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Comparison of Different Methods to Extract Lipid from *Sargassum Sp.* Macro Algae

Homira Agah*

Iranian National Institute for Oceanography and Atmospheric sciences, Iran

***Corresponding author:** Homira Agah, Iranian National Institute for Oceanography and Atmospheric sciences, 3 Etemadzadeh street, Fatemi Avenue Tehran Iran, Tel: +98 912 602 9555; Fax: +98 21 66 944 869; E-mail: aaagah_hom@yahoo.com

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Abstract

Sargassum sp. is one of the local macro algae, which possesses various medical well known applications. In this study, different methods of lipid extraction from this macro alga, including centrifugation, ultrasonic bath, and soxhlet were investigated. Results revealed that using Soxhlet method presented a good yield of lipid extraction. Soxhlet extractions were performed at three extraction times (6h, 9h and 12h) and with two solvents (*n*-hexane and methanol). Results showed that the yield of lipid extraction by methanol was higher than *n*-hexane and mixtures of these two solvents. Results would suggest that the quality of the extracted lipids at 6h (Resulted in 20 % lipid yield) was more appropriate to detect fatty acids by GC MS. The analysis of fatty acids demonstrated that palmitic acid, a- Linoleic acid, Docosahexaenoic acid, Myristic acid, Oleic acid, Arachidonic acid, Palmitoleic acid and Stearic acid were predominance fatty acids in total lipid of *sargassum* brown macro algae, respectively. Results of this assessment indicated that besides the presence of two Omega fatty acids (ω-3 and ω -6) in the analyzed macro algae, the ratios of ω-6/ω-3 was lower than 1, which verifies that extracted oil from *Sargassum* is good candidates for human consumption.

Keywords: Oil extraction; Fatty acids; *Sargassum* algae; Chabahar bay; Soxhlet

Abbreviations: DHA: Docosahexaenoic Acid; PUFAs: Polyunsaturated Fatty Acids; LA: Linoleic Acid; ALA: Alpha Linoleic Acid; GLA: Gamma Linoleic Acid; AA: Arachidonic acid; EPA: Eicosapentanoic Acid; FAMEs: Fatty Acid Methyl Esters; EI: Electron Impact Ionization

Introduction

In recent years, attempts have been made to discover algae which are rich in lipids; in this regard algae cultivation under harsh conditions, such as high temperature, pH, and salinity, is very important. So far, 300 algae and diatoms with these characteristics have been identified [1]. When feeding by sugar, algae can convert it into different types of lipids. Today attempts are made to find suitable alternatives feeding and fuel oil sources. In this favor, algae, especially macro algae (seaweed), are considered as a potential source, because they have short-term cultivation and low cost [2].

Sargassi sargassum as macro algae belonging to the family of Sargassaceae is vastly used as livestock feed [3]. Presence of different secondary metabolites and various vitamins, and occurrence of anti-viral compounds in this

alga has made it a good source for livestock feed. On top of that, this alga possesses different pharmaceutical properties encompassing anti-fungal, anti-parasitic activities; remove mucus and phlegm and thyroid disease [4,5]. Biological activities of the lipids from algae extracts have been probed in many studies. Algae contain a number of different lipids with many pharmaceutical applications [6]. Some of these compounds are vastly present in algae and fish, in much more amounts than plants and animals [6,7]. Wiraswati & Warganegara [2] indicated that lipid extract from *sargassum sp*. contains various fatty acids with many double bonds, especially oleic acid (ω-9 fatty acid), which shows the potential of *Sargassum sp*. to be utilized in pharmaceutical and food industries.

Experimental Details and Methodologies

Sargassum macro algae were collected manually from intertidal zone of Chabahar Bay (60◦39′13" and 25◦21′31" Tiss, Figure 1) in the 1st June 2016. In this study 9 samples were collected from 3 stations (Figure 1), transferred to plastic bags and kept in deep freezer.

