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Comparative study for extraction and characterization of oil from Nannochloropsis sp. and Spirulina sp. using Folch method and Soxhlet method

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ABSTRACT

Microalgal oil is an important source of high-value fatty acids. However, the quality and extraction yield is often affected by the oil extraction process. The quality of oil extracted decides its end use as biofuel or neutraceuticals. This study investigated the effect of two different extraction methods viz. Folch method and Soxhlet method on the quality as well as extraction yield of the oil from Nannochloropsis sp. and Spirulina sp. The chemical parameters of oils such as acid value, free fatty acid, peroxide value, and iodine value as well as the oil fatty acid composition analysis were carried out. The oil obtained from Soxhlet extraction showed lower acid value, free fatty acids content, and peroxide values in both species compared to the Folch method. However, the oil recovery (% oil yield) was higher (22% and 8%) in the Folch method as compared to the Soxhlet method (11% and 6%) from Nannochloropsis sp. and Spirulina sp. respectively. The fatty acid composition showed higher content of long-chain omega-3 fatty acids when extracted using Soxhlet method viz. 0.66% and 2.52% of EPA and DHA compared to 0.46 to 0.50% of EPA and DHA by Folch method from Nannochloropsis sp. and Spirulina sp respectively. The outcome of the study justifies the Soxhlet method of oil extraction as a superior method for oil extraction from microalgae with good quality and stability. Oil obtained in this way could be used in the food, feed and oil industry.

Keywords: *Folch method, Soxhlet method, Acid value, Peroxide value, Omega-3 fatty acids, Microalgae, algal oil*

Introduction

Microalgae are the primary producers on this planet. The per-hectare oil production rate obtained from these microalgae is 7-31 times higher than the oilseed crops [1]. Microalgal oil contains short-chain, medium-chain, and long-chain fatty acids [2] [3] [4]. The presence of SCFA makes it suitable for use as biofuel. The polyunsaturated fatty acids presence makes them suitable in the field of nutraceuticals. The quality as well as quantity of extracted oil depends on oil extraction methods. There are several methods for extracting oil from algal biomass *viz*. oil pressing, solvent extraction, microwave-assisted extraction, enzymatic extraction, ultrasound-assisted extraction, osmotic shock, oxidative stress, and electroporation [5]. The choice of extraction method depends on the desired oil yield, types of algal biomass, and expenses. The structure of cell walls and lipid content varies from one species to another species. The solubility of solvent with the lipids, extraction temperature, and pressure contribute to efficient oil extraction. The choice of solvent should be given paramount importance to minimize risk to the environment. Green extraction technologies use non-toxic and eco-friendly solvents *viz*. supercritical carbon dioxide, ionic liquids, organic carbonates, and bio solvents [6].

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Folch method is one of the popular methods of lipid extraction, which is based on biphasic solvent. Here chloroform: methanol is used in 2:1 ratio and water is used to form two layers: organic phase and aqueous phase. Lipids are dissolved in the aqueous phase. This method is easy and provides a high yield. However, it has the disadvantage of using toxic solvents and unsuitability for large samples. Another popular method of algal oil extraction is the Soxhlet method. It uses solvents for the extraction of a wide range of lipids [7]. In the present study, a comparative analysis of oil extraction methods *viz.*"Folch method" and Soxhlet extraction method were used to characterize algal oil from the microalgal cultures e.g. *Nannichloropsis* sp. and *Spirulina* sp. The oil quality-related parameters *viz*. peroxide value, acid value, free fatty acids, iodine value and saponification value, fatty acid profile, and fatty acids-related nutritional indices were estimated and compared.

Materials and Method

Microalgal cultures and growth condition

Two microalgal cultures namely *Nannochloropsis* sp. and *Spirulina* sp. (CCC* No. 480) (Figure 1)were obtained from the germplasm collection of Centre for Conservation and Utilisation of Blue Green Algae (CCUBGA), ICAR-Indian Agricultural Research Institute, New Delhi, India. For experimental purposes, all the cultures were cultivated in 100 mL medium contained in 250 mL Ehrlenmeyer flasks in a growth room at 28 ± 2 °C and light intensity of 40 µmol photons m^{-2} s^{-1} with 16h: 8h light-dark regime. *Nannochloropsis* sp. was grown in BG-11 media [8] and *Spirulina* sp. was grown in Zarrouk media [9]. All the estimations were carried out at the end of 21 days of cultivation.

