Development of a New Method for Determination of the Oil Content from Microalgae Lipid Fraction

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The purpose of this study was to develop a method for rapid quantitative and qualitative determination of the oil from microalgae lipid fraction obtained from Nannochloris sp biomass. The lipid fraction was first refluxed with 4% KOH in MeOH (60, 90, 120 min), followed by reaction with 20% BF_3 in MeOH, using different times of reflux (90,120, 150 min) for each time of reflux with 4% KOH in MeOH. The FAME samples were analyzed by GC-MS analysis. 120 min reflux with 4% KOH in MeOH, 90 min with 20% BF_3 in MeOH and a ratio lipid fraction: 4% KOH in MeOH: 20% BF_3 in MeOH=1:20:27, were required to obtain the higher percent of oil in the microalgae lipid fraction. The relevance of the method developed was proved by TGA analysis and by transesterification of a sunflower oil sample in the same conditions.

Keywords: microalgae lipid fraction, oil, transesterification, boron trifluoride

Microalgae are photosynthetic microorganisms that have the potential to produce more biomass than most crops [1]. Since the microalgal biomass is rich in lipid and proteins [2], has been investigated as a potential source of biofuels [3, 4], bioactive compounds [5], human food supplements, pharmaceuticals and animal feed [6]. Also, microalgae culture has been investigated for waste water and exhaust gas treatment [7]. Microalgae lipid content varied from one species to another and could range from 5 to 77% of dry weight. An important step in the production of biofuels and other value products from microalgae lipid is a precise characterization of its constituents. The microalgae lipid fraction contains generally (1) neutral lipids (acylglycerols, free fatty acids) – transesterified lipids; (2) polar lipids (phospholipids and glycolipids) and (3) other compounds soluble in organic solvents (hydrocarbons, sterols, ketones, pigments, wax sterols, steryl esters) [8, 9].

The aim of this study was to develop a new method for quantitative and qualitative determination of the oil (transesterified lipids) from microalgae lipid fraction. For this purpose, there were investigated the following parameters: - reflux time with 4% potassium hydroxide in methanol; - reflux time with 20% boron-trifluoride in methanol; - different ratio of lipid fraction to 20% BF_3 in MeOH. To our knowledge, quantitative determination of oil from microalgae lipid fraction, using a transesterification in two-step with KOH methanolic solution and BF_3 methanolic solution has not previously been reported.

Transesterification of microalgae lipid fraction with BF_3 methanolic solution has reported in the literature only for determining fatty acid composition of the algal oil [10]. Also, a two-step method for preparing methyl esters of the fatty acids from fish oil, using BF_3 solution in methanol was reported [11].

Experimental part

Materials

Boron trifluoride-methanol complex (20% solution in methanol) (Merck), potassium hydroxide (purity ≥85%), n-hexane and methanol reagent grade, min. 99.9% (Sigma Aldrich). Sodium chloride and chloroform (99%) (Chimreactiv SRL). All the substances were used as received, without further purification or distillation. Distilled water was used throughout all the experiments.

Algal biomass cultivation and harvesting

The algal biomass was obtained after cultivation of the strain *Nannochloris sp.* 424-1 in autotrophic conditions using the same apparatus and procedure described by Stepan et al. [3]. When the algal culture was in the stationary phase of grown, biomass was separated from the liquid medium by centrifugation at 8000 rpm for 10 min, using a Hettich Rotina 380R centrifuge. The wet algal biomass obtained, was dried at 60°C in an oven, until a constant weight has been reached.

Lipid fraction extraction

The lipid fraction was extracted from the dry algal biomass using a modified Folch [12] method combined to the use of ultrasounds. The extraction was performed in three steps, using as solvent a mixture of chloroform-to-methanol [2:1 (v/v)] in ratio dry algal biomass-to-solvent (1:4 w/v). The sample was weighted and transferred into a plastic tube, along with the solvent. The sample was held for 20 min in an ultrasonic water bath (Elma Ultrasonic) for lipid fraction extraction. The extraction products were separated from the algal biomass by centrifugation for 10 min at 8000 rpm. The liquid fraction was collected in a round-bottom flask. The described process was repeated twice for the same sample of algal biomass. All the liquid fractions were collected in the same round-bottom flask.

To determine the lipid fraction concentration, 30 mL of liquid fraction obtained after separation was measured and putted in a weighted round-bottom flask, the solvent was distilled and the lipid fraction was weighed. The data was used to calculate the lipid fraction obtained from algal biomass. The liquid fraction was keeping in the fridge at 4°C until the next use.