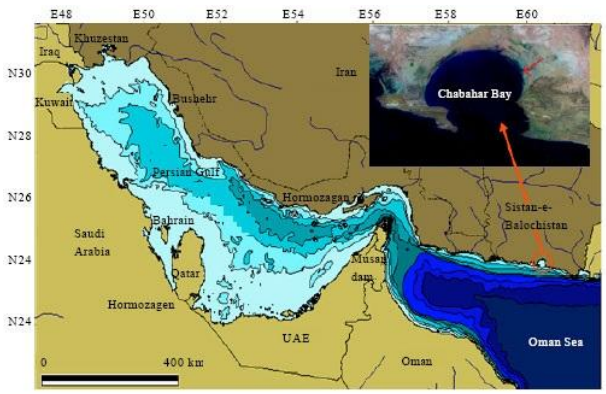

Figure 1: Geographical location of sampling site.

In the laboratory, the collected algae were washed thoroughly with fresh and subsequently distilled water to remove salt and sand particles. In order to determine water content, the algae were weighed before and after freeze drying (-50 Centigrade Vacuum degree, Operon bench Top Model OPR-FDB- 5503). The dried algae were ground in grinding mill (Wagtch international) and sieved to produce samples with particle size of less than 150µm. The sample was stored in deep freezer for further usage. Different extraction methods such as centrifugation (3000 rpm at room temperature, oilalgae.com) [8], ultrasonic-assisted extraction (at 40°C, [9] and Soxhlet [10] were used to extract lipid of 2 grams of milled *Sargassum sp.* macro algae by normal hexane (150 ml) in during 6 hours. In order to select appropriate condition for soxhlet extraction method, different solvents consisted of normal hexane (n-hexane), methanol, diethyl ether and mixture of normal hexane:methanol [11,12] and also different extraction times (6, 9, and 12 hours) were performed. The yield of lipid extraction was determined after removing the solvents by rotary evaporator device. Eventually, in order to ensure complete removal of solvent, nitrogen gas was blow on samples. The solvents used for Gas Chromatography and extraction were analytical grade (purity > 99%).

In order to determine fatty acids, 0.5 gr of the extracted lipid was dissolved in 1.5 ml BF3 (in10% methanol) for 30 min at room temperature. Then $NaBPh_4$ was added and transferred to a 15 ml vial to complete derivation process for 60 min at 80°c. Subsequently 5 mL water:Hexane (1:1) was added to control derivation process. The extraction of the fatty acid methyl esters (FAMEs) took place by adding 6 mL of hexane to the samples in three steps. The organic phase FAMEs was evaporated to 1 mL using nitrogen flushing and 1µL of it was injected into the gas chromatograph.

Fatty acids analysis was performed using GC MS equipped with electron impact ionization source under (EI) mode of 70 ev, and quadrupole mass analyzer as well as Chemstation data analysis system. Capillary column of HP-5 (5% diphenyl 95% dimethyl siloxane copolymer) with length of 30 m (320 µm internal diameter and film thickness of 0.1 µm) was used. Flow rate of Helium carrier gas (purity 99.999%) was 1.2 $mlmin^{-1}$. 0.5 µl of the extract (methyl ester derivatives of fatty acids) was injected to GC MS under 100:1 ration of split mode. Injection temperature was 200°C, oven temperature initiated at 60°C, then reached 300°C with ramp of 6°C min^{-1}, remaining at 300°C for 7 min [13].

FAMEs compound ranging from butyric methyl esters (C4:0) to lignoceric acid methyl esters (C24:0), were identified and quantified by comparing their retention times with those of standards. The values are expressed as a percentage of the total fatty acids mixture. Final result of triplicate injections was reported.

Results and Discussions

The results are expressed as the yield of crude extract: $Y_{extract}=100*\frac{\text{m extract}}{\text{m algae}}$, where $m_{extract}$ is the crude extract mass (gr) and m_{algae} is the extracted algae mass (gr). All experiments were triplicate for statistical evaluation. The yield of lipid extraction by different methods,

centrifugation, ultrasonic bath, and soxhlet is reported in Table 1. As it is apparent in the results, at similar extraction condition, soxhlet had higher yield of lipid extraction than the two other methods. Therefore, this method was selected as the optimum method for extracting lipids with higher yield. Ghada and coworkers also prepared lipid extract from *Sargassum Subrepandum* using soxhlet method with dichloromethane as the extraction solvent for 12 hours [14].

