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Celine Dejoye Tanzi, Maryline Abert, Farid Chemat. New procedure for extraction of algal lipids from wet biomass: A green clean and scalable process.. *Bioresource Technology*, 2013, 134, pp.271-275. 10.1016/j.biortech.2013.01.168 . hal-01328660

HAL Id: hal-01328660

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Submitted on 29 May 2020

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Accepted Manuscript

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PII: S0960-8524(13)00200-9
DOI: <http://dx.doi.org/10.1016/j.biortech.2013.01.168>
Reference: BITE 11332

To appear in: *Bioresource Technology*

Received Date: 13 December 2012
Revised Date: 25 January 2013
Accepted Date: 30 January 2013

Please cite this article as: Tanzi, C.D., Vian, M.A., Chemat, F., New procedure for extraction of algal lipids from wet biomass: A green clean and scalable process, *Bioresource Technology* (2013), doi: <http://dx.doi.org/10.1016/j.biortech.2013.01.168>

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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, α -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
8
9 can grow on non-arable, nutrient-poor land that cannot support conventional agriculture
10
11 (Singh and Gu, 2010). A recent life-cycle assessment (LCA) of biofuel production from
12
13 microalgae feedstocks mentioned that drying and n-hexane extraction accounted for up
14
15 to 90% of the total process energy (Lardon et al., 2009). The extraction of crude oil is
16
17 usually performed with petroleum solvents such as conventional n-hexane, chloroform
18
19 and methanol, with techniques are highly energy-consumption and environmentally
20
21 damaging (Halim et al., n.d.). Other extraction processes such as supercritical CO₂,
22
23 expelling, microwave-ultrasonic assisted extraction have also been reported (Cheng et
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25 al., 2011). Many processes have been investigated and reported for converting directly
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27 wet algae to crude biodiesel or biocrude (Anastasakis and Ross, 2011; Biller and Ross,
28
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
43
44 generally require long extraction times, petroleum-based solvents, dried biomass with
45
46 water content no more than 10% and high energy inputs. Upon harvesting, typical
47
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
2
3 lipid extraction step that eliminates biomass drying and petroleum solvent use could
4
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 (Virot 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,
23
24 2005).
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30 Extracted lipids obtained using this new procedure, conventional Soxhlet with *n*-
31
32 hexane and Bligh & Dyer method have been compared in term of total lipid content,
33
34 lipid classes distribution and fatty acid composition.
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36

37 **2. Materials and methods**

38 2.1. Strain, culture and harvesting conditions

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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
44
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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56 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 N₂ 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
4
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.
16
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
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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)

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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
43
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 CuSO₄ reagent (20 g CuSO₄, 200 ml methanol, 8 ml
4 H₂SO₄, and 8 ml H₃PO₄) 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.
14
15

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
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3 Liu, 2004; Liu and Mamidipally, 2005) have already noted this effect that might be due
4
5 to the slightly more polar nature of terpenes and much more polar nature of
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7 methanol/chloroforme mixture compared with *n*-hexane. In addition, in SDEP
8
9 procedure the matrix is in direct contact with the boiling solvent which is not the case
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11 with the conventional Soxhlet. A higher dissolving ability of terpenes for lipids might
12
13 also be pointed out by the higher temperature used to boil this solvent which could
14
15 produce a lower viscosity of the analytes in the matrix and, accordingly, a better
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17 diffusion rate of the solute from the solid phase to the solvent.
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22 3.2 Lipids class composition

23
24 Similar to higher plants, microalgal lipids are composed of neutral lipids and polar
25
26 lipids. Under nutrient stress conditions, many microalgae tend to accumulate neutral
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28 lipids (consist of triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols
29
30 (MAG) and free fatty acids (FFA)) and form lipid droplet localized in the cytoplasm as
31
32 a storage form of carbon and energy. Neutral lipids of the extracts were separated using
33
34 high-performance thin-layer chromatography (HP-TLC), quantified with densitometry
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36 and presented as % of lipid class in total lipids extracted with B&D, SDEP and Sox for
37
38 both microalgae. Four standards in a mixture of monoacylglycerol (MAG),
39
40 diacylglycerol (DAG), triacylglycerol (TAG) and free fatty acids (C18) were deposited
41
42 on the plate. For *N. oculata*, HP-TLC results confirm that TAG, DAG and FFA were the
43
44 main components of the extracted lipids, but they contributed to the total lipid content in
45
46 different amounts. As shown in Fig. 2, TAG made up an important percentage of total
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48 lipids (between 57 and 69% according to extraction method). Regarding *D. salina*, the
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50 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 α -pinene. Results obtained with the SDEP
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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*
16
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),
22
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
26
27 trace levels. No significant differences were detected according different extraction
28
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),
42
43 linoleic (C18:2) and linolenic (C18:3) acids were identified as the principal fatty acids
44
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
9
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
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24 current method to extract lipids, Soxhlet. Alternatively, the input power consumption
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26 was monitored using a separate Wattmeter at the entrance of electrical heater power
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28 supply (i.e. at the entrance of electrical heater, rotary evaporator and cooler power
29
30 supply). For Soxhlet extraction, drying biomass is required. So, in our calculation we
31
32 have taken into account the freeze-drying of microalgae for 48h. We have added the
33
34 energy consumption of all apparatus involved in each extraction technique. The energy
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36 requirement to perform the extraction, based on the power consumptions for 1g of
37
38 extracted lipids, was 8.84 kWh for Soxhlet while this value was 2.15 kWh for SDEP.
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40 This indicates a substantial saving in the extraction cost by SDEP compared to the
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42 conventional extraction technique, Soxhlet.
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49 4. Conclusion