Oil extraction methods extraction methods

Folch method

Lipid estimation was done by the Folch method (1975) with a slight modification in which 1 g of dry algal mass was ground using a mortar and pestle [10]. The chloroform and methanol were added in a ratio of 2:1, followed by an incubation of 30 min with intermittent gentle shaking. After the addition of 0.75 volumes of water, the sample was kept for phase separation. The lower chloroform phase was collected. This process was repeated twice, and then all solvent phases were combined. The solvent was evaporated in a rotary evaporator, and the collected lipid was then kept for oven drying. To the dried samples, 2 mL of hexane was added and again kept in the oven for the drying process. The percent lipid content was determined as follows:

$$\% \text{ Lipid} = \frac{\text{Weight of lipid (g)}}{\text{Biomass (g)}} \text{X } 100 (1)$$

Soxhlet Method

Five grams of dried sample was kept in a thimble and inserted in a Soxhlet extractor. 200 ml of petroleum ether was put into a pre-weighted round bottom flask and extraction was carried out at 40 °C for 4h. After extraction solvent was recollected in a rotary evaporator and the oil yield was determined as given in the equation [11].

$$\text{Oil yield } (\%) = \frac{\text{weight of oil (g)}}{\text{weight of dried algal biomass (g)}} * 100 \ (2)$$

Oil quality-related parameters

Peroxide value

The peroxide value (PV) of the oil samples (meq O_2/kg oil) was determined according to AOCS official methods (AOCS, 2004) and calculated using equation (3). 5g of oil sample was dissolved in 30 ml of chloroform: acetic acid (3:2) solution [12]. Then 0.5 ml of KI solution (15% saturated) was added and kept in the dark for 5 minutes. 30 ml of water was added before the titration with 0.1N sodium thiosulphate. 0.5 ml starch was used as an indicator.

$$\text{Peroxide value (meq } O_2\text{/kg sample)} = \frac{\text{S x N x 1000}}{\text{Weight of sample (g)}} \ (3)$$

Where S = ml of $Na_2S_2O_3$ (Test – Blank) and N = Normality of $Na_2S_2O_3$

Acid value and free fatty acid

The acid value (AV) of the oil samples was analyzed based on the AOCS official method [12]. 1 g oil was dissolved in 10 ml neutral solution (Choloroform: diethyl ether in ratio 2:1) and heated for 10 minutes. Phenolphthalein is used as an indicator. Titration is carried out against KOH until the endpoint is reached as the appearance of pink. The AV value was calculated by using equation (4) and expressed as milligrams of KOH/g of oil. The percentage of free fatty acids (FFAs) was calculated by using equation (5).

$$\text{Acid value (mg KOH/g)} = \frac{\text{Volume of KOH} * \text{normality of KOH} * 56.1}{\text{weight of oil sample (g)}} \ (4)$$

$$\% \text{ free fatty acid} = \frac{\text{Acid value}}{1.99} \ (5)$$

Iodine value

The iodine value (IV) is estimated based on AOCS (2004) [12]. 100 mg of oil is dissolved in 25 ml of carbon tetrachloride. The solution was kept in the dark for 45 minutes after the addition of 25 ml of Hanus solution. After that 10 ml of potassium iodide solution and 50 ml of distilled water were added. Titration is carried out with sodium thiosulphate. Starch is added as an indicator. The endpoint is noted from a purple color to colorless. The iodine value is calculated as follows and expressed as (g):

$$\text{Iodine value (g)} = \frac{(B-S) * \text{Normality of sodium thiosulphate} * 12.69}{\text{Weight of oil (g)}} \ (6)$$

Where, B - Volume of blank
S – Volume of sample

Estimation of saponification value

The saponification value (SAP) was analyzed based on the AOCS official method (AOCS, 2004) [12]. The SAP was calculated with the help of equation (7) and the value was expressed as milligrams of KOH/g of oil.

