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# New procedure for extraction of algal lipids from wet biomass: A green clean and scalable process

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## Abstract

A new procedure, called Simultaneous Extraction and Distillation Process (SDEP), for lipid extraction from wet microalgae (*Nannochloropsis oculata* and *Dunaliella salina*) was reported. This method does not require a pre-drying of the biomass and employs alternative solvents such as d-limonene,  $\alpha$ -pinene and p-cymene. This procedure has been compared with Soxhlet extraction (Sox) and Bligh & Dyer method (B&D). For *N. oculata*, results showed that SDEP-cymene provided similar lipid yields to B&D (21.45% and 23.78%), while SDEP-limonene and pinene provided lower yields (18.73% and 18.75% respectively). For *D. salina*, SDEP-Pinene provided the maximum lipid yield (3.29%) compared to the other solvents, which is quite close to B&D result (4.03%). No significant differences in terms of distribution of lipid classes and fatty acid composition have been obtained for different techniques. Evaluation of energy consumption indicates a substantial saving in the extraction cost by SDEP compared to the conventional extraction technique, Soxhlet.

**Keywords:** Wet lipid Extraction, Microalgae, *Nannochloropsis oculata*, *Dunaliella salina*, Terpenes

## 1. Introduction

Petroleum reserves depletion and global climate change have strongly encouraged the development of fuel production from various feedstocks such as

1 vegetable oils, waste cooking oils, animal fat and microalgae. Among these options,  
2  
3 microalgae have been recognized as potential good sources for biofuel production  
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5 because they synthesize and accumulate large quantities of neutral lipids (20–50 % dry  
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7 weight of biomass) and grow at high rates (Demirbas, 2008). In addition, microalgae  
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9 can grow on non-arable, nutrient-poor land that cannot support conventional agriculture  
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11 (Singh and Gu, 2010). A recent life-cycle assessment (LCA) of biofuel production from  
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13 microalgae feedstocks mentioned that drying and n-hexane extraction accounted for up  
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15 to 90% of the total process energy (Lardon et al., 2009). The extraction of crude oil is  
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17 usually performed with petroleum solvents such as conventional n-hexane, chloroform  
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19 and methanol, with techniques are highly energy-consumption and environmentally  
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21 damaging (Halim et al., n.d.). Other extraction processes such as supercritical CO<sub>2</sub>,  
22  
23 expelling, microwave-ultrasonic assisted extraction have also been reported (Cheng et  
24  
25 al., 2011). Many processes have been investigated and reported for converting directly  
26  
27 wet algae to crude biodiesel or biocrude (Anastasakis and Ross, 2011; Biller and Ross,  
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29 2011; Biller et al., 2011; Patil et al., 2011). But this biocrude differs from biodiesel that  
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31 we want to extract as it is composed primarily of hydrocarbons in contrast to biodiesel  
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33 which is composed of lipids in particular FAME after transesterification.  
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42 However, various conventional methods are available for oil extraction, but they  
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44 generally require long extraction times, petroleum-based solvents, dried biomass with  
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46 water content no more than 10% and high energy inputs. Upon harvesting, typical  
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48 microalgal concentrations in cultures range from about 0.1-1.0% (w/v) (Cooney et al.,  
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50 2009). This means that as much as 1000 times the amount of water per unit weight of  
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52 microalgae must be removed before attempting oil extraction. The microalgae paste  
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54 obtained from centrifugation (dewatering step) contains as much as *ca.* 80% water  
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1 content. Therefore energy consumption for drying microalgae is dramatically high. A  
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3 lipid extraction step that eliminates biomass drying and petroleum solvent use could  
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5 lead to significant energy and cost savings.  
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8 Here we propose a new procedure of lipid extraction from microalgae, such as  
9  
10 *Nannochloropsis oculata* (*N. oculata*) and *Dunaliella salina* (*D. salina*), that does not  
11  
12 require drying of the harvested microalgal biomass and employs bio-solvents  
13  
14 recognized as environmentally safer (Virost et al., 2008). Terpenes are natural solvents  
15  
16 existing both in the citrus fruits and in many other plants, with extraordinary technical  
17  
18 and chemical properties. Mamidipally and Liu recently demonstrated that the industrial  
19  
20 extraction of oil from rice bran was possible by using terpene such as d-limonene  
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22 instead of the regular *n*-hexane (Mamidipally and Liu, 2004; Liu and Mamidipally,  
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24 2005).  
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30 Extracted lipids obtained using this new procedure, conventional Soxhlet with *n*-  
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32 hexane and Bligh & Dyer method have been compared in term of total lipid content,  
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34 lipid classes distribution and fatty acid composition.  
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## 38 **2. Materials and methods**