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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
6
7 fatty acids distribution.
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10 11 **Acknowledgements**

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15 The authors are grateful for the financial support of the Salinalgue program (FUI-
16
17 France) coordinated by La Compagnie du Vent (Montpellier, France).
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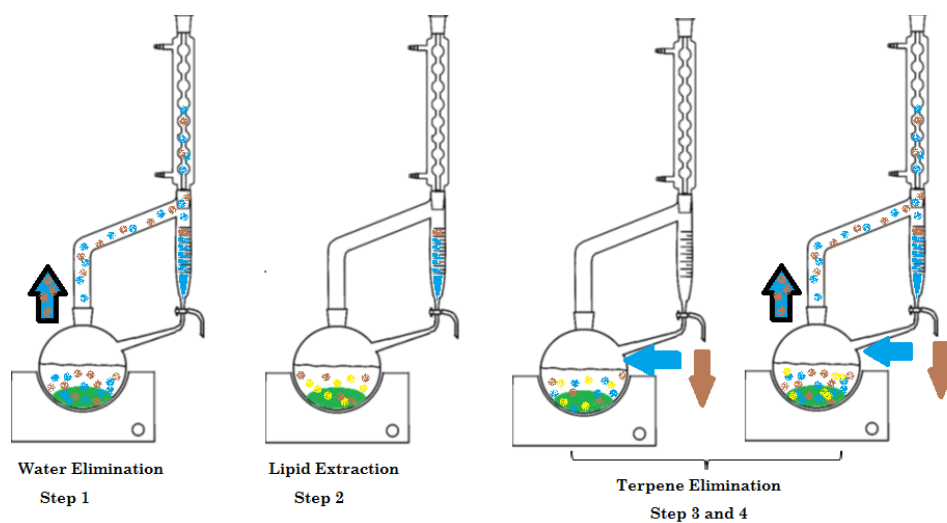


Fig. 1. Simultaneous Distillation Extraction Process (SDEP) (blue: water biomass, brown: terpene solvent, yellow: lipids, green: microalgae)

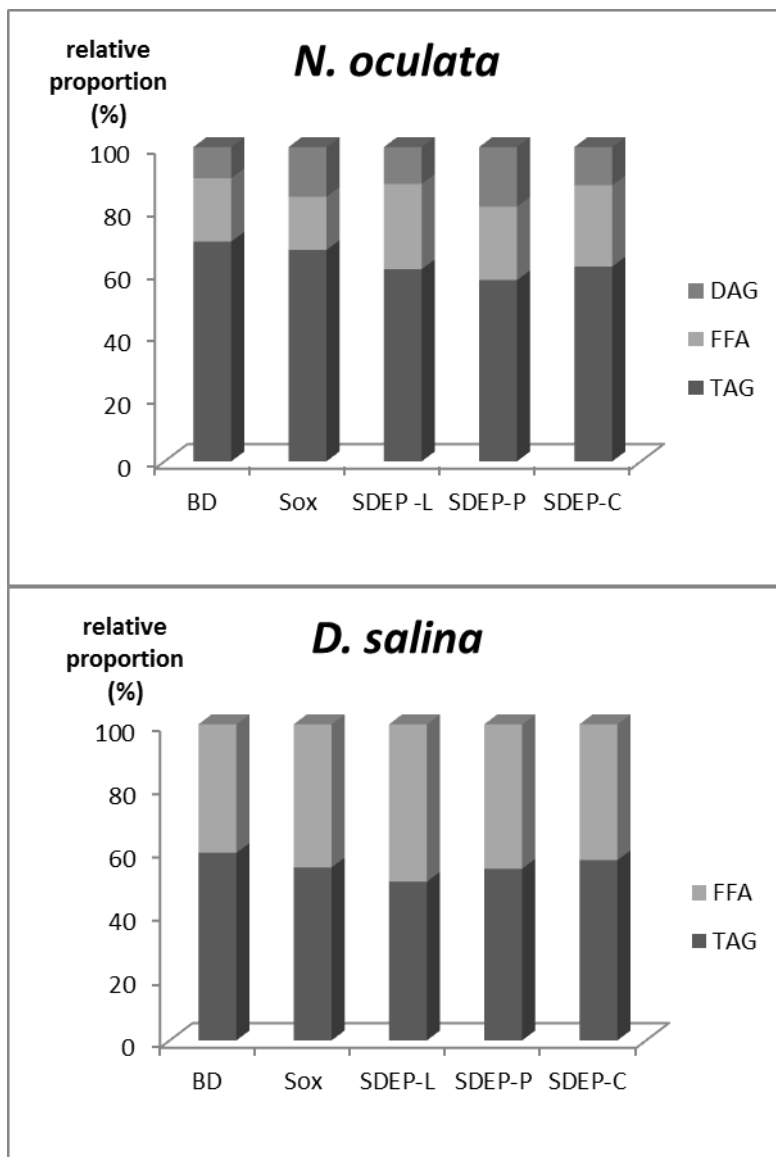


Fig. 2. Distribution of lipid classes by HP-TLC

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