$$\text{Saponification value} = \frac{56.1 \text{ X } (V2 - V1) \text{X Normality of HCl}}{\text{Weight of oil}} (7)$$

Where V_2= Volume of HCl used for blank
V_1= Volume of HCl used for oil sample

Gas chromatography (GC)

Preparation and analysis of Fatty Acid Methyl Esters (FAMEs)

FAME preparation was done using the method given by Ichihara and Fukubayashi (2010) [13]. Mild methanolysis was performed using the solution containing 8.0 % (w/v) HCl which was prepared using 9.7 mL of conc. HCl and 41.5 mL of methanol. The lipid sample was dissolved in 0.2 mL toluene followed by the addition of 1.5 mL methanol and 0.3 mL of 8.0 % HCl solution. After vortexing, the mixture was kept at 45°C overnight. Then 1 mL of hexane and 1 mL of water were added. The tube was vortexed, and the hexane layer containing FAME was taken for further analysis.

The fatty acid profile was determined by Gas Chromatography (Simadzu GC-2014, Serial no. C121652) using a Flame Ionization Detector (FID) fitted with a Restek Stabilwax column (30m, 0.25mm ID) and polyethylene glycol-modified nitroterephthalic acid stationary phase. Chromatography conditions used were: injection temperature, 230 °C, initial column temperature 120 °C with an equilibration time of 1 min which was held for 3 min, a 4 °C/min rise to 240 °C, held for 7 min, detector temperature, 250 °C and carrier gas, nitrogen at a flow rate of 1.20 mL/min. 1 µL of the esterified sample was injected. The total run time was 48 min. Fatty acids were identified by comparing the retention times with FAME standards (Sigma-Aldrich) and quantified by normalization of the area under relevant peaks using Varian Star software version 4.51. Lipid internal standard Supelco 37 component FAME mix was purchased from Sigma-Aldrich for GC analysis.

Fatty acids-related nutritional indices

The fatty acid profile obtained from gas chromatography was used for the determination of fatty acids-related nutritional indices *viz.*PUFA/SFA (polyunsaturated fatty acids: saturated fatty acids ratio, ω-6: ω-3 (omega-6 fatty acids: omega-3 fatty acids), AI- Atherogenicity index and TI-thrombogenicity index. AI and TI were calculated according to the equations stated below as given by Marques et al. (2023) [14].

$$\text{AI} = \frac{\text{C 12:0} + (4 * \text{C 14:0}) + \text{C 16:0}}{\sum \text{MUFA} + \sum \text{PUFA}} \ (8)$$

$$\text{TI} = \frac{\text{C 14:0} + \text{C 16:0} + \text{C 18:0}}{(0.5 * \sum \text{MUFA}) + (0.5 * \sum \omega - 6) + (3 * \sum \omega - 3) + (\omega - 6 \ / \ \omega - 3)} \ (9)$$

Where ∑PUFA & ∑ MUFA are the total polyunsaturated fatty acids and monounsaturated fatty acids, C 12:0, C 14:0, C 16:0, and C 18:0 are fatty acids with different carbon chain lengths, ω-3 & ω-6 fatty acids are omega-3 and omega-6 fatty acids.

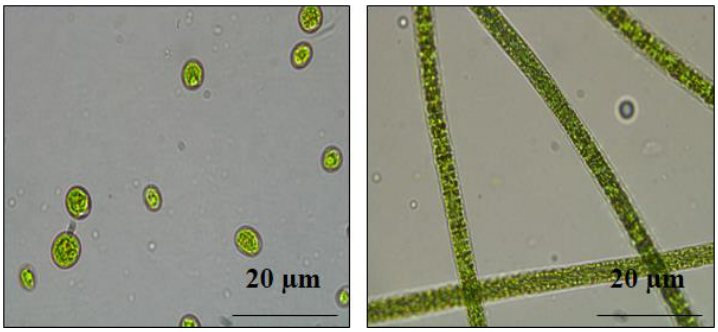


Fig.1 Micrographs of Nannochloropsis sp. and Siprulina sp.