### 39 2.1. Strain, culture and harvesting conditions

40  
41 *N. oculata* and *D. salina* were obtained by Greensea Company (Meze, France). *N.*  
42  
43 *oculata* was incubated in tubular reactor at ambient temperature under deficiency  
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45 conditions to obtain a high rate of lipids in the biomass. *D. salina* was grown in photo-  
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47 bioreactor at ambient temperature with good sunniness under favourable conditions. For  
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49 both, harvesting is being performed by centrifugation, resulting in a 20% dry weight  
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51 paste that is directly frozen (-25°C).  
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### 57 2.2. Extraction methods for total lipids

### 2.2.1 Bligh and Dyer method (B&D)

Total lipids content of both microalgae was determined using a modified B&D method (Bligh and Dyer, 1959) which is already described in Adam et al., 2012.

### 2.2.2 Soxhlet method (Sox)

Lipids were isolated from microalgae by means of Soxhlet extraction (F. Soxhlet, 1879). Lipids were extracted from 10 grams of dry microalgae for 8h using 300 mL of n-hexane. After the extraction, solvent was eliminated with a vacuum rotary evaporator. Extractions were performed in triplicate and the mean values were reported. Lipid extracts were dried under a stream of N<sub>2</sub> and re-suspended in solvent for HP-TLC or GC-FID analysis.

### 2.2.3 SDEP method

For SDEP extraction, 12 g ± 0.5 g of 20% dry weight microalgae paste were placed in a 500mL round-bottomed flask. 100mL of terpene solvent (p-cymene, d-limonene or α-pinene) was added in order to immerse the wet microalgae sample. The round-bottomed flask was surmounted by a modified Dean stark receiver with a 3-way valve and fitted with a condenser (Fig. 1). At the beginning of the experiment (step 1), the electrical heating was maximized until collection of the first droplets of microalgae water in the modified Dean stark receiver with a 3-way valve. Then the heating was adapted until most of the water had been distilled and it was continued to allow lipid extraction step (step 2) with terpene solvent. The extraction was performed for 30 min. Then, the terpene elimination took place (step 3 and 4) and for that water was re-introduced by adjusting the 3-way valve to form a binary water-terpene mixture. To eliminate d-limonene from the distillation flask, we used the property that terpenes are traditionally extracted from their matrix by using a technique called hydrodistillation

1 thus inducing the use of an azeotropic distillation to below the boiling point of terpene  
2  
3 under the boiling point of the water (boiling point of the azeotrope : 97.4 °C). Terpene  
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5 solvent was recovered from the water layer by phase separation in the modified Dean  
6  
7 stark receiver and the extracted lipids were recovered from the water layer by phase  
8  
9 separation in the distillation round-bottomed flask. Thus, the SDEP procedure was  
10  
11 allowed elimination of microalgae water, extraction of lipids and elimination of terpene  
12  
13 solvent in a single “in situ” step. Terpene solvent was recuperated at 100% and purity  
14  
15 levels show that it can be recycled for other uses, including other SDEP processes.  
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17  
18 Lipid extracts were analysed by GC-FID (against an external calibration with the pure  
19  
20 solvent) and did not contain contamination by solvent (less than 0.01% of solvent in  
21  
22 lipid extract). Extractions were performed in triplicate and the mean values were  
23  
24 reported. Lipid extracts were dried under a stream of nitrogen and re-suspended in  
25  
26 solvent without purification for HP-TLC or GC-FID analysis.  
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### 32 2.3. Analysis of total lipids

#### 33 2.3.1. Gravimetry

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35 Total lipid content was quantified by weight after total drying.  
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#### 38 2.3.2. High Performance Thin-Layer Chromatography (HP-TLC)