Extraction method	Time (h)	Solvent	Lipid % d.w
Centrifugation	6	n-hexane	0.23±0.012
Ultrasonic bath	6	n-hexane	0.30±0.011
Soxhlet	6	n-hexane	0.62±0.025

Table 1: The yields of lipids extracted from *Sargassum* by different extraction methods.

The extracted lipids were analyzed by GC MS analytical method. The injected lipids obtained from centrifugation, ultrasonic bath methods, showed no particular compound. In this study soxhelt extraction method was optimized using different solvents and extraction times (Table 2). In this order 2 gr of homogenized powder of *Sargassum* algae was transferred to soxhlet apparatus to be extracted by 150 ml of normal hexane during 6, 9, and 12 hours. Lipid extraction from 2 gr. *Sargassum* algae was performed during the same extraction times (6, 9 and 12 h) and conditions using methanol and mixtures of hexane:methanol (Ratios of 2:1and 1:2) [11,12] (Table 2). As diethyl ether is one of the appropriate solvents for lipids extraction [15] hence in this study lipid was extracted using Diethyl ether solvent from 2 gr *Sargassum* by Soxhlet during 2 hours [16], (ID 920.39, Table 2). The yield of extracted lipids for each method is shown in Table 2.

Solvent	Time(hours)	Lipid content %
n-hexane	6	0.62±0.025
n-hexane	9	1.24±0.06
n-hexane	12	1.79±0.09
Methanol	6	12.8±0.64
Methanol	9*	16.3±0.72
Methanol	12*	20.2±0.99
Hexane:Methanol (2:1)	6	5.1±0.25
Hexane:Methanol (2:1)	9	9.0±0.35
Hexane:Methanol (2:1)	12*	10.1±0.39
Hexane:Methanol (1:2)	6*	24±1.22
Hexane:Methanol (1:2)	9*	35.3±1.6
Hexane:Methanol (1:2)	12*	45.7±2.1
Diethyl ether	2	2.5±0.13

Table 2: The yields of lipids extracted from *Sargassum* sp. by soxhlet apparatus under different extraction conditions.

The quality of extracts (Jelly and cloudy) were not appropriated to be injected to GC MS. Although the mass of extracted lipid using methanol at 9 and 12 hours, Hexane:Methanol (2:1) at 12 hours and Hexane:Methanol (1:2) solvents in different times (6, 9 and 12 hours) were higher than other experiments, but the quality of extracts (Jelly and cloudy) were not appropriated to be injected to GC MS and it was necessary to pass different cleanup process. The results demonstrated that identification and characterization of the extracted lipids using methanol for 6 hours seems better.

The results of fatty acid compounds analysis of the total extracted lipids of *Sargassum sp.* macro algae in different conditions are shown in Table 3. The analysis of fatty acids was performed using GC MS. Myristic acid, Palmitoleic acid, Palmitic acid, Oleic acid, Stearic acid, a-Linoleic acid, Arachidonic acid, Docosahexaenoic acid (DHA) were detected in the extracted lipids.

Compounds	1*	2*	3*	4**	5***
Tetradecanoic acid (Myristic acid) methyl ester C14:0	3.8	4.6	2.9	3.9	4.2
9- Hexadecenoic acid (Palmitoleic acid) methyl ester C16:1	2.1	2.2	1.3	2.3	2.1
Hexadecanoic acid (Palmitic acid) methyl ester C16:0	43	37.2	34.6	48.2	47
9- Octadecenoic acid (Oleic acid) methyl ester C18:1	1.5	3.1	-	2.4	2.7

9,12.15- Octadecatrienoic acid (a- Linoleic acid) methyl ester C18:3 (n-3)	23.2	27.7	17.7	29.4	32
Octadecanoic acid (Stearic acid) methyl ester C18:0	1.6	1.7	4.2	1.4	1.2
5,8,11,14 Eicosatetraeoic acid (Arachidonic acid) methyl C20:4 (n-6)ester	3.9	3.6	1.9	3.6	3.4
(Probably , Docosahexaenoic acid (DHA)) C22:6	1.8	3.6	0.9	4.3	3.6
9,12- Octadecadienoic acid (linoleic acid) methyl ester C18:2	-	-	-	-	1.6
Other compounds detected (including Alkanes, Alkenes, Carboxylic acids etc.)	19.1	16.3	36.5	4.5	2.1

Table 3: Percentages of major fatty acids present in *Sargassum*, using different solvents in different extraction times.