Table 1 Crude oil yield from Nannochloropsis sp. and Spirulina sp. using Folch method and Soxhlet method

	Crude oil yield (%)	
	Folch method	Soxhlet method
Nannochloropsis sp.	22±0.03	11±0.04
Spirulina sp.	8±0.02	6±0.01

**Values are given as mean ± S.E (n=3)*

Results and discussion

Oil extraction Methods

Oil extraction methods play a key role in algal oil recovery and oil quality [15]. Two methods namely the Foch method and the Soxhlet method were used for oil extraction from *Nannochloropsis* sp. and *Spirulina* sp. (Figure 2). The crude oil yield was higher from the Folch method of oil extraction 22% and 8%. in *Nannochloropsis* sp. and *Spirulina* sp. respectively. However, the crude oil yield was 11% and 6 % in *Nannochloropsis* sp. and *Spirulina* sp. respectively from the Soxhlet method (Table 1). The oil appearance from the Folch method was dark green while from the Soxhlet method was a clear green color. The consistency of the oil was semisolid and viscous from the Folch method while from the Soxhlet method oil with free-flowing liquid-like consistency was obtained (Figure 2).

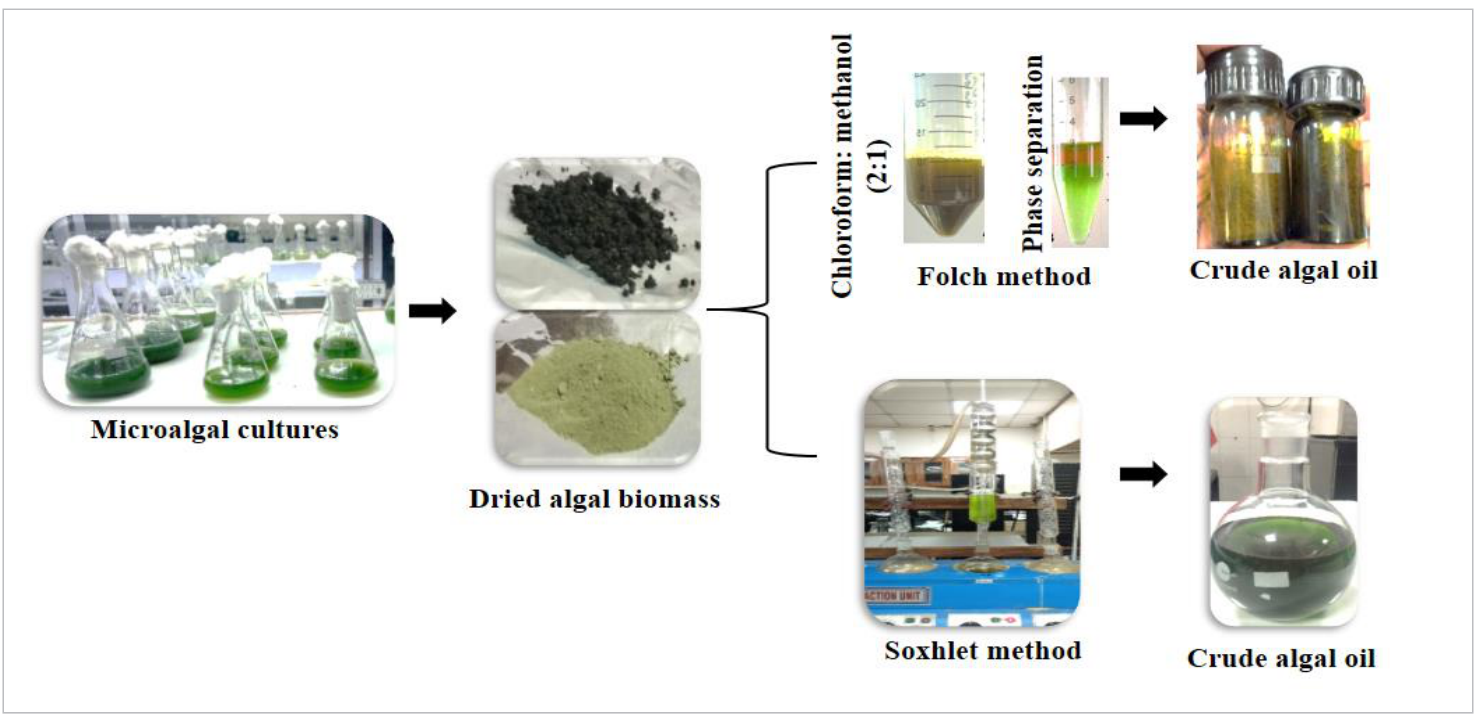


Fig. 2 Folch method vs. Soxhlet method of algal oil extraction

Oil quality