39  
40 Lipids were detected by charring and quantified using a CAMAG 3 TLC scanning  
41  
42 densitometer (CAMAG, Muttenz, Switzerland) with identification of the classes against  
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44 known polar and neutral lipid standards. Typically, Lipid extract was loaded as a spot  
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46 onto 20 × 10 cm silica gel 60 F254 HPTLC plates (Merck KGaA, Germany) using an  
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48 ATS 5 automatic TLC sampler (Camag, Switzerland). Plates were then developed in an  
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50 ADC2 automatic developing chamber (CAMAG, Switzerland) using first a methyl  
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52 acetate/isopropanol/chloroform/methanol/KCl (0.25% solution) (25:25:25:10:9) mixture  
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1 running to a height of 5.5 cm from the origin and then a n-hexane/diethyl ether/glacial  
2 acetic acid mixture (80:20:2) to a height of 8.5 cm from the origin. After dried, the plate  
3 was dipped for 6s in a modified CuSO<sub>4</sub> reagent (20 g CuSO<sub>4</sub>, 200 ml methanol, 8 ml  
4 H<sub>2</sub>SO<sub>4</sub>, and 8 ml H<sub>3</sub>PO<sub>4</sub>) then heated at 141 °C for 30 min on a TLC plate heater and  
5 finally scanned using a TLC Scanner 3 with WinCATs software (CAMAG). The  
6 densitometry data are reported as values which are expressed as percent of lipid class in  
7 total microalgae lipids.  
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### 10 2.3.3 GC Analysis

11 Fatty acids methyl esters (FAMES) were separated, identified and quantified by gas  
12 chromatography coupled with flame ionization detector (GC-FID) using the method  
13 already described by Adam et al., 2012.  
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## 16 3. Results and discussion

### 17 3.1 Recovery of Crude lipids

18 The measurement of lipids recovery was based on the total amount of FAMES in each  
19 sample. So it is a real yield in total fatty acids, and it is certainly a much more accurate  
20 value than a yield obtained by gravimetric method. Table 1 shows the results of both  
21 microalgae for each extraction method. Three extraction methods, B&D, SDEP and Sox  
22 were tested with different solvents. Use of B&D extraction is the commonly accepted  
23 method for lipid extraction from biological tissues due to its high lipid yields (Certik et  
24 al., n.d.). Therefore, the B&D method was selected as standard to compare other  
25 extraction technique. As mentioned in Table 1, B&D method yielded 23.78% and  
26 4.03% lipids content for *N. oculata* and *D. salina* respectively. Crude lipids of *N.*  
27 *oculata* and *D. salina* obtained by SDEP procedure with three terpenes were higher than  
28 Sox and slightly lower than B&D reference method. First, these results can be explained  
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1 by the difference of polarity between solvents; Liu and Mamidipally (Mamidipally and  
2 Liu, 2004; Liu and Mamidipally, 2005) have already noted this effect that might be due  
3 to the slightly more polar nature of terpenes and much more polar nature of  
4 methanol/chloroforme mixture compared with *n*-hexane. In addition, in SDEP  
5 procedure the matrix is in direct contact with the boiling solvent which is not the case  
6 with the conventional Soxhlet. A higher dissolving ability of terpenes for lipids might  
7 also be pointed out by the higher temperature used to boil this solvent which could  
8 produce a lower viscosity of the analytes in the matrix and, accordingly, a better  
9 diffusion rate of the solute from the solid phase to the solvent.  
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### 22 3.2 Lipids class composition