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Characterization of FAME and lipid fraction

Characterization of FAME was performed using GC–MS/MS TRIPLE QUAD (Agilent 7890 A) with DB-WAX capillary column (30 m length, 0.25 mm internal diameter, 0.25μm film thickness) and helium as carrier gas at a flow rate of 1 mL/min. The oven temperature was initially set at 70°C and then increased to 230°C with a rate of 4°C/min and hold time of 5 min. The GC injector and MS ion source temperatures were 250 and 150°C, respectively. The transfer line temperature was 280°C. The MS detector was operated in EI mode at 70 eV, with a m/z scanning range of 50–450. The FAME peaks in the sample were identified using NIST MS database. The determination of the FAME characteristics was done according to the following European standards: EN 14103 – ester content [3].

The lipids fraction obtained was characterized by Thermogravimetric analysis (TGA) using a DuPont Instruments *Thermal Analyst 2000/2100* coupled with a module *951 Thermogravimetric Analizer* on samples in range 3-5 mg in atmosphere inert gas (nitrogen) with a flow rate 50 mL/min and a heating rate of 10°C/min.

Fatty acids methyl esters (FAME) preparation

For determining quantitative the free fatty acids and triglycerides from the lipid fraction obtained after the extraction procedure, this fraction was transesterified starting from the procedure described in the international standard ISO 5509. The transesterification was conducted in two stage: 1) refluxing of the lipid fraction with 4% KOH in methanol, using different times of reflux (60, 90 and 120 min); 2) reaction with boron trifluoride-methanol complex (20% solution in methanol), using different times of reflux for each time of reflux (90,120, 150 min) with 4% KOH in MeOH. After refluxing the heating was stopped, 7 mL of hexane and 20 mL of 20% sodium chloride solution were added. The mixture was shacked vigorously, transferred in a centrifuge tube and separated by centrifugation at 5000 rpm for 20 min. After centrifugation, the organic layer was collected in a weighted flask and the hexane was evaporated. After complete evaporation of hexane, fatty acids methyl esters obtained (m_{esters}), were weighted and analyzed by GC-MS to establish the concentration (c_{esters}) of the FAME existing in the sample and their distribution. All the experiments were carried out in triplicate. For each experiment were used samples of lipid fraction obtained taking 40 mL from the mixture obtained lipid extraction. The solvent was removed and each time was obtained approximately 0.5 g of lipid fraction (m_{lipids}).

In order to determine the oil percent (free fatty acids and triglycerides) available in the lipid fraction was used the equation 1:

$$\%oil = \frac{c_{esters} \cdot m_{esters}}{m_{lipids}} \quad (1)$$

where:

c_{esters} = concentration of fatty acids methyl esters obtained by GC-MS analysis;

m_{esters} = mass of esters obtained after transesterification;

m_{lipids} = mass of lipid fraction used for transesterification.

Results and discussions

Influence of reflux time on oil percent determined

In order to investigate the influence of the reflux time on the percent of oil determined in the microalgae lipid fraction, the reflux time was varied between 60 and 120 min in 30 min increments for 4% KOH in MeOH and respectively between 90 and 150 min in 30 min increments for 20% BF_3 in MeOH for each reflux time with 4% KOH in MeOH. The ratio of lipid fraction to 4% KOH in MeOH and 20% BF_3 in MeOH was 1:20:27. Figure 1 show that percent of oil determined in the microalgae lipid fraction was influenced by the reflux time. The percent of oil determined increased with the reflux time of 4% KOH in MeOH. In case of an increased of the reflux time with 20% BF_3 in MeOH was observed a high percent of the oil determined only for 120 min of reflux and for a reflux time with 4% KOH in MeOH of 60 min. Using long time of reflux with 20% BF_3 in MeOH for each time of reflux with 4% KOH in MeOH was observed a slight decrease of the oil determined in the microalgae lipid fraction. This decrease of the oil determined is the results of the loss of some highly unsaturated fatty acids [13]. This was confirmed by FAME distribution, through which, for example in case of a reflux time of 120 min with 4% KOH in MeOH respectively 120 min with 20% BF_3 in MEOH, was observed a decrease of the percentage of linoleic acid (18:2 $\Delta^{9,12}$) and α-linolenic acid (18:3 $\Delta^{9,12,15}$) comparative with percentage for the same compounds in the sample obtained using 120 min reflux time with 4% KOH in MeOH and respectively 90 min with 20% BF_3 in MeOH. The highest percent of oil determined in the microalgae lipid fraction (39.53 ±0.475%) was obtained using 120 min reflux time with 4% KOH in MeOH and respectively 120 min reflux time with 20 % BF_3 in MeOH.