1, 2 and 3: n-hexane at 6, 9 and 12 h, respectively. **4: methanol at 6 hr. ***5: Hexane: Methanol (2:1) at 9 hr. Although the abundancy of essential fatty acids (ω-3 and ω-6, table 3) extracted by Hexane:Methanol (2:1) in during 9 hours was higher than other methods, but according to the yield of lipid extraction (table 2) the method using methanol for 6 hours can be a more appropriate. Figure 2 compares the percentages of extracted different fatty acids from *sargassum*, using different methods. The availability of important polyunsaturated fatty acids (PUFAs), such as linoleic acid (LA), alpha linoleic acid (ALA), gamma linoleic acid (GLA), arachidonic acid (AA), eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), with proven biomedical and nutraceutical applications, indicates their potential utilization in preparation of functional foods [17,18].

The predominance fatty acids in total lipid of *Sargassum* brown macro algae were in the following order: palmitic acid (16:0)(48.2%)>a- Linoleic acid (C18:3 (n-3)) (29.4%)> Docosahexaenoic acid (DHA) (C22:6, n-3) (4.3%)> Myristic acid (C14:0) (3.9%)>Oleic acid (18:1n-9)(2.4%)> Arachidonic acid (C20:4(n-6)) (3.6%)> Palmitoleic acid (C16:1) (2.3%)> Stearic acid (C18:0) (1.4%) (Figure 3).

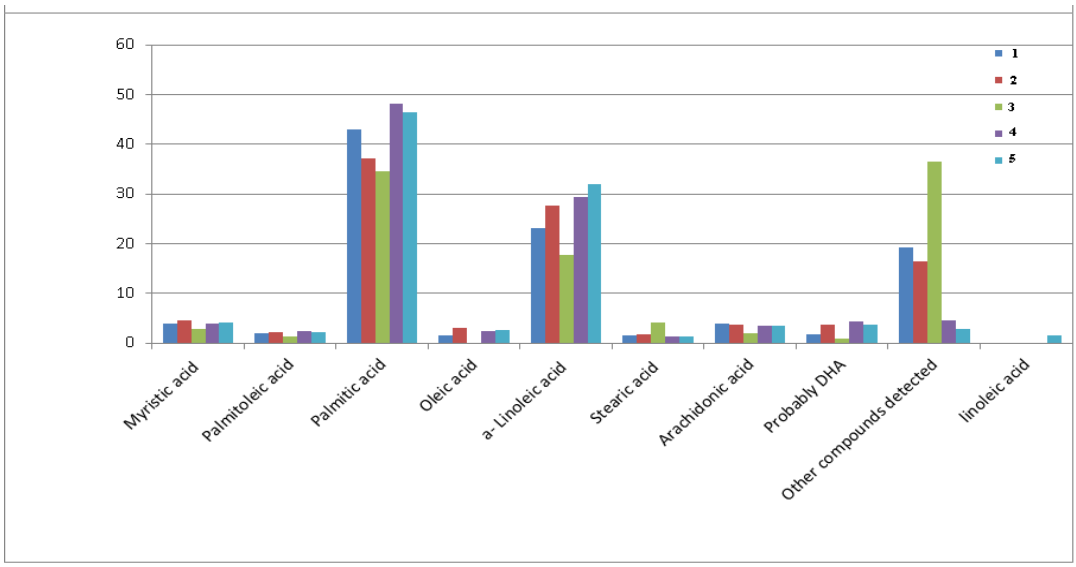

Figure 2: Comparison the percentages of extracted fatty acids from *Sargassum*, using Soxhlet apparatus and different solvents. 1, 2 and 3: n-hexane at 6, 9 and 12 h, respectively. 4: methanol at 6 hr. 5: Hexane: Methanol (2:1) at 9 hr.