23 Similar to higher plants, microalgal lipids are composed of neutral lipids and polar  
24 lipids. Under nutrient stress conditions, many microalgae tend to accumulate neutral  
25 lipids (consist of triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols  
26 (MAG) and free fatty acids (FFA)) and form lipid droplet localized in the cytoplasm as  
27 a storage form of carbon and energy. Neutral lipids of the extracts were separated using  
28 high-performance thin-layer chromatography (HP-TLC), quantified with densitometry  
29 and presented as % of lipid class in total lipids extracted with B&D, SDEP and Sox for  
30 both microalgae. Four standards in a mixture of monoacylglycerol (MAG),  
31 diacylglycerol (DAG), triacylglycerol (TAG) and free fatty acids (C18) were deposited  
32 on the plate. For *N. oculata*, HP-TLC results confirm that TAG, DAG and FFA were the  
33 main components of the extracted lipids, but they contributed to the total lipid content in  
34 different amounts. As shown in Fig. 2, TAG made up an important percentage of total  
35 lipids (between 57 and 69% according to extraction method). Regarding *D. salina*, the  
36 amount of TAG for B&D method is about 60%, 54% for Sox and ranging from 50 to  
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1 57% for SDEP procedures. The SDEP procedure with p-cymene solvent gives a higher  
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3 quantity of TAG than d-limonene and  $\alpha$ -pinene. Results obtained with the SDEP  
4  
5 method including different terpene were almost similar with those obtained by both  
6  
7 conventional Sox and B&D method.  
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### 10 3.3 Fatty acid compositions

11 Table 1 shows the classes' variability of FAMES in connection with the extraction  
12  
13 method used. The nature of FAMES composition in *N. oculata* and *D. salina* was  
14  
15 determined by GC analysis. According to Table 1, the main fatty acids for *N. oculata*  
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17 were palmitic (C16:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids.  
18  
19 These four fatty acids represent more than 80% of the total fatty acid composition of the  
20  
21 extracted oil. Other fatty acids such as myristic (C14:0), palmitoleic (C16:1),  
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23 palmitolenic (C16:2), hirigonic (C16:3) or stearic (C18:0) acids were also noted with a  
24  
25 less predominant peak area. Arachidic (C20:0) and behenic (C22:0) acids were found in  
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27 trace levels. No significant differences were detected according different extraction  
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29 methods and solvents. The fatty acid composition of *N. oculata* sp. displayed in Table 1  
30  
31 is different to the profile presented by Adam et al. (Adam et al., 2012). Despite similar  
32  
33 results regarding the respective proportions of C16:0 and C14:0, the authors report *N.*  
34  
35 *oculata* to contain particularly high levels of C16:1 and C25:3, with corresponding  
36  
37 reduction in C18:1, C18:2 and C18:3. Fatty acid profile of microalgal species is known  
38  
39 to be function of its culturing conditions and environmental conditions (Olofsson et al.,  
40  
41 2012). For *D. salina* sp., palmitic (C16:0), oleic (C18:1n9), hexadecatetraenoic (C16:4),  
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43 linoleic (C18:2) and linolenic (C18:3) acids were identified as the principal fatty acids  
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45 in *D. salina* extracted lipids. The data in table reveals that total unsaturated fatty acids  
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47 were about 45-60%, while saturated fatty acids were about 40-50%. Results obtained  
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1 with SDEP method were almost similar with those obtained by both conventional Sox  
2 extraction and B&D method. The sum percentages of saturated, mono- and poly-  
3  
4 extraction and B&D method. The sum percentages of saturated, mono- and poly-  
5  
6 unsaturated fatty acids were in line with those of several tables dealing with the fatty  
7  
8 acid composition of *N. oculata* and *D. salina* oil found in literature. As a conclusion, it  
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10 can be said that the proportion of the different fatty acids as well as the proportion of  
11  
12 SFAs, PUFAs, or MUFAs has not been affected by the unusual conditions used in our  
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14 experiment, in other words, the use of terpenes as solvent do not involve extraneous  
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16 effects and/or artefacts on the composition of the lipids extracted.  
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### 20 3.4 Energy consumption

21  
22 We conducted a comparison on energy consumption of SDEP process and the  
23 current method to extract lipids, Soxhlet. Alternatively, the input power consumption  
24  
25 was monitored using a separate Wattmeter at the entrance of electrical heater power  
26  
27 supply (i.e. at the entrance of electrical heater, rotary evaporator and cooler power  
28  
29 supply). For Soxhlet extraction, drying biomass is required. So, in our calculation we  
30  
31 have taken into account the freeze-drying of microalgae for 48h. We have added the  
32  
33 energy consumption of all apparatus involved in each extraction technique. The energy  
34  
35 requirement to perform the extraction, based on the power consumptions for 1g of  
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37 extracted lipids, was 8.84 kWh for Soxhlet while this value was 2.15 kWh for SDEP.  
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39 This indicates a substantial saving in the extraction cost by SDEP compared to the  
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41 conventional extraction technique, Soxhlet.  
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## 49 4. Conclusion

