lipids (TAG, FFA) and polar lipids (glycerophospholipids).

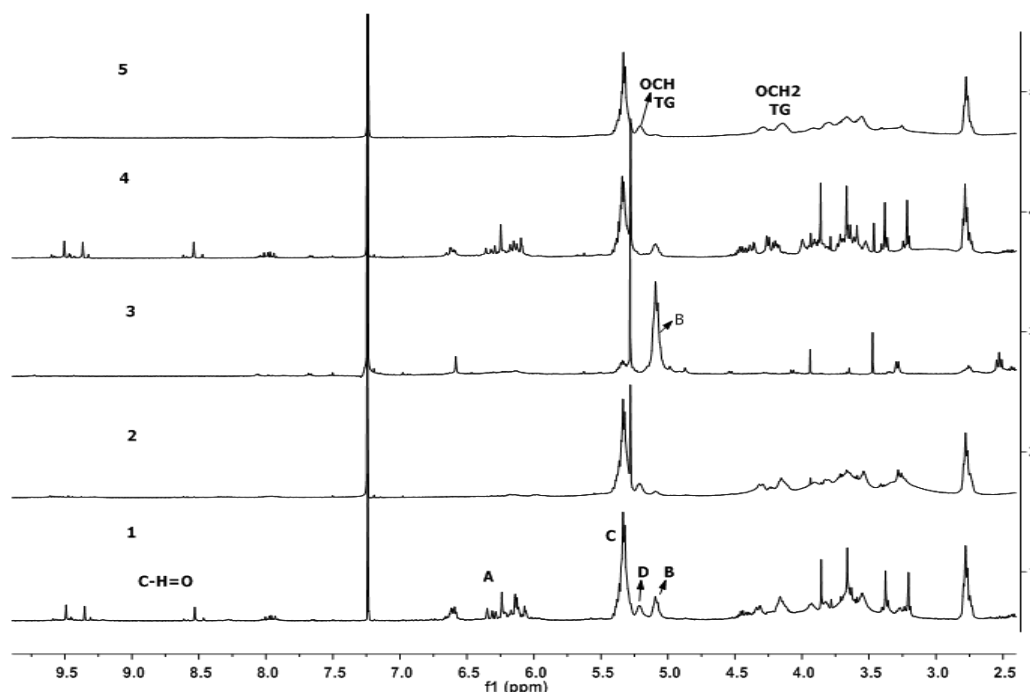
The spectrum of a 50% chloroform- 50% methanol fraction (spectrum marked 4) was found to be rich with oxo acids/esters as indicated by the intense signals shown in the expanded regions of 5.7 - 6.8 ppm (CH = CH) and 8 - 11.25 ppm (CH = O) as discussed in the preceding sections (Figure 21). The intense signals in the region of 4.5 - 3.0 ppm including the sharp signals were due to glyceroglyco/phospholipids. The spectrum was free from triglycerides as no signal was indicated at 5.26 ppm due to OCH protons of sn2 configuration. However, the fraction may contain signals due to mono and di glycerides overlapping with signals of glycerophosphates in the regions of 4.4 - 3.55 ppm. The spectral features of 10% chloroform-50% methanol fraction (spectrum 5) in Figure 20 match with the spectral features of mono, di and tri glycerides as evident from the appearance of signals at 5.26 ppm (marked A) due to OCH protons of sn1 configuration of triglycerides and OCH, CHOH and  $\text{CH}_2\text{OH}$  signals of glycerol part of mono



and di glycerides in the region of 4 - 3.5 ppm. This is more clearly shown along with the blend containing mono, di and tri glycerides (Figure 22). The  $^1\text{H}$  NMR analyses of column fractions of cyclohexane extracts of CYN and SN algae biomasses showed similar results.