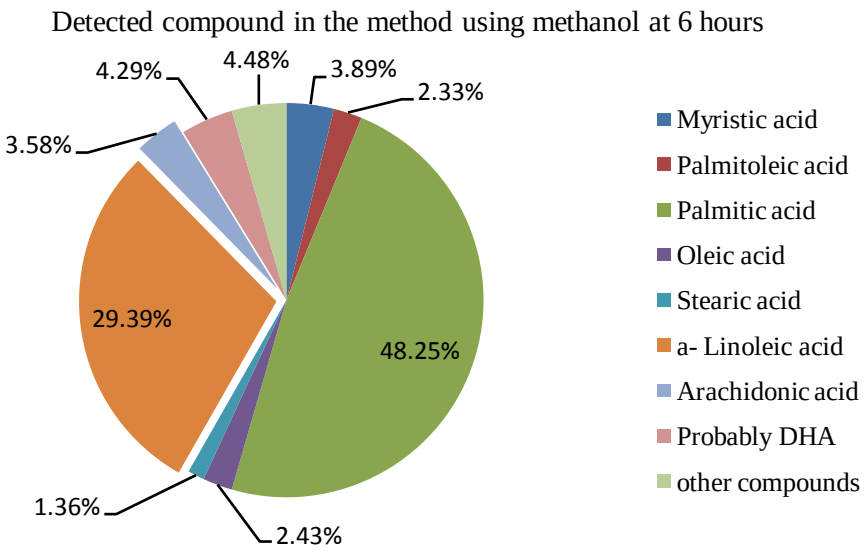

Figure 3: Different fatty acids extracted from *Sargassum* by Soxhlet apparatus and methanol solvent at 6 hours.

Conclusion

In this study different extraction methods using different laboratory grade solvents, diethyl ether, n-hexane, methanol and mixture of these two latter solvents for extraction lipid from *Sargassum* macro algae at different times, 6, 9 and 12 hours, were compared. The results showed that soxhlet extraction apparatus was more efficient than centrifuge and ultrasonic bath to extract lipid from *sargassum*. The experimental results demonstrated that oil yield using methanol was more than that using n-hexane. Although oil extraction yield at 12 hours was more than other experiments, but identification and characterization of the extracted oil by GC MS using methanol for 6 hours seems better. Among the saturated fatty acids, the major fatty acid was palmitic acid (c16:0), which is the main fatty acid in all trophic levels. In addition myristic and stearic acids were the next predominate fatty acids in the extracted lipid, respectively.

Based on qualitative analysis, the diversity of fatty acids identified into 8 types, which is consists of 3 types of saturated fatty acids (palmitic acid,; Myristic acid, Stearic acid, ΣSAFA: 53.5%), two types of monounsaturated fatty acids (Oleic acid, Palmitoleic acid ΣMUFA:4.7% and three types of polyunsaturated fatty acids (Linoleic acid, Docosahexaenoic acid, Arachidonic acid, ΣPUFA:37.3%)
It is revealed from Figure 3 that *Sargassum* sp. is rich in essential unsaturated fatty acids such as omega-3 (α-*linolenic acid*, docosahexaenoic acid) and omega-6 (arachidonic acid), which may reduce the risk of heart disease. Although the omega fatty acids (*n*−3 and *n*−6) are essential for human, but the ratio of ω−6 /ω−3 significantly influences human health.

Studies showed that the ratio of Omega-6/Omega-3 less than 1 in human diet can support human health [19,20,21] and can even reduce cancer cell activity. Results of this assessment indicated that besides the presence of two Omega fatty acids in the analyzed macro algae, the ratios of ω-6/ω-3 was lower than 1, which verifies that extracted oil from *Sargassum* is good candidates for human consumption. [22] revealed that *Sargassum* species are rich in ω-6 and ω-3 fatty acids. Based on these results it is expected that the results of this study can provide useful information for lipid extraction from *Sargassum* macro algae.

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