50  
51 The new procedure has undergone reproducible results in a substantially shorter  
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53 time. The efficiency of SDEP is considerably higher than the conventional procedure in  
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55 terms of lipid yield and if we take into account short distillation and extraction times  
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1 required, cost and energy used of the process. This study has clearly demonstrated that  
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3 this procedure can be used to extract lipid directly from wet microalgae with an  
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5 extraction yield similar to that of the B&D method, with only minor variation in the  
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7 fatty acids distribution.  
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## 10 11 **Acknowledgements**

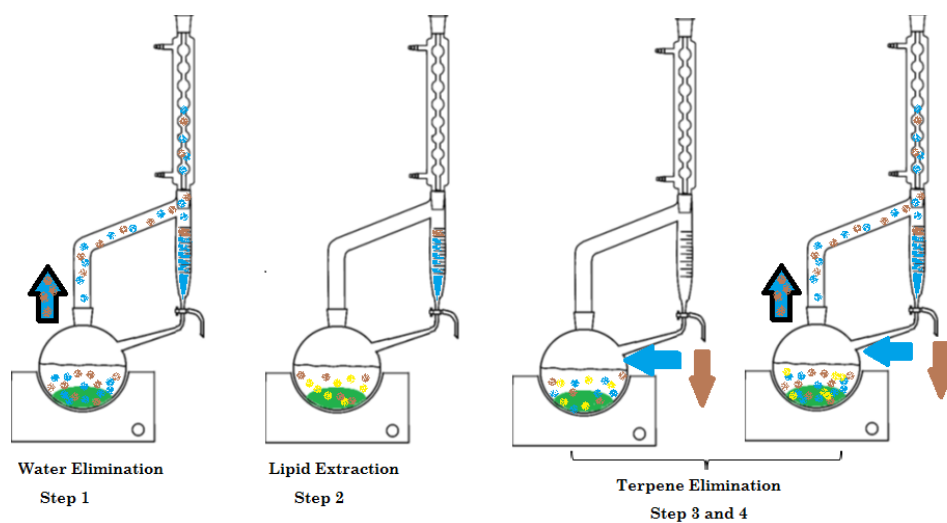
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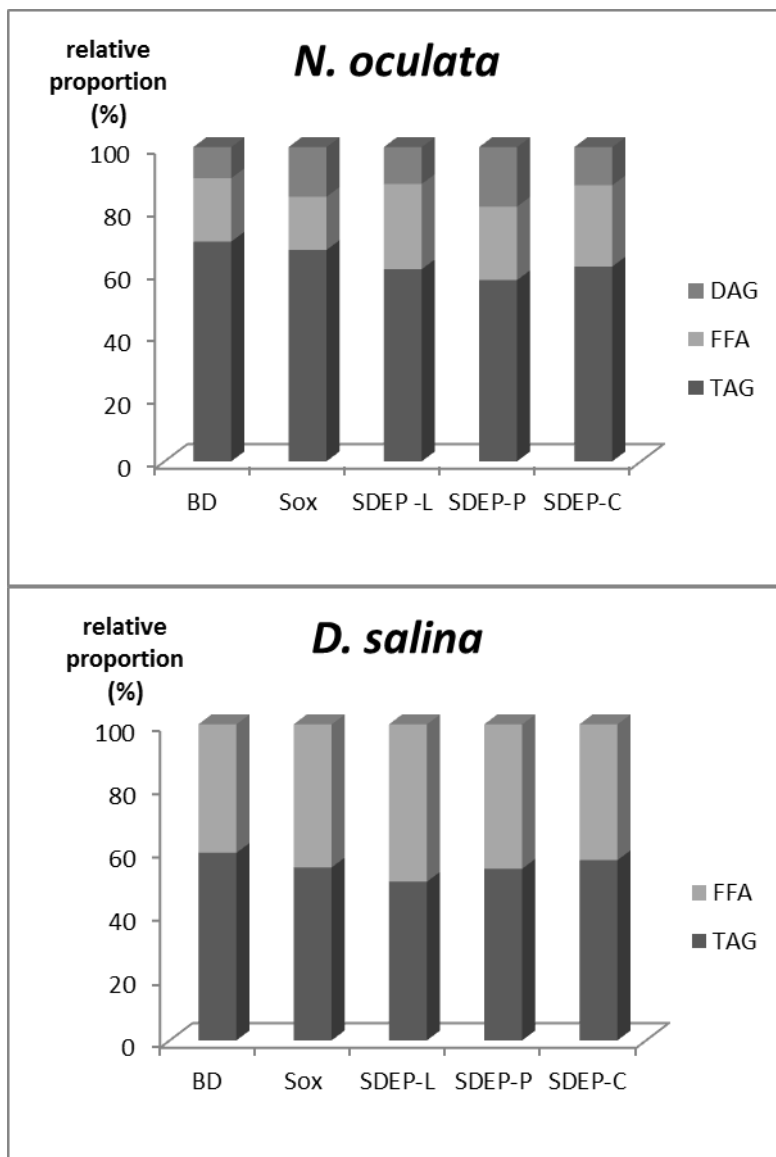
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**Fig. 1.** Simultaneous Distillation Extraction Process (SDEP) (blue: water biomass, brown: terpene solvent, yellow: lipids, green: microalgae)