The chemical parameters such as peroxide value (PV), acid value (AV), and free fatty acid (FFA) determine the oxidative stability of oil [16]. These values contribute to oil quality. The higher the PV, AV, and FFA lesser the oil shelf life. The PV, AV, and FFA were lesser in Soxhlet-extracted oil *viz.* 3.1 meq O_2/kg, 5.7 mg KOH/g and 4.7 % respectively in *Nannochloropsis* sp. and 3.3 meq O_2/kg, 8 mg KOH/g and 6.7 %respectively in *Spirulina* sp. However, higher values for these parameters were observed in both the species from the Folch method of extraction. The iodine value gives the information about unsaturation of fatty acids. The higher the unsaturation more the IV. The iodine values were 132 (g) and 152 (g) in *Nannochloropsis* sp. and *Spirulina* sp. from the Folch method and 119 (g) and 127 (g) in *Nannochloropsis* sp. and *Spirulina* sp. from the Soxhlet method. The SAP value gives an idea about the chain length as well as the molecular weight of fatty acids [17]. SAP value determines the soap-forming properties of the oil. SAP values were 179 (mg KOH/ g) and 182 (mg KOH/ g) from the Folch method of extraction and 178 (mg KOH/ g) and 171 (mg KOH/ g) from the Soxhlet method in *Nannochloropsis* sp. and *Spirulina* sp. respectively. Soxhlet method results in the removal of impurities that affect the oil stability-related parameters [18]. The quality of the oil obtained from the Soxhlet method appeared superior (Table 2).

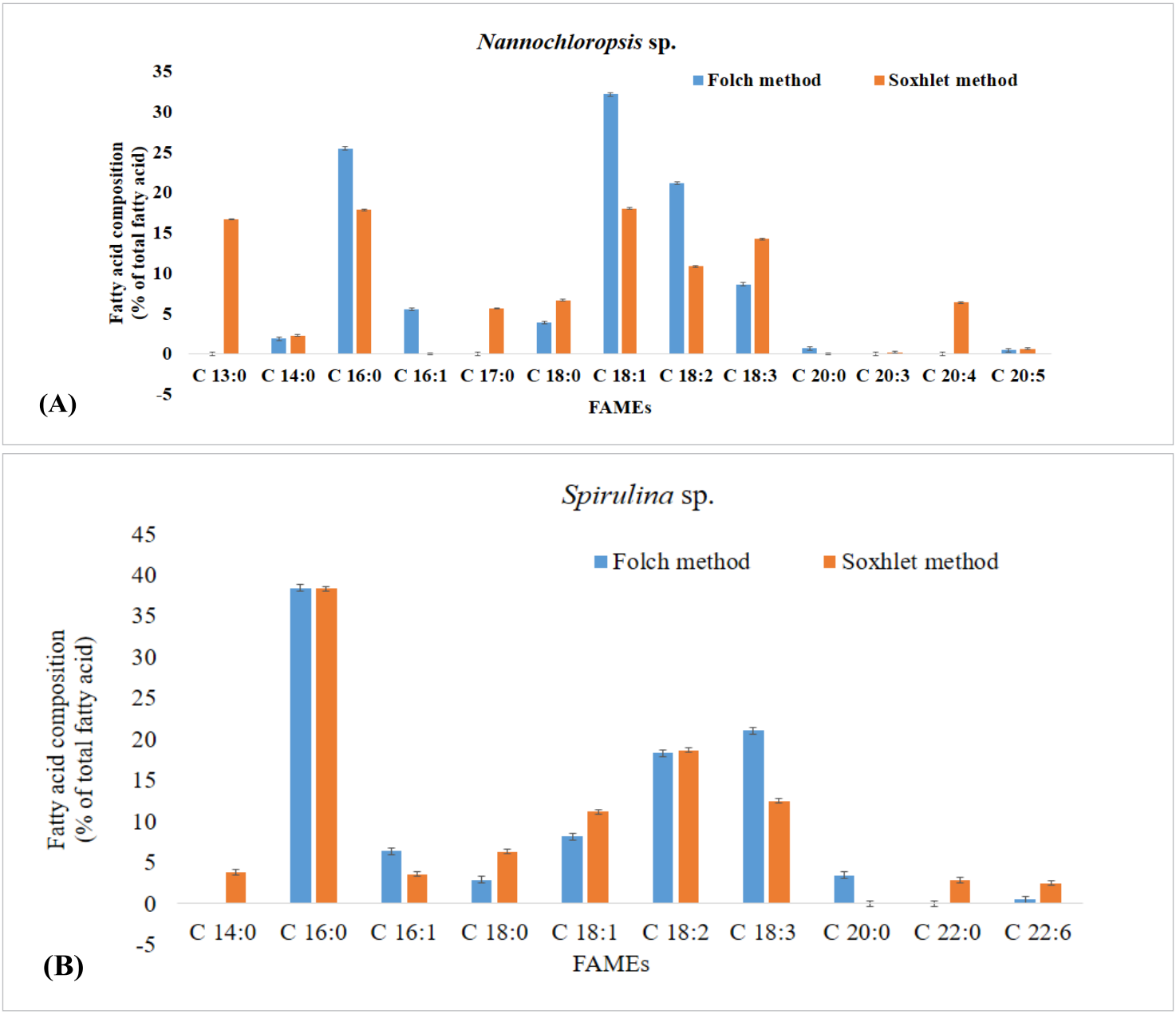
Table 2 Comparison of oil quality for the oils extracted by two different methods viz. Folch method and Soxhlet method from Nannochloropsis sp. and Spirulina sp.