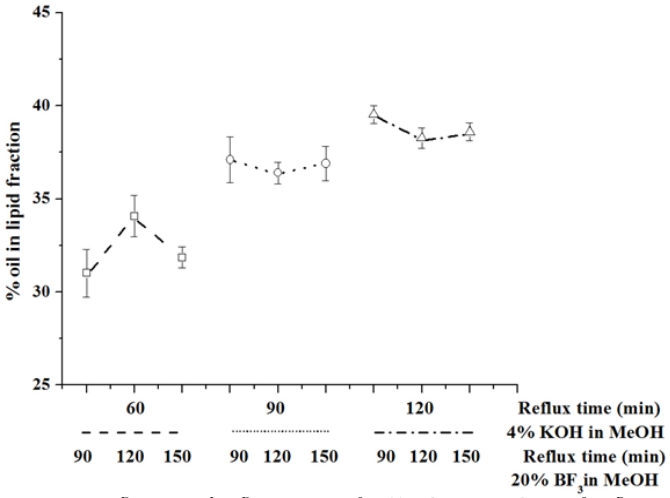

Fig.1. Influence of reflux time with 4% KOH in MeOH and reflux time with 20% BF_3 in MeOH on oil percent determined (ratio of lipid fraction to 4% KOH in MeOH and 20% BF3 in MeOH 1:20:27, mean ± S.D> (N = 3))

Influence of lipid fraction to 20% BF_3 in MeOH ratio on oil percent determined

In order to investigate the influence of different ratios of lipid fraction to 20% BF_3 in MeOH on oil percent determined in the lipid fraction, several ratio has been investigated (1:27, 1:33, 1:40), keeping constant the ratio of lipid fraction to 4% KOH in MeOH 1:20, reflux time with 4% KOH in MeOH 120 min and the reflux time with 20% BF_3 in MeOH 90 min. The obtained results are depicted in figure 2, and shows small increase of the oil determined using 1:33 as ratio, and a small decrease of the oil using 1:40 as ratio. The decrease of the oil determined, using a large volume of BF_3 may be the result of degradation of some fatty acid in presence of BF_3 [14].

TGA analysis of the lipid fraction compared with sunflower oil

Figure 3 shows a comparison of the TG and the derivative of the TG curves (DTG) of microalgae lipid fraction and sunflower oil. Thermal decomposition of sunflower oil presents one degradation step, which starts at 250°C, with

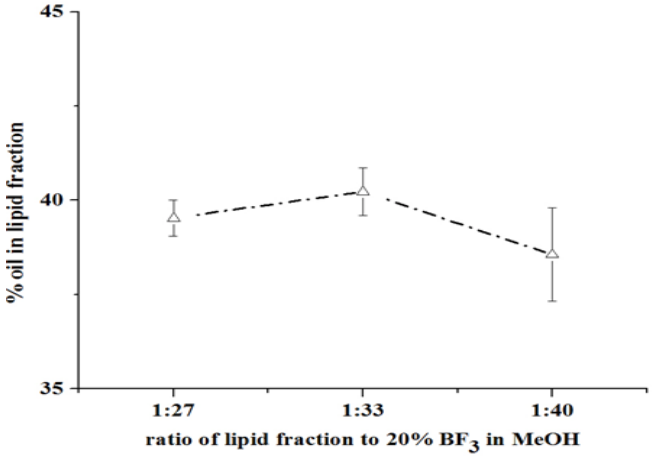

Fig. 2. Influence of different ration of lipid fraction to 20% BF_3 in MeOH on oil percent determined (reflux time 120 min with 4% KOH in MeOH and 90 min with 20% BF_3 in MeOH, ratio of lipid fraction to KOH in MeOH 1:20, mean±S.D. (n = 3))

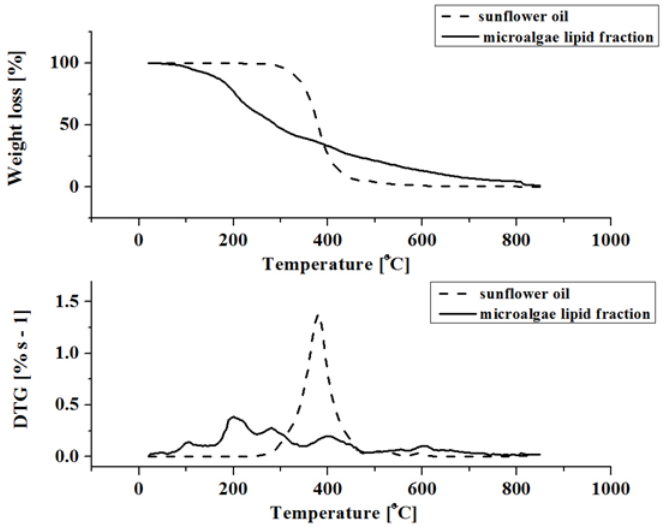

Fig. 3. TG/DTG of microalgae lipid fraction and sunflower oil. determined (ratio of lipid fraction to 4% KOH in MeOH and 20% BF_3 in MeOH 1:20:27, mean±S.D. (n = 3))