**Fig. 2.** Distribution of lipid classes by HP-TLC



**Table 1:** Crude lipids, distribution of lipid class and fatty acid composition of extracts obtained by different extraction methods. (PUFAs polyunsaturated fatty acids, MUFAs monounsaturated fatty acids, SFAs Saturated fatty acids)

	<i>N. oculata</i>									<i>D. salina</i>										
	B&D		SDEP		$\alpha$ -pinene		p-cymene		Sox	B&D		SDEP		$\alpha$ -pinene		p-cymene		Sox		
<b>Lipids Yield (%)</b>	23.78	$\pm 2.13$	18.73	$\pm 6.58$	18.75	$\pm 3.01$	21.45	$\pm 2.64$	8.31	$\pm 1.05$	4.03	$\pm 0.01$	2.94	$\pm 0.02$	3.29	$\pm 0.05$	2.99	$\pm 0.01$	1.90	$\pm 0.03$
<b>Lipid class composition</b>																				
FFA : Free fatty acid	20.21	$\pm 0.03$	27.12	$\pm 0.12$	23.42	$\pm 0.27$	25.84	$\pm 0.32$	16.92	$\pm 0.13$	40.64	$\pm 0.36$	49.77	$\pm 0.08$	47.55	$\pm 0.32$	42.95	$\pm 0.01$	45.25	$\pm 0.09$
TAG : Triacylglycerol	69.94	$\pm 0.16$	61.18	$\pm 0.05$	57.67	$\pm 0.65$	62.02	$\pm 0.82$	67.30	$\pm 2.68$	59.36	$\pm 0.02$	50.23	$\pm 0.20$	52.45	$\pm 0.05$	57.05	$\pm 0.24$	54.75	$\pm 0.04$
DAG: Diacylglycerol	9.85	$\pm 0.06$	11.71	$\pm 0.06$	18.91	$\pm 0.09$	12.15	$\pm 0.15$	15.78	$\pm 0.12$	-		-		-		-		-	
<b>Fatty acids composition</b>																				
<b>Saturated</b>																				
C14:0	1.20	$\pm 0.02$	1.52	$\pm 0.03$	1.43	$\pm 0.01$	0.72	$\pm 0.97$	1.37	$\pm 0.10$	3.37	$\pm 0.45$	6.74	$\pm 0.17$	3.69	$\pm 0.02$	3.61	$\pm 0.01$	1.01	$\pm 0.03$
C16:0	20.65	$\pm 0.09$	21.86	$\pm 0.41$	21.90	$\pm 0.01$	21.69	$\pm 0.03$	21.55	$\pm 0.28$	46.46	$\pm 0.31$	34.84	$\pm 1.05$	43.36	$\pm 0.15$	42.66	$\pm 0.06$	38.67	$\pm 1.65$
C18:0	1.62	$\pm 0.05$	1.84	$\pm 0.11$	1.75	$\pm 0.01$	1.88	$\pm 0.09$	1.72	$\pm 0.01$	1.26	$\pm 0.04$	3.13	$\pm 0.06$	1.01	$\pm 0.03$	1.17	$\pm 0.02$	0.81	$\pm 0.07$
<b>Mono-unsaturated</b>																				
C14:1	-		-		-		-		-		0.43	$\pm 0.01$	2.02	$\pm 0.02$	0.60	$\pm 0.01$	0.48	$\pm 0.02$	0.19	$\pm 0.02$