Parameters	Folch method		Soxhlet method	
	Nannochloropsis sp.	*Spirulina* sp.	*Nannochloropsis* sp.	*Spirulina* sp.
PV (meq O_2/kg)	5.60±0.03	6.60±0.02	3.10±0.01	3.30±0.04
AV (mg KOH/g)	1.00±0.02	25.00±0.04	6.70±0.02	8.00±0.03
FFA(%)	0.50±0.02	12.00±0.01	5.63±0.02	6.72±0.01
IV(g)	132±.0.01	152±0.03	119±0.03	127±0.02
SAP (mg KOH/ g)	179±0.04	182±0.02	178±0.01	171±0.02

**IV- Iodine value, AV –acid value, FFA- free fatty acids, PV peroxide value, SAP- Saponification value, Values are given as mean ± S.E (n=3)*

Fatty acid profile

The fatty acid profile showed the distribution of fatty acids from C 13:0 to C 22:6 in *Nannochloropsis* sp. and *Spirulina* sp. (Figure 3). The concentration of long-chain omega-3 fatty acids was increased in the Soxhlet method *viz*. 0.66% eicosapentaenoic acid; EPA and 2.52% docosahexenoic acid; DHA in *Nannochloropsis* sp. and *Siprulina* sp. while EPA and DHA content of 0.46% and 0.51 % in *Nannochloropsis* sp. and *Siprulina* sp. was obtained from Folch method of oil extraction (Table 3). In a recent study, the Soxhlet method was compared with the supercritical fluid extraction method, and higher EPA and DHA recovery (39.72 mgg^{-1} and 1.05mg^{-1}) was obtained by the Soxhlet method compared to the supercritical fluid extraction method (33.70 and 0.45 mgg-1 EPA and DHA respectively) from diatom biomass [19].



(B)Fig. 3 Comparision of fatty acid composition (% of total fatty acids) obtained by two different methods viz. Folch method and Soxhlet method (A) from Nannochloropsis sp. and (B) from Siprulina sp.

Table 3 Fatty acid profile of oil extracted by two different methodsviz. Folch method and Soxhlet methodfrom Nannochloropsis sp. and Siprulina sp.