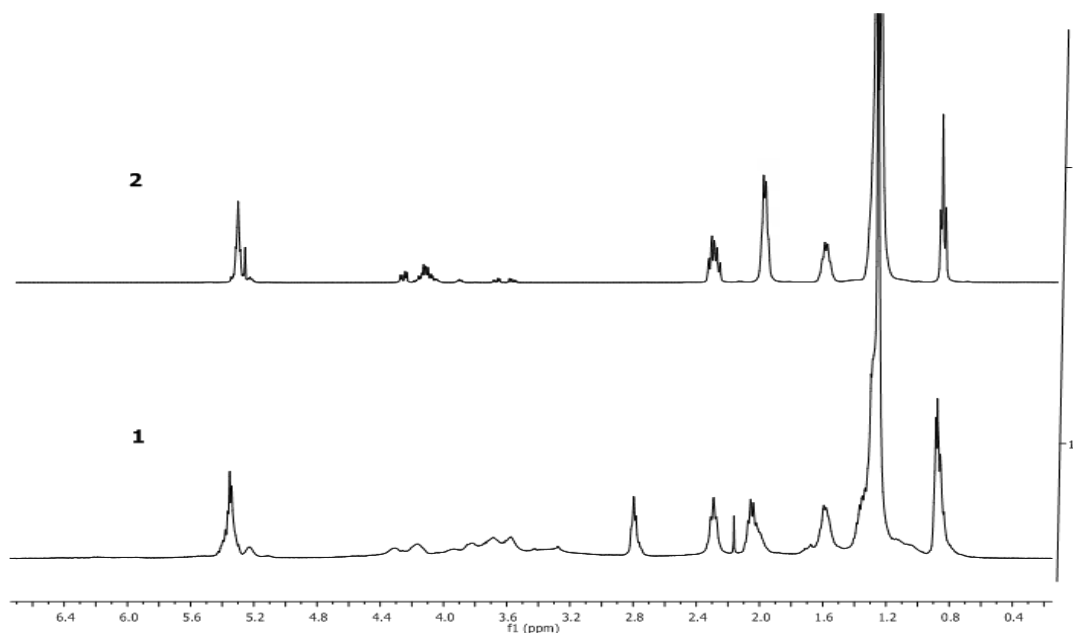
**Figure 20:**  $^1\text{H}$  NMR part spectra (0.5 - 6.6 ppm) of silica column fractions of cyclohexane extract of *Spirulina* (SPASCH1) by Accelerated Solvent extraction method. Sub spectra 1 = parent extract; 2 = hexane fraction 1; 3 = hexane fraction 2; 4 = 50% chloroform-50% methanol fraction; 5 = methanol fraction.



**Figure 21:**  $^1\text{H}$  NMR part spectra (2.5 - 10 ppm) of silica column fractions of cyclohexane extract of *Spirulina* (SPASCH1) by Accelerated Solvent extraction method. Sub spectra 1 = parent extract; 2 = hexane fraction 1; 3 = hexane fraction 2; 4 = 50% chloroform-50% methanol fraction; 5 = methanol fraction.

It is evident from the above detailed discussion that  $^1\text{H}$  NMR analyses of column fractions of CH extracts of SP, CYN and SN algae biomasses facilitated the exact assignment of chemical shift regions to specific functional groups corresponding to oxylipins, glycerides, hydrocarbons and glycerophospholipids. The hydrocarbons were clearly identified and their presence confirmed by the column fractions. Since the  $\text{OCH}_2$  groups (sn1, sn3) of mono, di and tri glycerides are overlapped with the signals of glycerophospholipid in the chemical shift region of 4 - 4.5 ppm, this region cannot be used for the identification and quantitative

analyses. Since the OCH (sn2) group of triglycerides at 5.26 ppm is free from the interferences of functional groups of other polar components, it can be used for the estimation of triglycerides. Since the  $^1\text{H}$  NMR spectral features of different solvent extracts of algae samples show similar characteristic signals due to various components of polar and non-polar lipids, the assigned chemical shift regions can be used to quantify these components as described in the succeeding section and Table 6.



**Figure 22:**  $^1\text{H}$  NMR spectra of a silica column fraction of cyclohexane extract of *Spirulina* (SPASH1) by Accelerated Solvent extraction method. Sub spectrum 1 = methanol fraction showing mixture of glycerides; 2 = blend of mono, di, and tri glycerides and FFA.

#### Quantitative analyses of neutral lipids (TAG, FFA, hydrocarbons), PUFA and Iodine values (IV) by NMR

The fatty acid profile by GC-FID and NMR analyses of combined cyclohexane, chloroform and methanol extracts of algae samples showed the majority of C16:0 and C18: N = 0-3 fatty acids/esters components. The higher fatty acids C20-24 are present in the range of 1 - 2.3% as given in the Table 3. The detailed  $^1\text{H}$  NMR spectral analyses of solvent extracts of the six algae biomasses including their column fractions have confirmed the presence of TG, FFA, PUFA (C18:3, C22:6) and polar lipids. The quantitative analyses of these quality parameters (TG, FFA, PUFA, IV) have been carried out by using the newly developed methods based on NMR spectroscopic techniques as described in our earlier published work<sup>[7,8,14,24,50]</sup>. The following equations have been used for the estimation of various quality parameters:

$$\text{TG} = 26.06 \times \text{ITG} - 0.62 \quad (4.25 - 4.34 \text{ ppm})^{[8]} \quad \text{Eq 1}$$

$$\text{FFA} = 23.57 \times \text{I}_{\text{FFA}} - 7.84 \quad (2.32 - 2.38 \text{ ppm})^{[8]} \quad \text{Eq 2}$$

$$\text{Iodine value(IV)} = 15.78 \times \text{I}_{\text{US}} \quad (5.05 - 5.65 \text{ ppm})^{[50]} \quad \text{Eq 3}$$

$$\text{PUFA} = 10.75 \times \text{I}_{\text{PF}} \quad (0.925 - 1.02 \text{ ppm})^{[7,8]} \quad \text{Eq 4}$$

Where ITG, I FFA, IUS and IPF are the integrated intensities in their respective chemical shift regions as mentioned in the equations 1 - 4 and Table 6. Results obtained by the above equations are compiled in the Table 5. The values of these quality parameters in the six algae biomasses have been compiled in Table 7.

**Table 7:** Values of iodine, triacylglycerides (TAG), free fatty acids (FFA), and polyunsaturated fatty acid (PUFA)(%w/w).

S.N	Algae biomass	TAG	Hydrocarbons	FFA	IV	PUFA
1	<i>Scenedesmus</i>	17.3	8.0	7 - 34	162 - 185	39.4 - 55.3*
2	<i>Chlorella</i>	9.0	6.0	6 - 50	137.0 - 147.8	32.6 - 37.15
3	<i>Spirulina</i>	5.9	10.0	5 - 59	69.8.2 - 112.7	2.3 - 17.1#
4	<i>SD Flakes</i>	10.5	4.0	80 - 85	117.7 - 144.7	37.2 - 49.8*
5	<i>Cyanothece</i>	4.6	3.0	73 - 85	104.9 - 109.8	31.2 - 38.6
6	<i>Chlamydomonas</i>	8.6	8.0	86 - 92	138.4 - 141.5	26.3 - 30.5*

Iodine value (IV) and PUFA ranges are the values of AS and SX cyclohexane extracts; TAG = Mono + Di + Triglycerides; \*(C18:3 + C22:6); #  $\gamma$ -C18:3.

## Precision of NMR methods

As reported previously, the precision of NMR methods for the determination of quality parameters TG, FFA, PUFA and IV are better or equivalent compared to methods based on GC, MS and other techniques<sup>[7,8,50]</sup>. The methods are direct and rapid and can be applied with ease and require no pre sample treatment. The Linear Regression analyses of TG and FFA content determined by methods based on NMR and GPC as given in the Table 5 show reasonable good agreement as indicated by regression coefficient ( $R^2$ ) in the range of 0.95 - 0.98. As already described, the extracts were comprised of complex mixtures of neutral and polar lipids, the newly developed equations were in a position to determine accurately the contents of TG, FFA and PUFAs.

## Conclusions

From the above discussion of chromatographic (GC, GC-MS, GPC) and NMR ( $^1\text{H}$  &  $^{13}\text{C}$ ) analyses, it is quite certain that scheme of solvent extraction of algae samples using different solvents, and analyses of their respective extracts or algal oils by the developed analytical protocol provided detailed information regarding the nature of neutral lipids and their fatty acids profile, nature of oxo acids, hydrocarbons, and the presence of the glyceroglyco/ phospho lipids. The results are summarized below:

- The assignment of  $^1\text{H}$  /  $^{13}\text{C}$  NMR chemical shift regions corresponding to specific functional groups has facilitated the identification and quantification of components of interest for biodiesel and value added PUFA/PUFE.
- The extracts or algal oils obtained by AS and X methods using CH, DCM and MeOH solvents were comprised of tri acyl glycerides (TAG; mono, di, tri), FFA and polar lipids. The fatty acid chain associated with these lipids was composed of both saturated and unsaturated types with majority of types of C16:0-C18:0, C18: N = 1-3 and C22:6 resembling the nature of fatty acid present in vegetable and fish oils. The fatty acids C20-24 were present in small amount in all the extracts. C18:3 and C16:0 were the predominant acids estimated by GC in the range of 14.4 - 36.8% w/w and 13.8 - 43.0% w/w, respectively, in all the extracts from Soxhlet compared to vegetable oils such as soybean, radish and others in the range of 7 - 10 % w/w and 12 - 16 % w/w, respectively.
- There was no trend observed between the two extractions methods used for extraction related to the content of neutral lipids as well as to their extraction efficiency using different solvents. The content and types of lipids extracted, and fatty acid profile were specific to solvent used for extraction and types of algae biomass.
- The analyses also indicated the presence of terpenes and hydrocarbons besides neutral lipids. The concentration was higher in CH extracts compared to DCM and MeOH extracts.
- The extraction efficiency of Accelerated Solvent (AS) and Soxhlet (X) extraction methods using solvents of CH and DCM were the same. However, the neutral lipid profile was different in fractions from these two methods. Cyclohexane solvent was found to extract quite higher amounts of FFA than TAG, and this trend was found to be consistent in all the extracts under study (Table 5).
- The Soxhlet extraction method provided higher amounts of neutral lipids (TAG+FFA) compared to the Accelerated Solvent extraction method.
- The TAG content were present in the range of 22.6 - 33.4% w/w and 15.4 - 27.1% w/w respectively in the cyclohexane extracts of SN and SP algae biomasses obtained by both the extraction methods. The mono glycerides content were the lowest in the range of 1 - 2.8% as determined by GPC in all the extracts.
- The SP extracts were found to contain higher amount of FFA (10.3 - 50.4 %w/w) compared to SN (7.4 - 34%) and CHL (6 - 50%). Surprisingly, SPASH1 and SPXH extracts were found to be completely free from FFA in contrast to other similar extracts.
- The algae biomasses CYN, CHM and FL were rich in FFA in the range of 72.8 - 84.9 %w/w, 86.4 - 92.4% and 80.2 - 84.7%w/w, respectively, in the CH and DCM extracts obtained by Accelerated Solvent and Soxhlet extraction methods.
- The MeOH extracts of all the extracts were found to contain only polar lipids, which were quite rich in C18:3 as confirmed by their NMR analyses.
- The SP extracts were found to contain  $\gamma$ -C18:3 (n-6) compared to SN, CHL, FL and CHM, which contained  $\alpha$ -C18:3 (n-3).
- The SP, SN, CHM and CHL extracts were found to contain higher amounts of hydrocarbons (6 - 10%) compared to FL and CYN (Tables 5 and 7).
- The polar solvents, DCM and MeOH, extracted higher amounts of oxylipins as evident from the integrated intensities of aldehyde and olefinic protons (ICH = O) given in Table 5. The CYN, CHM and FL contained lower amount of oxylipins compared to other algae samples (Table 5).
- The analyses of column fractions facilitated the exact assignment of chemical shift regions corresponding to glycerides, oxylipins and glyceroglyco/phospholipids. The NMR and GC-MS analyses were able to identify the types of oxylipins. Both saturated and unsaturated oxylipins containing CH=O groups in the carbon range of C8-C10 were identified.
- The Soxhlet extracts of SN, CHM and FL were found to be potential sources rich in PUFAs (24.9 - 36.8 % w/w) comprised of C18:3 and C22:6, which possess high nutritional values. Other extracts, CHL, SP and CYN, contained only C18:3 (14.4 - 28.4 % w/w) (Table 3). The PUFAs content were extraordinary high compared to present in soybean and radish oils (7 - 10 % w/w).
- The polar solvent DCM has shown higher preference for extraction of PUFAs (29.8 - 41.4% w/w compared to solvent CH (4.9 - 13.6 % w/w). Similar trend has been observed in case of extraction of saturates by CH, which has shown preference for saturates (43.5 - 57.0 % w/w) (Table 3a).
- There is a direct relationship between the iodine values, total unsaturated fatty acids, distribution of fatty acids and content of PUFA/PUFE as observed by NMR and GC analyses of samples (Tables 3, 5 and 7). The PUFA/PUFE content and iodine values were the highest in SN (39.4 - 55.3 % w/w; 135.4 - 185.1 gI<sub>2</sub>/100g) (Table 5) compared to other samples.
- The GC-FID and GC-MS analyses showed the absence of C22:6 in the FAMES of extracts of SN, CHM and FL in contrast to the

results of those extracts obtained by NMR. This was ascribed to the low conversion efficiency of glycerophospho lipids under the mild transesterification reaction conditions employed for the generation of FAMES. The glycerophospho lipids require high pressure conditions for their complete transesterification, particularly glycerophospho lipids containing C22:6 fatty acid chain as reported previously<sup>[7,8]</sup>.

- The NMR results of FFA and TG content showed a reasonably good agreement with the results by GPC in spite of some overlapping of signals in the respective regions considered for estimation.
- Such exhaustive spectroscopic study will be an effective aid to monitoring the cultivation process in the context of finding biodiesel and product potentials.

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