C16:1(n-9)	1.07	$\pm 0.01$	1.25	$\pm 0.05$	1.12	$\pm 0.01$	1.10	$\pm 0.01$	1.13	$\pm 0.02$	0.69	$\pm 0.09$	0.94	$\pm 0.09$	0.91	$\pm 0.01$	0.58	$\pm 0.01$	1.52	$\pm 0.58$
C16:1(n-7)	2.76	$\pm 0.02$	3.26	$\pm 0.01$	3.11	$\pm 0.01$	3.12	$\pm 0.05$	2.83	$\pm 0.11$	0.54	$\pm 0.01$	1.88	$\pm 0.34$	0.89	$\pm 0.01$	0.52	$\pm 0.01$	1.77	$\pm 0.24$
C18:1(n-9)	27.46	$\pm 0.04$	26.03	$\pm 0.02$	26.27	$\pm 0.11$	26.62	$\pm 0.73$	26.87	$\pm 0.36$	6.50	$\pm 0.14$	12.61	$\pm 0.19$	7.55	$\pm 0.03$	7.67	$\pm 0.01$	6.80	$\pm 0.27$
C18:1(n-7)	2.78	$\pm 0.02$	2.59	$\pm 0.24$	2.52	$\pm 0.11$	2.45	$\pm 0.15$	2.35	$\pm 0.04$	-		-		-		-		-	
<b>Poly-unsaturated</b>																				
C16:2	2.46	$\pm 0.01$	2.73	$\pm 0.13$	2.48	$\pm 0.01$	2.46	$\pm 0.03$	2.53	$\pm 0.03$	0.08	$\pm 0.01$	2.42	$\pm 0.08$	1.04	$\pm 0.01$	0.91	$\pm 0.01$	0.82	$\pm 0.06$
C16:3	4.76	$\pm 0.03$	4.84	$\pm 0.04$	4.75	$\pm 0.03$	4.74	$\pm 0.05$	4.90	$\pm 0.07$	1.18	$\pm 0.01$	1.64	$\pm 0.06$	0.37	$\pm 0.07$	0.27	$\pm 0.01$	0.91	$\pm 0.05$
C16:4	-		-		-		-		-		9.22	$\pm 0.17$	9.20	$\pm 0.03$	9.16	$\pm 0.04$	9.94	$\pm 0.01$	8.37	$\pm 0.22$
C18:2(n-6)	14.70	$\pm 0.01$	14.91	$\pm 0.09$	15.08	$\pm 0.03$	15.50	$\pm 0.64$	15.14	$\pm 0.20$	6.95	$\pm 0.09$	7.89	$\pm 0.99$	7.98	$\pm 0.15$	7.65	$\pm 0.17$	8.60	$\pm 0.50$
C18:3(n-3)	20.09	$\pm 0.07$	19.18	$\pm 0.36$	19.60	$\pm 0.01$	19.73	$\pm 0.24$	20.06	$\pm 0.32$	20.32	$\pm 0.10$	16.70	$\pm 0.10$	23.44	$\pm 0.09$	24.66	$\pm 0.05$	30.54	$\pm 0.14$
$\Sigma$ SFAs	23.47		25.22		25.08		24.28		24.64		51.09		44.71		48.06		47.44		40.49	
$\Sigma$ MUFAs	34.07		33.13		33.01		33.29		33.18		8.16		17.44		9.95		9.25		10.28	
$\Sigma$ PUFAs	42.01		41.66		41.90		42.43		42.62		37.75		37.85		41.99		43.43		49.24	

### Highlights

- A new extraction procedure of lipids from wet microalgae
- *Nannochloropsis oculata* and *dunaliella salina* were chosen as a microalgae
- Influence of operating parameters on the extraction yields will be studied
- Utilization of terpenes bio-solvents recognized as environmentally safer