	Folch method		Soxhlet method	
	Nannochloropsis sp.	*Spirulina* sp.	*Nannochloropsis* sp.	*Spirulina* sp.
C 13:0	0	0	16.68	0
C 14:0	1.88	0	2.29	3.845
C 16:0	25.46	38.45	17.87	38.343
C 16:1	5.55	6.43	0	3.588
C 17:0	0	0	5.65	0
C 18:0	3.91	2.91	6.68	6.373
C 18:1	32.17	8.18	18.04	11.215
C 18:2	21.18	18.35	10.88	18.69
C 18:3 (ALA)	8.64	21.08	14.26	12.519
C 20:0	0.69	3.49	0	0
C 22:0	0	0	0	2.898
C 20:3	0	0	0.21	0
C 20:4	0	0	6.37	0
C 20:5 (EPA)	0.46	0	0.66	0
C 22:6 (DHA)	0	0.51	0	2.528

**ALA, alpha linolenic acid, EPA, eicosapentenoic acid, DHA, docosahexaenoic*

Fatty acids-related nutritional indices

Fatty acids-related nutritional indices are PUFA/SFA, ω-6: ω-3 ratio, AI and TI. These indices give an idea about cardiovascular health, hypertension, and other diet-related disease conditions [20]. The PUFA/SFA ratio is recommended >1. In both the extraction methods *viz.* Folch method and Soxhlet method the PUFA/SFA was closer to 1. The ratio ω-6: ω-3 was 2.32 and 0.84 in oil extracted by the Folch method and 1.15 and 1.24 in oil extracted by the Soxhlet method from *Nannochloropsis* sp. and *Spirulina* sp. These values fall in the recommended range of ω-6: ω-3 ratio i.e. < 5-10:1 [21]. AI and TI values determine the effect of dietary fats on cardiovascular health. Lower values of AI and TI are found beneficial to cardiovascular health [22]. AI and TI values were 0.52 and 0.5 in oil extracted by the Folch method and 1.15 and 1.24 in oil in oil extracted by the Soxhlet method from *Nannochloropsis* sp. and *Siprulina* sp. Fatty acids-related nutritional indices showed a desirable range in oils extracted by both methods.

Table 4 Fatty acid profile related nutritional indices of oils extracted by two different methods viz. Folch method and Soxhlet method from Nannochloropsis sp. and Spirulina sp.

	Folch method		Soxhlet method	
	Nannochloropsis sp.	*Spirulina* sp.	*Nannochloropsis* sp.	*Spirulina* sp.
SFA	31.94	44.85	49.17	51.45
MUFA	37.72	14.61	18.04	14.80
PUFA	30.28	39.94	32.38	33.737
PUFA/SFA	0.94	0.89	0.65	0.65
ω-6	21.18	18.35	17.25	18.69
ω-3	9.1	21.59	14.92	15.04
ratio	2.32	0.84	1.15	1.24
AI	0.48	0.7	0.53	1.1
TI	0.52	0.5	0.42	0.76

** SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; ω-6, omega-6 fatty acids; ω-3, omega-3 fatty acids; AI, Atherogenicity index; TI, thrombogenicity index.*

Conclusion

The comparative analysis of two different methods *viz.* Folch method and Soxhlet method of oil extraction were done in *Nannochloropsis* sp. and *Spirulina* sp. Oil yield was greater in the Folch method, however, Soxhlet-extracted algal oil showed better chemical parameters than the Folch method. The Soxhlet extracted oil showed lesser PV, AV, FFA, and desirable values for IV and SAP. The fatty acid profile showed higher EPA and DHA content (0.66% and 2.52%) by the Soxhlet method than the Folch method (0.46% EPA and 0.55%) from *Nannochloropsis* sp. and *Spirulina* sp. The fatty acids-related nutritional indices PUFA/SFA, ω-6: ω-3 ratio, AI, and TI were found in the desirable range for both extraction methods. This study will help in selecting oil extraction methods for improving microalgal oil quality and stability.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships.

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Conflict of interest: The authors declare no competing interests.

Ethics approval: No ethical clearance was required for this study

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