Peak Number	Compound name	Algal oil	Sunflower oil
1	C14:0	0.34	-
2	C15:0	0.42	-
3	C16:2n	16.14	-
4	C16:3n	4.74	-
5	C16:1n (9)	2.42	-
6	C16:1n (7)	1.58	-
7	C16:0	16.72	9
8	C16:1n (2)	0.34	-
9	C18:2n (9,12)	31.18	66
10	C18:3n (9,12,15)	20.9	1
11	C18:1n	1.79	20
12	C18:0	0.5	4
13	C20:2n	1.69	-
14	C22:0	1.24	-

Table 1
FAME COMPOSITIONS OF MICROALGAE LIPID FRACTION AND SUNFLOWER OIL

a maximum weight loss at 380°C and end at 500°C. In case of microalgae lipid fraction was presented three decomposition stages. Stage one which starts at 170°C and end at 250°C, release 20-25% of the total mass which may represent decomposition of the compounds formed through autoxidation during the extraction or by enzyme catalyzed during storage time (ex. ketones and aldehydes) or intermediate compounds in lipids biosynthesis [2,15]. The stage II from 250 to 500°C, presents two main peaks of weight loss, first with maximum weight loss at 300°C and second with maximum weight loss in the interval 400-410°C. The percentage weight loss was 42-45% and represents decomposition of the major lipid constituents such as triglycerides and free fatty acids [16]. This stage corresponds with the thermal degradation interval for sunflower oil, which confirms the idea that in this range were released the major components of the lipid. Also, the percentage obtained in this interval, are approximately the same with the maximum percent of oil obtained by transesterification, this think confirming the suitability of the developed method [16]. Stage III, between 500°C and 800°C, corresponding to solid residue decomposition [16] and the weight loss observed was 18-23%, which means that in the microalgae lipid fraction extracted are presented a high quantity of other compounds like sterols, phospholipids and glycolipids [8].

The fatty acid compositions of the microalgae lipid fraction

The fatty acid compositions of the microalgae lipid fraction and sunflower oil are presented in table 1. In case of sunflower oil was obtained the well known distribution. In case of microalgae lipid fraction a wider spectrum of fatty acid are presented, which include linoleic acid (18:2 $\Delta^{9,12}$) and α-linolenic acid (18:3 $\Delta^{9,12,15}$) with 31% and respectively 21%, as major constituents. Also presents a high levels of palmitic acid (16:0) and hexadecadienoic acid (16:2 $\Delta^{7,10}$) with 17% and respectively 16%. The microalgae oil obtained from Nannochloris sp. contained over 80% of polyunsaturated fatty acids and 17% of saturated fatty.

Transesterification of a sunflower oil sample

A sunflower oil sample of was transesterified using the same conditions in which was determined a maximum percent of oil in microalgae lipid fraction, 120 min reflux with 4% KOH in MeOH, 90 min reflux with 20 % BF_3 in MeOH and a ratio of lipid fraction: 4% KOH in MeOH: 20% BF_3 in MeOH =1:20:27, in order to verified the developed

method. It was taken into account the use of the same ratio of oil: 4% KOH in MeOH: 20% BF_3 in MeOH. Because the maximum percent of oil determined in the lipid fraction was 40%, reporting the used volumes of the 4% KOH in MeOH and respectively of the 20% BF_3 in MeOH at 40% from the lipid fraction was obtained a ratio of oil: 4% KOH in MeOH: 20% BF_3 in MeOH = 1:50:66. This ratio was used for the transesterification of the sunflower oil. Performing the same steps as for transesterification of the lipid fraction, was obtained 93.52 ±1.23% oil in the sunflower oil sample. Considering the experimental errors this value is very close to 100%, which proves that the method developed for quantitative determination of oil (transesterified lipids) from the microalgae lipid fraction is suitable.

Conclusions

In the present work, a method for a rapid quantitative and qualitative determination of the oil (transesterified lipids) from the microalgae lipid fraction was developed. The maximum percent of transesterified lipids obtained in the lipid fraction was 39.53±0.475%, using as reaction conditions: a ratio of lipid fraction: 4% KOH in MeOH: 20% BF_3 in MeOH =1:20:27, 120 min reflux with 4% KOH in MeOH and respectively 90 min reflux with 20% BF_3 in MeOH. The oil content was proved performing the TGA analysis on the same sample of microalgae lipid fraction. The TG and DTG curves for lipid fraction and compare with the TG and DTG curves for sunflower oil, revealed a weight loss in the interval characteristic for lipids, very close to the value obtained using the developed method, 42-45%. By transesterification of a sunflower oil sample in the same conditions in which was determined the maximum percent of oil, was obtained a 93.45% oil in the sample, that think proved again the suitability of the developed method.

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